UNIVERSIDAD DE EXTREMADURA

DEPARTAMENTO DE ZOOTECNIA

Desarrollo de nuevos transformados cárnicos cocidos a partir de carne, hígado y grasa de cerdo Ibérico con antioxidantes naturales

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Development of novel cooked products using livers, muscles and adipose tissues from Iberian pigs with natural antioxidants

Memoria de Tesis Doctoral presentada por el Licenciado Mario Estévez García.

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El Dr. Ramón Cava López, Profesor Titular de la unidad de Tecnología y Bioquímica de los Alimentos de la Facultad de Veterinaria de la Universidad de Extremadura,

INFORMA:

Que la Tesis Doctoral presentada por el Licenciado Mario Estévez García, "Desarrollo de nuevos transformados cárnicos cocidos a partir de carne, hígado y grasa de cerdo Ibérico con antioxidantes naturales", ha sido realizada bajo mi dirección en la Unidad de Tecnología y Bioquímica de los Alimentos de la Facultad de Veterinaria. Hallándose concluida y reuniendo a mi entender las condiciones necesarias, autorizo su presentación para su defensa ante el tribunal pertinente.

Cáceres, Julio de 2005

Fdo. Ramón Cava López

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"No había piedad en ellos [...]. Frailes, juez, escribano y verdugos se comportanban con una frialdad y un distanciamiento tan rigurosos que producían más pavor, incluso, que el sufrimiento que eran capaces de infligir: la helada determinación de quien se sabe respaldado por leyes divinas y humanas"

"Porque en las cárceles secretas de Toledo pude aprender que no hay nada más despreciable, ni peligroso que un malvado que cada noche se va a dormir con la conciencia tranquila [...]. En especial, cuando viene parejo con la ignorancia, la superstición, la estupidez o el poder; que a menudo se dan juntos"

"No soy amigo de dar consejos –a nadie lo acuchillan en cabeza ajena- , mas ahí va uno de barato: desconfíen siempre vuestras mercedes de quien es lector de un solo libro"

Iñigo Balboa

Index

RESUMEN/SUMMARY.....19
INTRODUCTION.....33
I. IBERIAN PIG.....35
I.A. Production of Iberian pigs.....36
I.B. Products from Iberian pigs: quality traits.....37
II. OXIDATION AND ANTIOXIDANT STRATEGIES.....47
II.A. Lipid oxidation.....47
II.B. Protein oxidation.....53
II.C. Antioxidant strategies.....57
II.C.1. Dietary means.....59
II.C.2. Added antioxidants.....61
III. REFERENCES.....64
JUSTIFICATION AND OBJECTIVES.....81
MATERIAL AND METHODS.....85
I. MATERIALS.....87
I.A. Biological material.....87
I.B. Chemicals.....88
I.C. Equipment.....88
II. METHODS.....89
II.A. Manufacture of cooked products.....89
II.A.1. Manufacture of the liver pâté.....89
II.A.2. Refrigeration of the liver pâté.....91
II.A.3. Manufacture of the frankfurters.....92
II.A.4. Refrigeration of the frankfurters.....94
II.B. Analytical methods.....95
II.B.1. Moisture.....95
II.B.2. Protein content.....95
II.B.3. Fat content.....96
II.B.4. Ash.....97
II.B.5. Total iron.....97
II.B.6. Non-heme iron.....97

II.B.7. Phenolic compounds content.....98
II.B.8. Tocopherol content.....98
II.B.9. Fatty acid profile.....99
II.B.10. Volatiles profile.....100
II.B.11. Objective colour measurement.....102
II.B.12. Instrumental hardness of liver pâtés.....102
II.B.13. Texture profile analysis of frankfurters.....103
II.B.14. pH.....104
II.B.15. TBA-RS.....104
II.B.16. Protein oxidation.....105
II.C. Data analysis.....106
III. REFERENCES.....107
CHAPTERS.....109
I. EXTENSIVELY REARED IBERIAN PIGS VERSUS INTENSIVELY REARED WHITE PIGS FOR THE MANUFACTURE OF LIVER.....111
I.1. Abstract.....113
I.2. Keywords.....113
I.3. Introduction.....113
I.4. Material and methods.....115
I.5. Results and discussion.....117
I.6. Conclusions.....125
I.7. Acknowledgements.....125
I.8. References.....126
II. LIPID AND PROTEIN OXIDATION, RELEASE OF IRON FROM HEME MOLECULE AND COLOUR DETERIORATION DURING REFRIGERATED STORAGE OF LIVER PÂTÉ.....137
II.1. Abstract.....139
II.2. Keywords.....139
II.3. Introduction.....139
II.4. Material and methods.....141
II.5. Results and discussion.....143
II.6. Conclusions.....148
II.7. Acknowledgements.....149
II.8. References.....149
III. PUFA DEGRADATION, TBA-RS AND LIPID-DERIVED VOLATILESIN REFRIGERATED STORED LIVER PÂTÉ: EFFECT OF NATURAL AND SYNTHETIC ANTIOXIDANTS.....159
III.1. Abstract.....161
III.2. Keywords.....161

III.3. Introduction.....161
III.4. Material and methods.....163
III.5. Results and discussion.....167
III.6. Conclusions.....171
III.7. Acknowledgements.....171
III.8. References.....172
IV. PROTEIN OXIDATION AND COLOUR AND TEXTURE CHANGES IN REFRIGERATED STORED LIVER PÂTÉ: EFFECT OF NATURAL AND SYNTHETIC ANTIOXIDANTS.....181
IV.1. Abstract.....183
IV.2. Keywords.....183
IV.3. Introduction.....183
IV.4. Material and methods.....185
IV.5. Results and discussion.....188
IV.6. Conclusions.....193
IV.7. Acknowledgements.....194
IV.8. References.....194
V. ANALYSIS OF VOLATILES IN PORCINE LIVER PÂTÉS WITH ADDED SAGE AND ROSEMARY ESSENTIAL OILS BY USING SPME-GC-MS.....203
V.1. Abstract.....205
V.2. Keywords.....205
V.3. Introduction.....205
V.4. Material and methods.....207
V.5. Results and discussion.....210
V.6. Acknowledgements.....217
V.7. References.....217
VI. EFFECTIVENESS OF NATURAL AND SYNTHETIC ANTIOXIDANTS AS INHIBITORS OF LIPID AND PROTEIN OXIDATION: CONTRADICTORY EFFECTS IN DIFFERENT TYPES OF PORCINE LIVER PÂTÉS.....229
VI.1. Abstract.....231
VI.2. Keywords.....231
VI.3. Introduction.....231
VI.4. Material and methods.....233
VI.5. Results and discussion.....238
VI.6. Conclusions.....244
VI.7. Acknowledgements.....245
VI.8. References.....245
VII. EXTENSIVELY REARED IBERIAN PIGS VERSUS INTENSIVELY REARED WHITE PIGS FOR THE MANUFACTURE OF FRANKFURTERS.....261
VII.1. Abstract.....263
VII.2. Keywords.....263

VII.3. Introduction.....263
VII.4. Material and methods.....265
VII.5. Results and discussion.....269
VII.6. Conclusions.....277
VII.7. Acknowledgements.....277
VII.8. References.....278
VIII. OXIDATION OF LIPIDS AND PROTEINS IN FRANKFURTERS WITH DIFFERENT FATTY ACID COMPOSITION AND TOCOPHEROLS AND PHENOLICS CONTENT.....289
VIII.1. Abstract.....291
VIII.2. Keywords.....291
VIII.3. Introduction.....292
VIII.4. Material and methods.....294
VIII.5. Results and discussion.....298
VIII.6. Acknowledgements.....305
VIII.7. References.....306
IX. PROTEIN OXIDATION IN FRANKFURTERS WITH DIFFERENT LEVELS OF ADDED ROSEMARY ESSENTIAL OIL: EFFECT ON COLOUR AND TEXTURE DETERIORATION.....317
IX.1. Abstract.....319
IX.2. Keywords.....319
IX.3. Introduction.....319
IX.4. Material and methods.....321
IX.5. Results and discussion.....324
IX.6. Conclusions.....330
IX.7. Acknowledgements.....330
IX.8. References.....330
X. ANALYSIS OF VOLATILES IN PORCINE FRANKFURTERS WITH INCREASING LEVELS OF ADDED ROSEMARY ESSENTIAL OIL BY USING SPME-GC-MS.....341
X.1. Abstract.....343
X.2. Keywords.....343
X.3. Introduction.....343
X.4. Material and methods.....345
X.5. Results and discussion.....349
X.6. Acknowledgements.....358
X.7. References.....358
XI. EFFECTIVENESS OF ROSEMARY ESSENTIAL OIL AS INHIBITOR OF LIPID AND PROTEIN OXIDATION: CONTRADICTORY EFFECTS IN DIFFERENT TYPES OF FRANKFURTERS.....369
XI.1. Abstract.....371
XI.2. Keywords.....371
XI.3. Introduction.....371

XI.4. Material and methods.....373
XI.5. Results.....377
XI.6. Discussion.....380
XI.7. Conclusions.....383
XI.8. Acknowledgements.....383
XI.9. References.....383
GENERAL DISCUSSION.....397
I. EXTENSIVELY REARED IBERIAN PIGS VS INTENSIVELY REARED WHITE PIGS FOR THE MANUFACTURE OF LIVER PÂTÉS AND FRANKFURTERS.....399
I.A. Proximate composition.....399
I.B. Iron.....399
I.C. Instrumental colour.....401
I.D. Fatty acid composition.....402
I.E. Antioxidants.....405
I.F. Oxidative stability of lipids.....407
I.G. Oxidative stability of proteins.....411
I.H. Volatile compounds profile.....413
II. EFFECT OF THE ADDITION OF PLANT ESSENTIAL OILS ON THE OXIDATIVE STABILITY OF LIVER PÂTÉS AND FRANKFURTERS.....417
II.A. Essential oils as inhibitors of oxidative reactions.....417
II.A.I. Antioxidant effect on lipids.....417
II.A.2. Antioxidant effect on proteins.....419
II.A.3. Effects on instrumental colour.....420
II.A.4. Effects on instrumental texture.....422
II.B. Contradictory role of added essential oils on cooked products: antioxidant versus prooxidant effects.....424
III. REFERENCES.....427
CONCLUSIONES/CONCLUSIONS.....437
ATTACHED PAPERS.....443
I. PHYSICO-CHEMICAL PROPERTIES AND OXIDATIVE STABILITY OF LIVER PÂTÉ AS AFFECTED BY FAT CONTENT.....445
I.1. Abstract.....447
I.2. Keywords.....447
I.3. Introduction.....447
I.4. Material and methods.....449
I.5. Results and discussion.....453
I.6. Conclusions.....459
I.7. Acknowledgements.....459
I.8. References.....459
II. CHARACTERISATION OF A TRADITIONAL FINISH LIVER SAUSAGE AND DIFFERENT

TYPES OF SPANISH LIVER PÂTÉS: A COMPARATIVE STUDY.....471
II.1. Abstract.....473
II.2. Keywords.....473
II.3. Introduction.....473
II.4. Material and methods.....475
II.5. Results and discussion.....478
II.6. Conclusions.....485
II.7. Acknowledgements.....486
II.8. References.....486

Resumen/Summary

Título: "Desarrollo de nuevos transformados cárnicos cocidos a partir de carne, hígado y grasa de cerdo Ibérico con antioxidantes naturales".

Hipótesis del Trabajo

Sobre la base de estudios preliminares, los tejidos de cerdos Ibéricos criados en un sistema de producción en extensivo y de cerdos blancos criados en intensividad son considerablemente diferentes en relación a la composición en ácidos grasos y contenidos en hierro y tocoferoles, y por lo tanto, los productos cocidos elaborados a partir de materias primas de cerdo blanco deberían presentar diferencias con aquellos elaborados con materias primas de cerdo Ibérico, aunque esta hipótesis no ha sido hasta el momento científicamente probada. El uso de sustancias naturales con actividad antioxidante en productos cárnicos podría ser una interesante opción al uso de antioxidantes sintéticos, los cuales se han asociado, en ciertas ocasiones, al desarrollo de enfermedades y que provocan rechazo por parte del consumidor. El estudio comparativo de la efectividad de ciertos antioxidantes naturales (aceites de salvia y romero) y la de un antioxidante sintético (BHT) sobre la oxidación de lípidos y proteínas en productos cocidos no se ha llevada a cabo con anterioridad.

Objetivos

1. Desarrollo y evaluación nutritiva y tecnológica de patés de hígado y salchichas cocidas elaboradas con tejidos de cerdos Ibéricos criados en extensivo y cerdos blancos criados en intensivo.
2. Evaluación del efecto de la adición de antioxidantes naturales (aceites esenciales de salvia y romero) y artificiales (BHT) sobre la estabilidad oxidativa de los productos cárnicos cocidos.

Material y Métodos

Material:

Tejidos (hígados, músculos y tejidos adiposos) de cerdos Ibéricos criados en extensivo y cerdos blancos (Large-White x Landrace) criados en intensivo

fueron utilizados para el desarrollo de patés de hígado y salchichas cocidas. Los cerdos Ibéricos fueron alimentados con recursos naturales (bellotas y pasto) mientras que los cerdos blancos se alimentaron con un pienso compuesto. El antioxidante sintético (BHT) se obtuvo de Sigma-Aldrich (Steinheim, Alemania) mientras que los antioxidantes naturales (aceites esenciales de salvia y romero) fueron suministrados por Soria Natural S.L. (Soria, España).

Métodos:

La caracterización química de los productos cocidos se llevó a cabo mediante métodos AOAC. La esterificación de ácidos grasos previo al análisis por cromatografía gaseosa (CG) se realizó de acuerdo con el método descrito por López-Bote *et al*. (1997).

La cuantificación de α- and γ-tocoferol se llevó a cabo de acuerdo con el método de Rey *et al*. (1997) usando HPLC.

La cuantificación de compuestos fenólicos en tejidos y productos cocidos se realizó mediante espectrofotometría empleando el método de Folin Ciocalteau.

Los compuestos volátiles fueron aislados del espacio de cabeza de los productos cocidos mediante microextracción en fase sólida (MEFS) y analizados mediante cromatografía gaseosa y espectrometría de masas (CG-EM). Las medidas de color instrumental (CIE L* a* b*; CIE, 1976) de las salchichas y los patés se llevaron a cabo usando un Colorímetro Minolta CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ, USA).

La textura instrumental fue medida usando un texturómetro Universal TA-XT2i (Stable Micro Systems, Godalming, UK).

La cuantificación de las sustancias reactivas al ácido tiobarbitúrico (TBA-RS) se llevó a cabo siguiendo el método de Rosmini *et al*. (1995) mediante espectrofotometría.

El análisis de compuestos volátiles derivados de oxidación lipídica (hexanal) fue realizada usando MEFS/CG-EM siguiendo el método descrito por Estévez *et al*., (2003).

La evaluación de la oxidación de proteínas se llevó a cabo conforme al método descrito por Oliver *et al*. (1987) para cuantificar mediante espectrofotometría el contenido total de carbonilos procedentes de la oxidación proteica.
La evaluación de la estabilidad de los pigmentos hemínicos durante la refrigeración de los productos cocidos se realizó mediante la estimación de la liberación del hierro del grupo hemo midiendo el incremento del contenido en hierro no hemínico (Miller *et al*., 1994).
Los análisis de datos fueron llevados a cabo con el paquete estadístico SPSS.

Resultados más Relevantes

Tanto factores extrínsecos (sistema de producción, alimentación, edad…) como intrínsecos (genéticos) influyeron sobre la composición química de los tejidos porcinos analizados y por lo tanto sobre las características de los patés de hígado y las salchichas cocidas. Las salchichas de cerdo Ibérico presentaron un mayor contenido de hierro que las de cerdo blanco. Los patés y salchichas de cerdo Ibérico presentaron un mayor contenido en ácidos grasos monoinsaturados (AGMI) y menor de saturados (AGS) y poliinsaturados (AGPI) que los mismos productos de cerdo blanco. La diferente composición de los alimentos con que los cerdos Ibéricos y blancos fueron alimentados influyó sobre el perfil de ácidos grasos de sus tejidos, que reflejaron la composición en ácidos grasos de dichos alimentos. Los tejidos y productos cocidos de cerdo Ibérico presentaron un mayor contenido de antioxidantes (α- and γ-tocoferoles) que los tejidos y productos de cerdo blanco. Se han aportado igualmente datos novedosos sobre el contenido total de compuestos fenólicos en tejidos de cerdo y en productos cárnicos cocidos, encontrando que aquellos procedentes de cerdos Ibéricos presentan un mayor contenido que los de cerdos blancos. La presencia de estas sustancias antioxidantes en los tejidos animales está supeditada al consumo de las mismas con la dieta, y por tanto, el mayor contenido de dichas sustancias en los tejidos y productos de cerdo Ibérico se explicaría por el consumo de alimentos con alto contenido en tocoferoles y compuestos fenólicos como el pasto y las bellotas.

Tras 60 días de almacenamiento en refrigeración, los productos cocidos de cerdo blanco sufrieron una mayor pérdida de AGPI y generaron un mayor contenido de productos de oxidación de lípidos (TBA-RS, aldehídos volátiles) y proteínas (compuestos carbonílicos), sugiriendo que los productos de cerdo blanco sufrieron con mayor intensidad procesos de deterioro oxidativo que los de cerdo Ibérico. La diferente composición en ácidos grasos y la presencia de un mayor contenido de sustancias con actividad antioxidante en los tejidos y productos de cerdo Ibérico ayudan a explicar los resultados obtenidos.

Los antioxidantes añadidos inhibieron satisfactoriamente las reacciones de oxidación en los productos cocidos de cerdo Ibérico. La actividad antioxidante de los aceites esenciales de salvia y romero fue similar a la que presentó el BHT, sugiriendo la posibilidad de que los antioxidantes naturales pudieran ser usados como alternativos al uso de antioxidantes sintéticos. Sin embargo, los antioxidantes naturales no fueron tan eficientes en los productos de cerdo blanco, donde presentaron incluso un efecto prooxidante, lo que sugiere que algunos componentes presentes en los productos cárnicos podrían afectar a la actividad antioxidante de los compuestos fenólicos de los aceites esenciales. Es conocida la interacción existente entre diferentes tipos de antioxidantes como, por ejemplo entre compuestos fenólicos y tocoferoles habiéndose descrito mecanismos de sinergismo y regeneración. La presencia de un cierto contenido de antioxidantes endógenos (tocoferoles) en los tejidos podría influir sobre la actividad de compuestos fenólicos directamente añadidos en los productos cocidos que producirían efectos globales antioxidantes o prooxidantes. El contenido significativamente más alto de tocoferoles en tejidos y productos de cerdo Ibérico en comparación con los de cerdo blanco apoyaría esta hipótesis. En el presente trabajo, se han descrito mecanismos por los cuales le desarrollo de reacciones de oxidación proteica podría influir sobre las características de color y textura de productos cocidos arrojando luz sobre un aspecto poco estudiado en el ámbito de la bioquímica de alimentos. La liberación de hierro del grupo hemo podría evidenciar la degradación oxidativa de la mioglobina muscular produciendo en consecuencia una decoloración de los productos cárnicos: pérdida del color rojo e incremento de

la luminosidad. La oxidación de las proteínas cárnicas durante la refrigeración probablemente causó un incremento en la dureza de los productos cocidos debido a que las proteínas oxidadas pierden su estructura nativa y tienden a sufrir procesos de agregación y a establecer enlaces cruzados entre ellas.

Expectativas para Futuras Investigaciones

El desarrollo de nuevos experimentos podría confirmar algunas de las hipótesis propuestas en el presente trabajo. La presencia de compuestos fenólicos procedentes de la ingesta de pasto y bellotas debería confirmarse mediante i) la evaluación de la proporción de polifenoles procedentes de dichos vegetales supuestamente contenidos en el heterogéneo grupo de compuestos fenólicos detectados en los tejidos de cerdos Ibéricos y cerdos blancos, ii) la descripción del perfil de compuestos fenólicos lo que podría contribuir a dilucidar si dichos compuestos se acumulan realmente en los tejidos animales y iii) la comprobación de que dichos compuestos contribuyen significativamente a mejorar la estabilidad oxidativa de los tejidos.

Por otra parte, nuevos experimentos serían interesantes para arrojar luz sobre las posibles interacciones entre los compuestos fenólicos de antioxidantes añadidos y ciertos componentes cárnicos y para evaluar específicamente la influencia de la composición química de la carne y otros tejidos en relación al contenido en tocoferoles y perfil de ácidos grasos sobre el efecto de antioxidantes añadidos. El estudio de las interacciones entre tipos de antioxidantes en modelos más simples como liposomas o emulsiones podría ser de gran utilidad.

Los mecanismos propuestos mediante los cuales la oxidación de proteínas afecta la calidad de los productos cocidos en términos de color y textura deberían ser confirmados en futuras investigaciones: nuevos experimentos en diferentes tipos de productos cárnicos podrían ser llevados a cabo. El conocimiento de la susceptibilidad de diferentes proteínas musculares (miofibrilares, sarcoplasmáticas y tejido conectivo) a sufrir reacciones de oxidación podría ser de interés para además evaluar el efecto que la oxidación

de cada uno de estos tipos de proteína tiene sobre diversos aspectos de la calidad de la carne.

Title: "Development of novel cooked products using livers, muscles and adipose tissues from Iberian pigs with natural antioxidants".

Hypothesis of Work

Based on preliminary research, tissues from free-range reared Iberian pigs and intensively reared white pigs are considerably different in terms of fatty acid composition, iron and α-tocopherol levels, and therefore, cooked products from Iberian and white pigs should be different though this extent has never been investigated. The use of plant materials as antioxidants in muscle foods could be an interesting alternative option to the use of synthetic antioxidants linked to health risks and rejected by consumers. The comparison between the effects of added natural (rosemary and sage essential oils) and synthetic antioxidants (BHT) on the oxidative stability of lipids and proteins from cooked products has never been carried out.

Objectives

1. The manufacture and the nutritional and technological evaluation of liver pâtés and frankfurters manufactured using tissues from free-range reared Iberian pigs and intensively reared white pigs.
2. Evaluation of the effect of the addition of natural (sage and rosemary essential oils) and synthetic (BHT) antioxidants on the oxidative stability of the cooked meat products.

Material and Methods

Material:

Tissues (livers, muscles and adipose tissues) from free-range reared Iberian pigs and intensively reared white pigs were used for the manufacture of liver pâtés and frankfurters. Iberian pigs were fed outdoors on natural resources (grass and acorns) whereas white pigs were fed in confinement on a commercial mixed diet.

The synthetic antioxidant (BHT) was purchased from Sigma-Aldrich (Steinheim, Germany) whereas natural antioxidants (sage and rosemary essential oils) were provided by Soria Natural S.L. (Soria, Spain).

Methods:

The chemical characterisation of cooked products was carried out using AOAC methods. The esterification of fatty acids into fatty-acid methyl esters (López-Bote *et al*., 1997) was carried out prior to analysis with gas chromatography (GC). The quantification of α- and γ-tocopherol was accomplished according to Rey *et al*. (1997) using reverse HPLC. The quantification of total phenolics in tissues and cooked products was carried out using the Folin Ciocalteau method by spectrophotometry.

Volatile components on cooked products were isolated and analysed using solid phase microextraction (SPME) coupled to gas chromatography and mass spectrometry (GC-MS).

Instrumental colour (CIE L* a* b*; CIE, 1976) was measured on the surface of frankfurters and liver pâtés using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ, USA).

Instrumental texture of cooked products was measured with a Universal TA-XT2i texture analyzer (Stable Micro Systems, Godalming, UK).

Thiobarbituric acid reactive substances (TBA-RS) were quantified following the method of Rosmini *et al*. (1995) using espectrophotometry.

Lipid-derived volatiles (i.e. hexanal) were quantified using SPME coupled to GC-MS following the method of Estévez *et al*. (2003).

The evaluation of protein oxidation was assessed following the method described by Oliver *et al*. (1987) in order to quantify the total amount of protein carbonyls.

The evaluation of heme pigments stability was carried out by assessing the release of iron from the heme molecule through the quantification of non-heme iron during the refrigerated storage of cooked products (Miller *et al*., 1994).

Statistical analyses were carried out with SPSS software.

Most Relevant Results

Extrinsic (production system, feeding background, age…) and intrinsic (genetic) factors influenced chemical composition of porcine tissues and therefore, the characteristics of liver pâtés and frankfurters. Frankfurters from Iberian pigs contained higher amounts of iron than those from white pigs. Frankfurters and liver pâtés from Iberian pigs contained significantly higher amounts of monounsaturated fatty acids (MUFA) and smaller of saturated (SFA) and polyunsaturated fatty acids (PUFA) than those from white pigs. The different feeds given to the animals likely affected the fatty acid composition of porcine tissues which tended to reflect the fatty acid composition of the feeds. Tissues and cooked products from Iberian pigs had higher amounts of antioxidants (α- and γ-tocopherols) than those from white pigs. We have originally reported data on the amount of total phenolic compounds in porcine tissues and cooked products. Tissues and cooked products from Iberian pigs contained higher total phenolics than those from white pigs. The presence of some particular antioxidants in porcine tissues such as tocopherols and phenolic compounds is dependent on the intake of such substances with the diet. The intake of natural resources by Iberian pigs could explain the significantly higher amount of these substances in their tissues.

After 60 days of refrigerated storage, cooked products from white pigs lose higher amounts of polyunsaturated fatty acids and contained significantly higher amounts of lipid and protein oxidation products such as TBA-RS, saturated volatile aldehydes such as hexanal, octanal and nonanal and protein carbonyls, suggesting that suffered the development of oxidative reactions to a higher extent than cooked products from Iberian pigs did. The different fatty acid composition and the presence of different amounts of tocopherols in tissues and products from both Iberian and white pigs would explain the results obtained.

Added antioxidants successfully inhibited the development of oxidative reactions in cooked products from Iberian pigs. The antioxidant activity of added natural antioxidants (sage and rosemary essential oils) was similar to that displayed by the synthetic one (BHT) suggesting the possibility that

natural antioxidants could be used as an alternative option to synthetic ones. Natural antioxidants were, however, not so efficient in products from white pigs, showing even a prooxidant effect, suggesting that some components of the meat product could affect the activity of phenolic compounds from essential oils. It is known the interactions between types of antioxidants such as those between plant polyphenol compounds and tocopherols. For instance, synergist and regeneration mechanisms have been reported by other authors. The presence of a certain amount of endogenous antioxidants (tocopherols) in porcine tissues could influence the activity of exogenous polyphenols directly added to the meat product leading to antioxidant or prooxidant effects. The significantly higher amount of tocopherols in tissues and meat products from Iberian pigs compared to those from white pigs support this hypothesis. In the present thesis, reasonable mechanisms have been reported by which the development of protein oxidation would affect the colour and texture characteristics of cooked products, shedding light on a particular topic that have been poorly studied in the field of food biochemistry. The release of iron from the heme molecule could be a reflection of the oxidative damage of the muscle myoglobin leading to discolouration of meat products: loss of redness and increases of lightness. The oxidation of muscle proteins probably caused the increase of hardness during refrigerated storage of cooked products, since oxidised proteins loose their native structure and functionality and tend to aggregate and cross-links between them are generated.

Expectations for Future Experiments

Upcoming experiments should confirm some of the hypothesis proposed so far.

For instance, the presence of plant phenolics in porcine tissues as a consequence of the intake of grass and acorns should be confirmed by i) measuring the proportion of plant polyphenols supposed to be contained in the heterogeneous group of phenolics compounds detected in the porcine tissues, ii) describe the polyphenol profiles which could contribute to elucidate if those compounds are certainly accumulated in animal tissues as a result of

the intake of natural resources and iii) evaluate if these compounds certainly contribute to enhance the oxidative stability of the tissues. In addition, further experiments would be interesting to shed light on the specific interactions between added plant essential oils and meat components and to evaluate the influence of the chemical composition of meat in terms of fatty acids and tocopherols on the activity of these substances. The investigation of the interactions between types of antioxidants in more simple models such as emulsions or liposomes could be an interesting option.

The mechanisms proposed by which protein oxidation affects the quality of muscle foods in terms of colour and texture deterioration should be also confirmed with further research: new experiments in different types of meat models should be carried out. Investigations about the susceptibility of the different muscle proteins (myofibrillar, sarcoplasmatic and connective tissue) to undergo oxidative reactions would be of interest as well to evaluate the effect derived from the oxidation of each type of protein on meat quality.

Introduction

I. IBERIAN PIG

The Iberian pig is a rustic breed which has been free-range reared in the southwest of the Iberian Peninsula since ancient times. This breed is perfectly adapted to the Mediterranean evergreen oak forests called 'Dehesa' and decisively contributes to the preservation of such ecosystem. Iberian pigs are fed making use of the natural resources provided by the environment, mainly acorns from evergreen oaks (*Quercus ilex* and *Quercus rotundifolia*) and pasture (López-Bote, 1998). Fifty years ago, industrial genotype pigs with lean carcasses and high growth rates were introduced in the Iberian Peninsula from foreign European countries to partly substitute the foodstuff obtained from this rustic breed. In those years, meat and meat products from Iberian pigs were despised by Spanish consumers for the reason of their high fat content and the presence of some sanitary scandals such as the African Swine Fever (ASF) that led to the almost disappearance of this breed (Diéguez, 2001).

Nowadays, Iberian pig breed and its livestock farming is supported by the fact that the meat and meat products traditionally obtained from this breed (dry-cured products) are considered by Spanish consumers as high quality foodstuff, being always more appreciated than those from industrial genotype pigs (Ventanas *et al.*, 2001; Cava *et al.*, 2003). Due to the high prices of Iberian dry-cured products, the traditional production schemes used for Iberian pigs are still profitable. In fact, the Iberian pig is one of the few non-selected pig breeds which have survived the modern techniques of pig production. However, this traditional feeding system is not always possible and alternative rearing systems involving feeding mixed diets in semi-intensive conditions have been proposed in order to minimise costs (López-Bote, 1998). Nevertheless, the outdoors traditional feeding system has been considered one of the main aspects to take into consideration for the achievement of high-quality products and in fact, products obtained from Iberian pigs raised in the traditional conditions are largely preferred by consumers (García *et al.*, 1996; Ventanas *et al.*, 2001).

I.A. Production of Iberian pigs

The whole productive cycle of Iberian pigs is planned so that they are able to take advantage of the natural resources from the environment 'La Dehesa', mainly acorns and pasture. López-Bote (1998) stated the perfect adaptation between Iberian pigs and their environment. The necessity of low temperatures in the first stages of the manufacture of Iberian dry-cured products to avoid microbial spoilage required that the slaughter of animals would take place around January, when the lowest temperatures during the year in the southwest of the Iberian Peninsula are reached. That perfectly fits with moment of the maturation of acorns which occurs during early November to late February. During that time, Iberian pigs are fattened outdoors for around two months to increase their live weight from 85-90 kg up to 140-160 kg (López-Bote, 1998). The association 'Dehesa'-Iberian pigs largely affects the quality of the Iberian dry-cured products since the intake of pasture and particularly acorns has been claimed to be essential to obtain high-quality products with very specific properties. However, to perform the traditional production procedure is not always possible since it is dependent on the natural resources provided by the environment and it is frequently modified to find less seasonal-dependable and more profitable production systems. In consequence, there is a diversity of production systems depending on the genetic (crossbreeds with Duroc) and feeding (partial supplementation or total feeding with mixed diets) background (López-Bote, 1998; Buxadé, 2000). This variety of production systems lead to a diversity of Iberian dry-cured products of different qualities: products obtained from non-pure Iberian pigs and supplemented with mixed diets are not so appreciated and reaches lower prices in the market (López-Bote, 1998).

The current census of Iberian pigs is estimated in around 2 millions of animals mainly located in 4 south western Spanish regions (Extremadura, Andalucía, Castilla León and Castilla la Mancha) (AICE, 2005). Based on indirect estimations by Cava *et al*. (2004), during the year 2000, around 600,000 free-range reared Iberian pigs were slaughtered in Spain. Iberian pig production largely contributes to the development of the regional meat

Industry in the southwest of the Iberian Peninsula as long as this sector supplies 30% of the total agricultural production (AICE, 2005). In addition, the consumption and production of dry-cured hams and loins has increased 25% in the last 4 years, with the profits from this trade being estimated around € 750 millions (AICE, 2005).

I.B. Products from Iberian pigs: quality traits

The production of Iberian pigs has been traditionally associated to the manufacture of dry-cured hams and loins. In the Mediterranean area, the environmental conditions throughout the year, with cold winters and hot and dry summers, allowed the production of self-stable meat products when other preservation technologies such as refrigeration were not available. Nowadays, the manufacture of dry-cured products is still the most relevant industrial activity derived from Iberian pigs with the Iberian dry-cured ham being, between them, the most valuable product (López-Bote, 1998; Ventanas *et al.*, 2001). Recent studies have compared the quality characteristics of meat from Iberian pigs for fresh consumption with those displayed by meat from industrial genotype pigs concluding that the former presented better nutritional and technological properties than the latter (Estévez *et al.*, 2003a,b). The distinctive sensory features of meat and dry-cured products from Iberian pigs derive from both intrinsic (genetic) and extrinsic (environmental) factors which have little in common with those from improved genotype pigs raised under intensive systems (different genetic and feeding background, slaughter weight, processing, etc.). The fat content and fatty acid composition, the myoglobin and iron contents and the deposition of high tocopherol levels from pasture and acorns in muscle membranes are considered the most important quality traits of Iberian pig's muscles affecting the high nutritional, technological and sensory properties of the Iberian cured products.

Fat content

The marbling of Iberian pig meat is typically abundant and evident, much more intense than in the meat from commercial genotypes (Estévez *et al.*, 2003a). This is a direct consequence of the high intramuscular fat (IMF) content of Iberian pig meat, with levels as high as 10% fresh matter (Mayoral *et al.*, 1999), while common levels described for selected breeds strangely are above 2% (Fernández *et al.*, 1999; Estévez *et al.*, 2003a). Not only the IMF content but the total fat content of the carcass is much higher than in commercial pigs. For example, backfat thickness in Iberian pigs commonly reaches 8cm (Mayoral *et al.*, 1999), while in commercial pigs strangely is above 2cm (Sather *et al.*, 1999).

The high fat content in muscles and meat products from Iberian pigs is explained by several factors. Iberian pigs are usually slaughter at high weights (around 160 kg), which implies that fat deposition takes place during a longer time than in industrial genotype pigs, usually slaughtered between 50-80 kg. In addition, restricted feeding in the first stages of fattening is deliberately used to increase fat deposition in the finishing phase where the growth of the tissue reaches the highest potential (Lawrie, 1998). Finally, Iberian pig is an anabolic and fast maturing breed with a high tendency to accumulate fat (López-Bote, 1998; Serra *et al.*, 1998). Therefore, by the time animals are being fattened (12-14 months old), most of the ingested calories are directed to fat synthesis.

The high intramuscular fat content of Iberian meat has several consequences on the technological properties of the meat for the dry-curing processing, and is also one of the main factors leading to the high sensory quality of the derived dry-cured products. Both, high levels of IMF and thick backfat, contributes to control moisture losses and the rate of sodium chloride diffusion during the initial steps in Iberian dry-cured ham processing, aimed to allow dehydration but also to permit salt distribution through the whole piece (Wood, 1966).

IMF content is closely related also to the juiciness of Iberian dry-cured products (Ruiz *et al.*, 2000). Intramuscular fat stimulates saliva secretion and

contributes directly to juiciness by coating the tongue, teeth and other parts of the mouth (Lawrie, 1998). Since processing of dry-cured products involves strong dehydration, the moisture from saliva and the direct contribution of IMF play a very important role in juiciness.

Together with juiciness, flavour intensity has been reported as the main factor influencing consumer's acceptability of Iberian dry-cured products (Ventanas *et al.*, 2001). IMF acts as a substrate for the generation of lipid-derived flavour compounds and influences, as well, the release of volatile compounds to the mouth, mainly retaining non-polar compounds (Seuvre *et al.*, 2000).

Concerning health implications, meat is perceived as the major dietary source of fat and especially of saturated fatty acids (SFA) in developed countries (Wood *et al.*, 2004). According to the recommendations of the World Health Organization (WHO, 2003) fat should provide between 15 and 30% of the calories in the diet and saturated fat should not provide more than 10% of these calories. Nowadays, consumers prefer low-fat meat products and meat consumption is recommended in moderation, thus during recent decades much efforts has been done in reducing fat content of carcasses in order to fulfil consumer's demands for leaner meat. Though the high IMF content in meat and meat products from Iberian pigs is not in agreement with the current tendencies of meat consumption, it is essential in order to assure a correct processing and to obtain dry-cured meat products with appropriate sensory attributes. However, the lipid content of Iberian cured products is in general lower than that of other meat products which are more frequently consumed, such as sausages (16-20%) (Piironen *et al.*, 2002) or hamburgers (12-17%) (Fernández, 2000).

Table 1: Percentages of IMF and fatty acids in muscles from different types of pigs.

%	Industrial genotype[1]	Corsican[2]	Light Iberian[3]	Heavy Iberian[4]
IMF	2.7	8.1	4.8	8.1
C16:0	23.8	24.9	24.3	23.3
C18:0	11.9	10.7	13.8	8.8
C18:1	39.6	51.7	43.7	54.7
C18:2	15.5	4.5	9.8	5.3
C18:3	0.4	0.3	0.4	0.4
C20:4	4.5	0.8	1.2	0.3

[1] Main feed during fattening: soya and corn oil; Intensively reared; m. *L. dorsi* (Hernández *et al*., 1998).
[2] Main feed during fattening: chesnuts; Free-range reared; m. *B. femoris* (Coutron-Gambotti *et al*., 1998)
[3] Main feed during fattening: cereals y soya; Free-range reared and slaughtered at 90 kg live weight; m. *L. dorsi* (Cava *et al*., 2003)
[4] Main feed during fattening: acorns and grass; Free-range reared and slaughtered at 150 kg; m. *B. femoris* (Tejeda *et al*., 2002)

Fatty acid composition

Fatty acid composition in porcine tissues is greatly influenced by the proportion and composition of lipids coming from the feed (direct deposition) and those produced endogenously (*de novo* synthesis) (Nawar, 1996; Gandemer, 1998). Traditional rearing system for Iberian pigs involved the free rearing during the fattening, with free availability of acorns and grass. Acorns show a high fat content (up to 7%) and high proportions of oleic acid (around 60-70%), while grass shows a high proportion of linolenic acid (Ruiz *et al*., 1998; Daza *et al*., 2005). Consequently, muscle lipids and back fat from Iberian pigs are rich in oleic acid (up to 55-60%), (Ruiz *et al*., 1998; Andrés *et al*., 2001) and show slightly higher proportions of n-3 fatty acids than pigs fed on mixed diets (Muriel *et al*., 2002). Iberian pigs fed on mixed diets show a different fatty acid profile depending on the feeding composition (Cava *et al*., 1997; Ruiz *et al*., 1998; Muriel *et al*., 2002). The effects of the intake of natural resources (grass and acorns) by free-range reared Iberian pigs on muscle lipids have been deliberately imitated through the development of mixed diets with high levels of oleic acid and lower n-6/n-3

ratios in order to improve the fatty acid composition of porcine tissues and meat products (Muriel *et al.*, 2004a).

The particular fatty acid profile of tissue lipids from Iberian pigs reared outdoors largely affects technological, sensory and nutritional quality of meat, since it determines the physical state of the fat (liquid or solid), its prone to get oxidized, and the nutritional and metabolic effects on the consumer. Fat consistency, which depends on the fatty acid profile, strongly influences the appearance, the feasibility for manipulation and the dehydration of meat products (Ruiz *et al.*, 2000).

The fatty acid composition of muscle foods largely influences their oxidative stability since the susceptibility of fatty acids to undergo oxidative degradation increases with higher number of double bonds, with the polyunsaturated fatty acids (PUFA), which are considerably more unstable than monounsaturated fatty acids (MUFA) or SFA.

Though deep oxidation of unsaturated lipids may lead to rancid flavour in Iberian meat products, several compounds from lipid oxidation and from interaction between amino compounds and compounds from lipid oxidation play a key role in dry-cured products' flavour (Andrés *et al.*, 2001; Carrapiso *et al.*, 2002). Since the fatty acid profile of the raw material highly influences the profile of volatile flavour compounds of processed meats, the high levels of oleic acid in tissue lipids of Iberian pigs, appears as a very interesting approach for reducing the amount of compounds showing rancid notes (those coming from PUFA), and at the same time, increasing those, such as octanal and nonanal, mainly originated from the autoxidation of oleic acid which show pleasant flavour, or at least not so intense rancid aroma notes (Ruiz *et al.*, 1999; Martín *et al.*, 2000).

Finally, fatty acid composition plays an important role on the diet-health relationship, since each dietary fatty acid affects the plasmatic lipids levels differently and has different effects on atherogenic and trombogenic processes**.** SFA increase blood total-, low-density lipoprotein (LDL)-cholesterol and the high density lipoprotein HDL/LDL ratio, which imply a risk factor for cardiovascular diseases. Myristic (C14:0) and palmitic (C16:0) fatty acids,

commonly found in pork are the main SFA behind the cholesterol elevating effect (Mattson & Grundy, 1985). The presence of MUFA and PUFA in the diet reduces the level of plasma LDL-cholesterol, although PUFA also depress the HDL-cholesterol (Mattson & Grundy, 1985), which has a positive effect in preventing from cardiovascular diseases. The intake of MUFA has been inversely associated with the risk of cardiovascular heart disease, although the correlation is weaker than for PUFA (Hu *et al.*, 1997). In accordance to recommendations (WHO, 2003) the ratio PUFA/SFA should be above 0.4. Meat and dry-cured products from Iberian pigs contains high levels of MUFA and particularly of oleic (C18:1) acid since it is presented in acorns, as aforementioned. Traditionally, in Mediterranean countries, the sources of MUFA in the diet have been vegetable origin fats such as olive oil which contains 78% oleic acid. Meat and meat products from Iberian pigs might be an alternative source of MUFA, which have a positive effect in order to prevent from coronary diseases. On the other hand, numerous strategies for achieving healthier meat and meat products involve replacing part of the animal fat with another more suited to humans needs, i.e. with less SFA and more MUFA (oleic acid) or PUFA (Jímenez-Colmenero *et al.*, 2001). It has been proved that the substitution of saturated by unsaturated fat is more effective in the decrease of risk of cardiovascular disease than only reduction of total fat intake (Hu *et al.*, 2001). Consequently, fat from Iberian pigs might be a healthy fat source due to his optimal fatty acids composition especially taking into account the high levels of oleic acid (50-58% in subcutaneous fat) (Flores *et al.*, 1988; Ruiz *et al.*, 1998). Currently, nutritionists have focussed on the type of PUFA and the balance in the diet between n-3 and n-6 fatty acids. α-linolenic acid (C18:3) can be elongated in porcine tissues to generate long chain n-3 PUFA, which have been found to improve the status of cardiovascular system, by reducing platelet aggregation and serum triglycerides and cholesterol levels, and also regulate the immune response control (*revised by* Wood & Enser, 1997). On the other hand, a higher proportion of long chain n-6 PUFA derived of linolenic acid (C18:2) results in a pro-inflammatory status. Therefore an inappropriate ratio n-6/n-3 is a risk

factor in cancers and coronary heart disease (Enser, 2001), so it is recommended maintained this ratio below 4 (Wood & Enser, 1997; WHO, 2003). Mainly due to the intake of grass with high level of C18:3 (Ruiz *et al*., 1998), Iberian pig tissues contain relatively low n-6/n-3 ratios (Muriel *et al*., 2002).

Myoglobin and iron contents

Meat from Iberian pigs has been considered an excellent source of high available iron for humans (Cava *et al*., 2003; Estévez *et al*., 2003a). Several studies have reported higher concentrations of heme pigments and total iron in muscles from Iberian pigs compared to those from commercial genotype pigs (Serra *et al*., 1998; Estévez *et al*., 2003a). Accordingly, Forero (2003) described higher levels of iron in Iberian dry-cured hams (32 µg iron/g) than in those elaborated with raw material from selected pig breeds (22 µg iron/g). In fact, muscles from Iberian pigs contain similar iron levels than those from other animal species such as beef or ostrich meat, habitually considered the best sources of iron for humans as far as muscle foods is concerned (Lombardi-Boccia *et al*., 2002). The high levels of heme pigments and iron in muscles from Iberian pigs is mainly explained by the peculiar genetic characteristics of the non-selected rustic pig breeds. It is known that muscles from commercial pig breeds selected for fast growth contain higher content of fast fibres (glycolytic IIB) than muscles from rustic breeds (Weiler *et al*., 1995) which affects muscle heme pigments concentration and therefore, iron levels. In fact, Andrés *et al*. (2000) reported higher proportion of oxidative fibres in muscles from Iberian pigs (around 48%) than those found in other works studying muscles from selected pig breeds such as Large-White, Landrace or Yorkshire in which the proportion of such oxidative fibres did not exceed 31% in the same muscles (Bellati *et al*., 1996). Accordingly, Ruusunen & Puolanne (2004) and Lindahl *et al*. (2001) reported higher pigment concentrations in muscles from rustic pig breeds or wild pigs than in those from commercial genotype pigs. Some other extrinsic factors associated to the traditional procedures of Iberian pig's rearing system affect myoblogin

and iron contents in muscles. In order to obtain heavy and fatty carcasses, Iberian pigs are traditionally slaughtered with 12-14 months of age whereas considerably shorter times are used for commercial genotype pigs (around 5 months). The concentrations of myoglobin and iron in muscles are known to increase with age (Lawrie, 1998). In fact, Mayoral *et al*. (1999) observed increasing amounts of myoglobin in muscles from Iberian pigs throughout the animal lifetime. In addition, Pearson (1990) described a positive impact of physical exercise on myoglobin content in muscles through the increase of the proportion of oxidative fibres (Petersen *et al*., 1997). Dworschak *et al*. (1995) reported higher amount of iron in muscles from free-range reared pigs than in those from intensively reared pigs. The positive effect of physical exercise on myoglobin and iron contents in muscles from Iberian pigs has been suggested in several papers (Mayoral *et al*., 1999; Andrés *et al*., 2000, Estévez *et al*., 2003a).

Iron is considered a potent promoter of oxidative reactions in muscle foods (Kanner, 1994) and therefore, the presence of high iron levels in muscles from Iberian pigs could enhance their oxidative instability. In fact, Estévez *et al*. (2003b) suggested the prooxidant role played by iron in refrigerated stored loin muscles and found a significant correlation between heme iron content and TBA-RS numbers. Consequently, the high levels of iron in muscles from Iberian pigs could affect their oxidative stability. However, the accurate knowledge of the proportion between the chemical forms of iron is of great interest since non-heme iron is thought to have more ability to promote oxidative reactions than heme iron (Kanner, 1994). Considering that particular point, there is no information available concerning the proportion of heme and non-heme iron in muscles and products from Iberian pigs and therefore, there is a lack of knowledge on the impact of the different pools of iron on the oxidative stability in muscle foods from Iberian pigs.

From a sensory point of view, the high concentrations of myoglobin and iron in muscles from Iberian pigs have a direct impact on their colour traits due to the relationship established between those parameters (Warris *et al*., 1990). The redness (a*-values) described in muscles from Iberian pigs (Cava *et al*.,

2003; Muriel *et al*., 2004b) are considerably higher than those reported in the same muscles from commercial genotype pigs (Leseigneur-Meynier & Gandemer, 1991). Comparing the colour characteristics of fresh loin chops from Iberian and commercial genotype pigs, Estévez *et al*. (2003a) reported that muscles from Iberian pigs displayed a more intense colour with low hue values than those from commercial pigs. The colour standards displayed by meat from Iberian pigs are preferred by consumers who appreciate intense red colours in fresh pig meat (Brewer *et al*., 1998). In fact, Ruiz (1996) & Cava (1997) found positive and significant correlations between instrumentally measured redness in Iberian dry-cured hams and panellist's acceptability.

Antioxidants

The influence of the traditional feeding system for Iberian pigs on the chemical composition and oxidative stability of their tissues has been profusely studied concluding that the high quality of Iberian pigs' products can be mainly attributed to this feeding regime (Ruiz *et al*., 1998; Cava *et al*., 2000; López-Bote & Rey, 2001). Acorns provide high levels of MUFA (mainly oleic acid) and γ-tocopherol to Iberian pigs whereas the grass is a recognised source of ω-3 fatty acids (mainly linolenic acid) and α-tocopherol (Ruiz *et al*., 1998; Cava *et al*., 2000; López-Bote & Rey, 2001). According to Cava *et al*. (2000) and Daza *et al*. (2005) meat from Iberian pigs fed on natural resources (grass and acorns) contained similar or even higher tocopherol levels than those fed with diets supplemented with α-tocopherol up to 200 mg/Kg. Furthermore, the presence of γ-tocopherol is almost restricted in tissues from pigs fed with acorns, being detected in tissues from animals fed on α-tocopherol-supplemented diets at considerably lower levels (Rey *et al*., 1998; Daza *et al*., 2005).

Recent studies have considered the possibility that some other substances accumulated in tissues from Iberian pigs as a consequence of the intake of natural resources could also contribute to enhance their oxidative stability. Gonzalez *et al*., (2004) have recently reported significantly higher amounts of

total phenolic compounds in adipose tissue from Iberian pigs fed exclusively on natural resources (grass and acorns) than those fed with a mixed diet (9.11 vs 6.74 mg caffeic acid equivalents kg^{-1}). These authors suggested that the high oxidative stability attributed to Iberian pigs products could be explained not only by the presence of tocopherols but also by the likely protective role of phenolic compounds. Consistently, Cantos *et al*. (2003) have recently reported elevated polyphenol levels in acorns which could explain the results obtained by Gonzalez *et al*., (2004). So far, the enhancement of the oxidative stability of tissues from pigs fed outdoors with access to pasture has been exclusively referred to the increase of tocopherols levels. Therefore, if this data were supported by further results, the presence of plant phenolics in porcine tissues as a consequence of the intake of grass and other natural materials would have been described for the first time in Iberian pigs though further studies to support this hypothesis would be of interest.

II. OXIDATION AND ANTIOXIDANT STRATEGIES

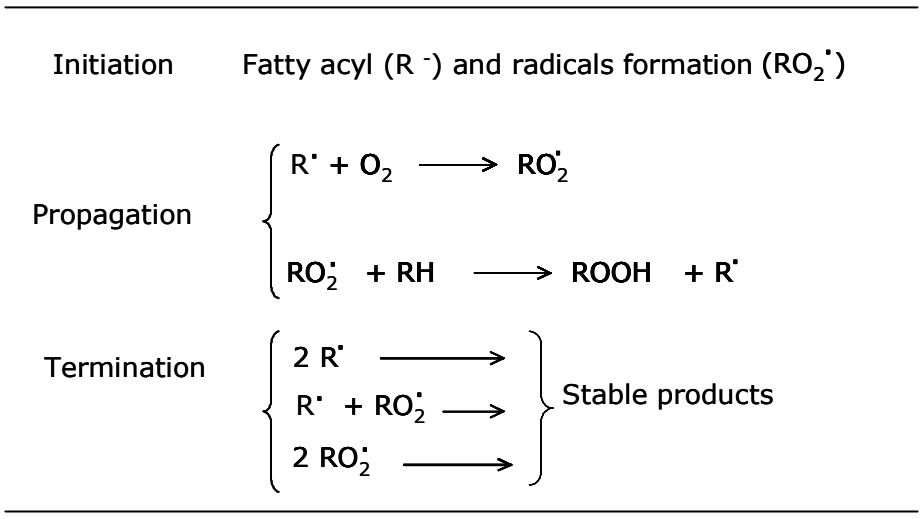
II.A. Lipid oxidation

Regardless of microbial spoilage, lipid oxidation is the main factor reducing the quality of meat and meat products (Morrissey *et al.*, 1998). Though lipid oxidative reactions contribute to certain desirable quality attributes such as the development of a pleasant flavours in cooked meats (Kanner, 1994), the overall effect of lipid oxidation is negative leading to adverse effects on sensory traits, nutritional value and healthiness of muscle foods (Gray *et al.*, 1996; Morrissey *et al.*, 1998).

Mechanism of lipid oxidation

The overall mechanism of fatty acid oxidation is generally a free radicals process including initiation, propagation and termination stages (Frankel, 1984). The first step of lipid oxidation involves the removal of a hydrogen atom from a methylene carbon in a fatty acid (RH) to generate fatty acyl ($R^{\cdot}$) and peroxyl radicals ($RO_2^{\cdot}$). The initiation step needs to be catalysed and the role of iron in promoting the generation of species capable to abstract a hydrogen atom from an unsaturated fatty acid has been profusely described (Kanner, 1994; Gray *et al.*, 1996). The radicals previously generated propagate the chain reaction to other fatty acids which are consequently oxidised. The reaction between fatty acids and peroxyl radicals lead to the formation of new peroxyl radicals and lipid hydroperoxides (ROOH) (Morrissey *et al.*, 1998). Radicals and hydroperoxides (primary lipid oxidation products) are finally decomposed to generate stable molecules with small molecular weight (secondary lipid oxidation products) such as hydrocarbons, aldehydes, ketones, acids, esters, lactones and a large variety of nitrogen and sulphur containing compounds (Frankel, 1984; Morrissey *et al.*, 1998).

Mechanism of lipid oxidation (Frankel, 1984).



As far as muscle foods contain unsaturated fatty acids and prooxidant components are prone to suffer oxidative reactions. Between the muscle lipid fractions, the higher sensitivity of the polar lipid fraction to oxidation is mainly explained by the facts that polar lipids from cellular membranes contain a higher proportion of unsaturated fatty acids and are, additionally, in close relationship with oxidation promoters located in the aqueous phase of the muscular cell (Gandemer, 1998). As opposed to the neutral lipid fraction, the polar fraction is primarily responsible for lipid oxidation in muscle foods (Igene *et al.*, 1980). Though muscle tissues have endogenous antioxidant mechanisms to control lipid oxidation *in vivo* such as antioxidant enzymes and lipid-soluble tocopherols and β-carotenes (Sies, 1986; Chan & Decker, 1994), their effectiveness is largely diminished with increasing time *post-mortem*. In fact, the third phase of lipid oxidation in muscle foods frequently occurs during handling, processing, storage and cooking processes.

Factors influencing lipid oxidation

As partially described above, there are several aspects affecting the occurrence and intensity of lipid oxidative reactions in muscle foods. The amount and characteristics of the muscle lipids and the presence of

prooxidant (i.e. iron, sodium chloride) and antioxidant (i.e. tocopherols, sodium ascorbate and nitrite) factors are known to be largely influential (Kanner, 1994; Gray *et al*., 1996; Morrissey *et al*., 1998).

The amount and composition of muscle lipids largely determine the oxidative stability of a muscle food. Jo *et al*. (1999), Sasaki *et al*. (2001) and Estévez *et al*. (2003b) reported significant positive correlations between fat content and lipid oxidation suggesting that the higher amount of total lipids, the higher substrate to undergo oxidative reactions. The sensitivity of fatty acids to oxidative reactions increases with the number of double bounds and therefore, PUFA are more prone to be oxidised than MUFA or SFA (Frankel, 1984; Morrissey *et al*., 1998). High levels of PUFA in muscle foods have been previously associated with high oxidative instability during meat cooking and subsequent storage (Bloukas & Paneras, 1993; Jeun-Horng *et al*., 2002; Estévez *et al*., 2004; Cortinas *et al*., 2005). As described above, amongst the meat components, the role of iron and heme pigments in the promotion of lipid peroxidation has been well established (Schaich, 1992, Kanner, 1994). Iron, free and protein bound, heme and non-heme, oxidised or reduced has the ability to promote the oxidation of unsaturated fatty acids in meat but the relative contribution of each chemical form has not been assigned (Gray *et al*., 1996). According to Schaich (1992), the mechanisms by which iron could promote the initiation of lipid oxidative reactions can be classified in three types of reactions: i) a direct initiation by higher valence state iron (Fe^{3+}) or by reactive oxygen species (ROS) produced by a metal autoxidation process, ii) an indirect initiation by hypervalent iron complexes such as those in heme protein and porphyrin compounds and iii) an indirect initiation-propagation of lipid oxidation through the decomposition of preformed hydroperoxides into peroxyl radicals.

In addition to the intrinsic pro- and antioxidant components of the muscle itself, a number of extrinsic factors influence lipid oxidation in muscle foods. The manufacture of meat products involves the addition of non-meat ingredients and the application of technological processes that can modify the oxidation status of the muscle foods.

Meat cooking enhances the development of oxidative reactions since the reaction between molecular oxygen and muscle lipids increases with increasing temperatures (Nawar, 1996). In fact, cooking process leads to a dramatic increase in lipid oxidation in muscle foods and the development of the 'warmed-over' flavour of refrigerated cooked meats (Tim & Watts, 1958). The acceleration of lipid oxidation following cooking has been attributed to heat-induced changes in muscle components including disruption of cellular compartmentalisation and exposure of membranal lipids to a prooxidative environment, thermal activation or release of catalytic free iron from myoglobin (Kristensen & Andersen, 1997) and thermal inactivation of antioxidant enzymes (Lee *et al*., 1996). The intensity of lipid oxidation is dependent on the cooking temperature since the formation of Maillard reaction products with antioxidant activity at temperatures above 100ºC, would inhibit the development of oxidative reactions to some extent (Gray & Pearson, 1987).

The manufacture of meat products includes a number of technological processes such as cutting, mincing or flaking which involves some physical disruption which leads to the exposure of muscle lipids to the prooxidative environment (Monahan, 2000). The vacuum packaging and modified atmosphere packaging (i.e., 70% N_2 : 30% CO_2) are, on the contrary, successful strategies to minimise lipid oxidation in raw and cooked meats (Ahn *et al*., 1992, Kingston *et al*., 1998).

The use of certain additives enhances the susceptibility of muscle lipids to oxidative reactions. Sodium chloride is commonly used in meat products to reduce water activity and inhibit microbial spoilage and contributes, as well, to saltiness. Sodium chloride can promote lipid oxidation possibly through displacement of iron from heme proteins (Kanner *et al*., 1991). On the other hand, sodium nitrite exhibit antioxidant effect in cured meats and several mechanisms have been proposed including: i) the formation of stable complex with heme pigments, ii) the chelation of free iron released from heme pigments following heating or iii) stabilisation of unsaturated fatty acids (Freybler *et al*., 1993). Phosphates are widely used in comminuted cooked

meat products to increase water binding capacity and also act as antioxidants through metal chelation (Tim & Watts, 1958).

Other antioxidant strategies are also deliberately used to inhibit the adverse effect of lipid oxidation in muscle foods including the modification of the muscle lipid characteristics through dietary means and the direct addition of synthetic and natural antioxidants. These points will be stated below.

Adverse effects of lipid oxidation

The deterioration of certain quality traits in muscle foods including flavour and colour changes, loss of nutritional value and safety risks, have been precisely attributed to lipid oxidation (Pearson *et al.*, 1983; Gray & Pearson, 1987; Kanner, 1994).

Flavour problems associated to lipid oxidation are particularly pronounced in cooked meats, particularly if these meats are reheated (Lyon & Ang, 1990). The increase of off-flavour notes during refrigerated storage of cooked meat is now called 'meat flavour deterioration' (St. Angelo, 1996; Monahan, 2000) in substitution of the term 'warmed-over' flavour which has been traditionally used (Tim & Watts, 1958). The effects on meat flavour are caused by secondary lipid oxidation products since lipid peroxides are colourless and tasteless (Mottram, 1998). Some of these compounds impart rancid, fatty, pungent and other off-flavour characteristics to muscle foods (Chang & Peterson, 1977). Among them, the most important aroma compounds are aldehydes and several unsaturated ketones and furan derivatives including C3-C10 aldehydes, C5-C8 unsaturated ketones and pentyl or pentenyl furans (Shahidi, 1994; Mottram, 1998). On the other hand, saturated and unsaturated aldehydes have been extensively used as markers for lipid oxidation in food (Shahidi & Pegg, 1993). Some of them, such as 2,4-decadienal or hexanal impart rancid or pungent flavours, which are believed to be negative for the overall flavour of meat products (Shahidi, 1994; Mottram, 1998; Chevance & Farmer, 1999; Carrapiso *et al.*, 2002). Some other compounds from fatty acid oxidation could positively affect the flavour of meat products. For example, 1-octen-3-ol, an unsaturated alcohol that

could be generated from autoxidation of either linoleic or arachidonic acids, shows an intense mushroom odour (Chevance & Farmer, 1999; Carrapiso *et al*., 2002). Lactones, which may also have a lipid oxidation origin, show very interesting aromatic notes and very low detection thresholds (Slaughter, 1999). Hept-(*E*)-4-enal, which has been highlighted as a contributor to off-flavour in pâté, imparting metallic notes, is also a compound from the autoxidation of linoleic acid or arachidonic acids (Im *et al*., 2004).

Oxidation of the oxymyoglobin muscle pigment to metmyoglobin leads to the discolouration of red meats. There is a clear relationship between pigment and lipid oxidation since heme oxidation generates hypervalent iron complexes which promote unsaturated fatty acids oxidation (Kanner & Harel, 1985). On the other hand, radicals and hydroperoxides from lipid decomposition can catalyse heme pigment oxidation (Ying & Faustman, 1993). In fact, both lipid and heme pigment oxidation are affected by similar pro- and antioxidant factors. Lipid soluble tocopherols inhibit oxymyoglobin oxidation as well as oxidative decomposition of PUFA (Faustman *et al*., 1989; Yin *et al*., 1993). Discolouration of cooked meats during storage has been associated to the degradation of nitrosopigments caused by oxidative processes, though no precise mechanisms were reported (Carballo *et al*., 1991; Perlo *et al*., 1995; Jo *et al*., 2000; Fernández-Ginés *et al*., 2003). Some other authors linked the discolouration of cooked products with lipid oxidation (Akamittath *et al*., 1990; Jo *et al*., 1999). It is reasonable that the colour changes in cooked products are caused by oxidative reactions since the addition of substances with proven antioxidant activity inhibit to some extent the discolouration of frankfurters and other meat products (Sebranek *et al*., 2005). On the other hand, the release of the iron from heme pigments following meat cooking and storage has been related to the development of lipid oxidative processes since the increase of non-heme iron in cooked meats was reported to occur at the expense of heme iron when the heme protein was exposed to lipid hydroperoxides (Kristensen & Andersen, 1997). These chemical changes in the heme group could have a reflection of muscle food colour though this extent has never been proved.

High importance has been given to lipid oxidation processes in relation to their health implications. Particularly interesting is the intake of oxidised lipids and cholesterol oxidation products (COPs) since those have been associated to the development of coronary heart diseases and cancer (Sevanian & Peterson, 1984; Hubbard *et al*., 1989). The evidence of the protective role of dietary antioxidants against the onset of certain pathological conditions in humans supports that statement (Jialal & Fuller, 1996). Certain COPs such as 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol have been found in several meat products such as dry-cured ham, salami, mortadela and processed turkey meat products (Novelli *et al*., 1998; Zanardi *et al*., 2000; Petrón *et al*., 2003; Baggio *et al*., 2005).

II.B. Protein oxidation

The major concerns regarding the occurrence of oxidative processes in muscle foods are related to the adverse effect of those on certain quality traits. Whereas the undesirable oxidative changes in muscle foods have been extensively studied, the precise mechanisms of alteration have not been accurately identified (Xiong, 2000). For instance, colour, flavour and texture changes during refrigerated storage of meat coincide with the development of lipid oxidation and in many cases, the oxidation of unsaturated fatty acids have been highlighted as the main cause of these adverse changes. Some other changes in muscles such as those related to the loss of texture-forming ability and water holding capacity have been associated to a loss of protein functionality though no exact mechanism has been elucidated yet (Xiong, 2000).

Though recent studies of protein oxidation in biomedical sciences have shed light on the mechanisms by which extracellular and membrane proteins can be affected by ROS leading to adverse biological effects (Butterfield & Stadtman, 1997; Chevion *et al*., 2000; Stadtman, 2001), hardly any work devoted to the study of protein oxidation in muscle foods has been carried out. Recent studies on model and food systems have pointed out that the oxidative damage of proteins leads to alterations in gelation, emulsification,

viscosity, solubility and hydratation (Wang *et al.*, 1997; Wang & Xiong, 1998). Little is known, however, about protein oxidation in muscle foods concerning the precise chemical mechanism of protein oxidation, the characterisation of the protein oxidation products, the adverse effects on meat quality and the effectiveness of different antioxidant strategies against protein oxidation.

Mechanism of protein oxidation

In muscle foods, the occurrence of protein oxidation can be linked to any of the prooxidant factors traditionally associated to lipid oxidation (Xiong, 2000). Therefore, proteins can be oxidised by similar oxidation promoters capable to oxidise unsaturated fatty acids such as heme pigments, transition metal ions and various oxidative enzymes (Xiong, 2000). In fact, in the presence of oxidising lipids, protein oxidation is manifested by free radical chain reactions similar to those for lipid oxidation, including initiation, propagation and termination stages (Gardner, 1979; Schaich, 1980). Theoretically, all aminoacids are susceptible to be oxidised by free radicals and non-radical ROS but, actually, some of them are particularly vulnerable, with the cysteine and other aminoacids with reactive side chains (sulphydryl, thioether, amino group, imidazole or indole ring) being the most susceptible to undergo oxidative reactions (Gardner, 1979; Xiong, 2000).

The attack of reactive oxygen species (ROS) on muscle proteins leads to the loss of sulphydryl groups and the generation of carbonyl compounds (Xiong, 2000). The generation of carbonyls (aldehydes and ketones) has been highlighted as one of the most relevant changes in oxidised proteins (Xiong, 2000). In fact, measuring carbonyl concentration is an accurate and easy method for assessing protein oxidation (Levine *et al.*, 1990). Protein carbonyls can be generated via four possible pathways: (i) direct oxidation of amino acid side chains, (ii) fragmentation of the peptide backbone, (iii) reactions with reducing sugars and (iV) binding non-protein carbonyl compounds (Xiong, 2000). Nevertheless, the deamination reaction is considered the commonest way of protein carbonyls formation (Xiong & Decker, 1995).

Mechanism of protein oxidation (RH: fatty acid; P: Protein) (adapted from Xiong, 2000).

Initiation	$RH \longrightarrow R^{\bullet}$
Propagation	$R^{\bullet} + O_2 \longrightarrow RO_2^{\bullet}$
Hydrogen abstraction	$RO_2^{\bullet} + P \longrightarrow ROOH + P^{\bullet}(-H)$
Addition	$RO_2^{\bullet} + P \longrightarrow ROOP^{\bullet}$
Complex	$ROOP^{\bullet} + P + O_2 \longrightarrow RO_2PO_2P^{\bullet}$
Polymerization	$P\text{-}P^{\bullet} + P^{\bullet} + P \longrightarrow P\text{-}P\text{-}P^{\bullet} + P\text{-}P\text{-}P$

Some studies have demonstrated that ROS can cause meat protein polymerisation by formation of protein-protein cross-linked derivatives by the following mechanisms: i) by the oxidation of cysteine sulphydryl groups to form disulphide linkages, ii) by the complexing of two oxidised tyrosine residues, iii) by the interactions of an aldehydes group in one protein with the amine group of a lysine residue in another protein, iV) by the crosslinking of two amine groups (lysine residues) in two different proteins through a dialdehyde (i.e. malondialdehyde) and V) by the condensation of protein free radicals (Pokorny *et al.*, 1990; Howell *et al.*, 2001; Srinivisan & Xiong, 1997). Finally, peptide scission can take place concurrent with formation of polymers. According to Stadtman & Berlett (1997) free radicals can abstract a hydrogen atom from the α-carbon of a polypeptide backbone leading to the generation of new radicals which finally undergo the peptide bond scission. The protein degradation products include those that contain the reactive carbonyl groups described above.

Adverse effects of protein oxidation

Several studies have demonstrated that meat protein polymerisation and degradation leads to a decrease in protein solubility and functionality in model systems (Pokorny *et al*., 1990; Howell *et al*., 2001). In meat and meat products, the effects of protein oxidation on specific quality traits have been poorly studied. The most important functional properties of proteins in muscle foods include those that contribute to the textural characteristics and structural properties of cooked products. Thus, gelation and meat particle binding (which result from protein-protein interactions and protein matrix-water interactions), emulsification (which is dictated by protein-lipid interactions), and hydratation and or water-binding (which is regulated by protein-water interactions) are all important functionalities in muscle foods. Factors that affect these functional properties, including oxidation, could be responsible for the deterioration of certain quality attributes in cooked products. In fact, Fernández-Ginés *et al*. (2003) and Fernández-López *et al*. (2004) described a hardness increase during refrigerated storage of frankfurters and other food emulsions and associated this change to the process of emulsion destabilization due to water and fat separation from the protein matrix. Those authors did not consider the possibility that the loss of protein functionality could be caused by an oxidative damage of muscle proteins during refrigeration. Recently, Rowe *et al*., (2004) have suggested the influence of post-mortem protein oxidation on certain quality deterioration associated to texture changes in refrigerated beef.

The oxidation of meat pigments has been traditionally referred to the chemical states of iron and the effect of the oxidative reactions on meat discolouration has been profusely described (Faustman & Cassens, 1990; Ledward, 1992; Renerre, 2000). Little attention has been paid to the oxidative damage of the myoglobin itself, though it could lead to changes in its structure and therefore to colour changes. Carballo *et al*. (1991), Perlo *et al*. (1995), Jo *et al*. (2000) and Fernández-Ginés *et al*. (2003) reported that colour deterioration during refrigerated storage of cooked meats is explained

by the degradation of certain nitrosopigments caused by oxidative processes, though no precise mechanisms concerning protein oxidation were reported.
Finally, oxidative changes in muscle proteins could affect the nutritional value of meat and meat products. Aminoacid destruction would constitute a significant nutritional impact on meat products. In addition, free radical-initiated protein denaturation usually results in enhanced proteolytic susceptibility due to protein unfolding and the increased accessibility of peptide bonds to proteases (Meucci *et al.*, 1991; Agarwal & Sohal, 1994). On the other hand, the influence of oxidation on the digestibility of muscle protein depends on the specific conditions under which proteins are physically and chemically modified, as well as on the conditions in which proteins are digested. Muscle proteins oxidised with relatively mild conditions (i.e. do not promote excessive cross-linking or formation of insoluble aggregates) are more easily digested (Liu & Xiong, 1997).

II.C. Antioxidant strategies

Though the positive effect of lipid oxidation on the development of desirable aroma characteristics in cooked meat (Elmore *et al.*, 1999; Estévez *et al.*, 2003c), and dry-cured products (Ruiz *et al.*, 1999; Carrapiso *et al.*, 2002) has been reported, the development of oxidative reactions in muscle foods has been generally associated with loss of quality including aroma, colour and texture deterioration and generation of toxic compounds (Morrissey *et al.*, 1998; Gandemer, 1998). Therefore, the use of antioxidants in muscle foods has been conventionally performed in order to enhance the oxidative stability of porcine tissues, reduce the development of oxidative reactions during processing of meat products and minimise the adverse effects on meat quality. According to Ingold (1968), antioxidants can be classified into two groups: primary or chain breaking antioxidants, which can react with lipid radicals to convert them into more stable products, and secondary or preventive antioxidants which reduce the rate of chain initiation by a variety of mechanisms. Most of primary antioxidants act as donators of hydrogen atoms to lipid radicals according with the reaction:

$$RO^{\bullet} + AH \rightleftharpoons ROH + A^{\bullet}$$

In addition to the hydrogen donation, the radical derived from the antioxidant must be more stable than the lipid radical, or is converted to other stable products (Gordon, 1990). The presence of aromatic rings in most primary antioxidants allows the delocalisation of the unpaired electron leading to several valence bond isomers of considerably stability (Gordon, 1990). A large variety of synthetic and natural compounds act as primary antioxidants such as gallates, tocopherols, carotenes, quinones and other flavonoids and phenolic compounds.

Secondary antioxidants operate by a variety of mechanisms including metal binding, oxygen scavenging, hydroperoxides decomposition, UV radiation absorption and singlet oxygen deactivation. Citric acid, ethylenediaminetetetraacetic acid (EDTA) and phosphoric acid derivatives may extend the shelf life of lipid foods to a great extent by their metal chelating properties (Gordon, 1990). Other compounds such as ascorbic acid or sodium erythorbate act as oxygen scavengers and reducing agents. The ascorbic acid is particularly effective in combination with primary antioxidants such as tocopherols since it is able to regenerate the tocopherol reducing the tocopheroxyl radical (Nawar, 1996). Some primary antioxidants can also exhibit antioxidant activity using mechanisms of secondary antioxidants. For instance, certain flavonoids are considered potent free-radical scavengers and in addition, are able to bind metals reducing their prooxidant activity (Rice-Evans *et al.*, 1997).

The use of antioxidants to inhibit the adverse effect of oxidative reactions in muscle foods can be approached from different strategies. The enhancement of the oxidative stability of porcine tissues through dietary means and the direct addition of antioxidants in muscle foods are the commonest.

II.C.1. Dietary means

The improvement of the oxidative stability of porcine meat products through dietary means involves not only the supplementation with substances with proven antioxidant activity but also the modification of the fatty acid composition of the tissues, commonly focussed in reducing PUFA percentages (Ruiz & López-Bote, 2002). In order to achieve this purpose, pigs are fed on diets with high MUFA/PUFA ratios and supplemented with α-tocopherol at levels between 100 and 200 mg kg^{-1}. (Monahan *et al.*, 1990; Jensen *et al.*, 1997; Rey *et al.*, 2004 ; Mason *et al.*, 2005). The dietary supplementation with α-tocopherol has been successfully used as well in other animals such as poultries (Nam *et al.*, 1997; Botsoglou *et al.*, 2003a), turkeys (Botsoglou *et al.*, 2003b), lambs (Lauzurica *et al.*, *in press*), calves (Yang *et al.*, 2002; Descalzo *et al.*, 2005) and rabbits (Corino *et al.*, 1999; Lo Fiego *et al.*, 2004). The supplementation with supranutritional levels of α-tocopherol leads to higher levels of such compound in muscles and other tissues since the deposition of α-tocopherol in porcine tissues is dependent on the concentration of that compound in the feed (Ruiz & López-Bote, 2002). Particularly interesting is the fact that α-tocopherol is a lipid soluble compound which is thought to be accumulated in cellular membranes where the initiation of oxidative processes in muscle foods takes place (Ruiz & López-Bote, 2002). α-Tocopherol inhibits the free radical oxidation by reacting with peroxyl radicals to stop the chain propagation and prevent, in addition, from the hydroperoxides decomposition decreasing the generation of aldehydes and other lipid oxidation products (Frankel, 1996). The slowness of reaction between the tocopheroxyl radical and oxygen is one of the main reasons explaining why tocopherols appear to be the nature's major lipid soluble chain breaking antioxidant (Burton *et al.*, 1998). In addition, the tocopheroxyl radical could react with other peroxyl radicals yielding stable products (Frankel, 1996). Though α-tocopherol is principally added in mixed diets with antioxidant purposes, some other substances have been tested such as β-carotene with or without tocopherols (Yang *et al.*, 2002; Lo Fiego *et al.*, 2004), catechins (Mason *et al.*, 2005) and plant essential oils (Botsuglou

et al., 2002; Botsuglou *et al*., 2003b). The beneficial effects of dietary α-tocopherol supplementation on meat quality include lower lipid oxidation, lower generation of toxic compounds such as COPs or nitrosamines, enhanced colour stability and lower drip loss during storage (Monahan *et al*., 1990; Ashgar *et al*., 1991; Faustman *et al*., 1998; Rey *et al*., 2001). Nevertheless, the effect of antioxidant strategies (i.e. supplementation with antioxidants in animal diets) on the quality and functionality of muscle proteins has been poorly studied. Mercier *et al*. (1998) reported decreased protein carbonyls formation in muscle from turkey fed vitamin E.

The traditional procedures used for Iberian pig's feeding is in absolute agreement with the current strategies carried out in order to enhance the oxidative stability of porcine muscles. The intake of acorns (with high levels of MUFA and γ-tocopherol) and pasture (rich in α-tocopherol) decisively influences on the chemical composition and oxidative stability of Iberian pig's tissues. In fact, Iberian pigs reared in confinement are fed using MUFA-enriched diets with α-tocopherol supplementation up to 200 mg/Kg in order to imitate the effects of the traditional free range feeding system (Ruiz & López-Bote, 2002). The use of such mixed diets for feeding Iberian pigs rapidly spread out as a result of the publication of papers in which MUFA and tocopherols were recognised as the major enhancers of oxidative stability in tissues and meat products from Iberian pigs (Rey *et al*., 1997; Rey *et al*., 1998; Ruiz *et al*., 1998; Cava *et al*., 2000; Isabel *et al*., 2003; Daza *et al*., 2005). The protective role of tocopherols against lipid oxidation in dry-cured Iberian ham is manifested by a reduction of the generation of lipid oxidation products during ripening and improving some particular sensory characteristics such as flavour and odour intensity (Cava *et al*., 1999). The persistence of relatively high tocopherols levels in cellular membranes after Iberian ham processing allows the protection against lipid oxidation beyond the ripening stage: ham slices from supplement pigs showed lower discolouration and lower weight losses than those from pigs fed on control diets when stored imitating the conditions of marketing display (Isabel *et al*., 1999).

Table 2. α- and γ- tocopherol contents and oxidation parameters in m. *Longissimus dorsi* from Iberian pigs with different feeding background (Adapted from Daza *et al.*, 2005).

	α-tocopherol[1]	γ-tocopherol[1]	Iron-induced oxidation[2]	Hexanal[3]
Control[4]	2.57^{b}	0.13^{b}	4.93^{a}	$4.21^{a} \times 10^{3}$
Supplemented[5]	4.79^{a}	0.15^{b}	4.02^{a}	$2.24^{b} \times 10^{3}$
Free-range[6]	4.58^{a}	0.38^{a}	2.57^{b}	$2.46^{b} \times 10^{3}$

[1] mg Tocopherol/g muscle
[2] mmols MDA/ mg protein. Iron-induced oxidation in microsomes extracts from muscles.
[3] mg hexanal/ kg muscle
[4] Reared in confinement and fed basal α-tocopheryl acetate diet (40 mg /kg diet).
[5] Reared in confinement and fed supplemented α-tocopheryl acetate diet (200 mg /kg diet).
[6] Free-range reared and fed on acorns and pasture.

II.C.2. Added antioxidants

The direct addition of antioxidants in muscle foods is one of the most effective and most frequently used methods to minimise the unpleasant effect of lipid oxidation. In the last 25 years, special attention has been given to the use of natural antioxidants because of the worldwide trend to avoid the use of synthetic food additives (Frankel *et al.*, 1996). In fact, the image of some particular synthetic substances used as antioxidants in foods such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl, octyl, and dodecyl gallates (PG, OG, DG) has been worsened by the findings linking the use of those compounds to health risks (Clayson *et al.*, 1986). Consequently, substances derived from the plant kingdom such as dried herbs and essential oils have been successfully used to reduce lipid oxidation in meat products (Chen *et al.*, 1999; McCarthy *et al.*, 2001; Formanek *et al.*, 2001). Furthermore, increasing expectations have arisen as a result of the evidence that natural antioxidants in foods could have additional benefits because of their anticarcinogenetic effects and their ability to inhibit biologically harmful oxidative reactions in the body (revised by Frankel,

1996). Still nowadays high scientific efforts are exerted to find substances from the plant kingdom to be used as antioxidants in muscle foods and other foodstuffs (Hinneburg *et al*., *in press*; Proestos *et al*., *in press*).

Amongst the natural antioxidants, rosemary and sage have been widely accepted as some of the spices with highest antioxidant activity (Hopia *et al*., 1996; Kähkönen *et al*., 1999; Ibáñez *et al*., 1999). The antioxidant activity of sage and rosemary extracts is primarily related to two phenolic diterpenes: carnosic acid and carnosol (Cuvelier *et al*., 1996; Ibáñez *et al*., 1999) which are considered two effective free-radicals scavengers (Aruoma *et al*., 1992). The antioxidant activity of these phenolic diterpenes has been compared to that from other recognised antioxidant substances and Richheimer *et al*., (1999) indicated that the activity of the carnosic acid was approximately seven times higher than that of BHT and BHA. Essential oils and extracts from rosemary and other *Labietae* herbs have been successfully used to reduce oxidative deterioration in a large variety of foods including frozen pork patties (McCarthy *et al*., 2001), refrigerated turkey products (Yu *et al*., 2002), refrigerated beef (Djenane *et al*., 2003), seed oils (Abdalla & Roozen, 1999), bread (Frutos & Hernández-Herrero, 2005), cooked sausages (Sebranek *et al*., 2005) and deep-fat-fried potatoes (Che Man & Tan, 1999).

However, recent studies have described the complexity associated to the use of phytochemical components of plant extracts as inhibitors of oxidative reactions (Kähkönen *et al*., 1999; Zheng & Wang, 2001). The antioxidant activity of these substances is affected by many factors including i) the system: composition of oil/emulsion (Hopia *et al*., 1996; Huang & Frankel, 1997), interaction with other active compounds (Meyer *et al*., 1998), temperature (Sature *et al*., 1995) and pH (Huang *et al*., 1996); and ii) the antioxidant: total number and location of hydroxyl groups on aromatic rings, the nature of the extracts and their concentration (Kähkönen *et al*., 1999*,* Zheng & Wang, 2001; Škerget *et al*., 2005). Kähkönen *et al* (1999) suggested that the antioxidant activity of plant phenolics could be also affected by the oxidation conditions and lipid characteristics of the system whereas Wong *et al*. (1995) and Škerget *et al*. (2005) reported that phenolic compounds from

plants can interact with other substances such as tocopherols leading to synergist effects. Furthermore, plant phenolics have shown unexpected prooxidant properties in biological materials and food systems (Laughton *et al*., 1989; Yen *et al*., 1997).

Yet again, the effect of the addition of antioxidants in meat products on the oxidation of muscle proteins is unknown. Viljanen *et al*. (2004a,b) described the protective role of berry phenolics, anthocyanins and other phenolic compounds against the oxidation of proteins from liposomes. Phenolic compounds could inhibit the oxidation of proteins by retarding the lipid oxidative reactions and by binding to the proteins and forming complexes with them (Siebert *et al*., 1996).

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Justification and objectives

Pigs from industrial genotypes (white pigs) are selected for high growth rates and traditionally reared intensively under controlled conditions and fed with concentrated diets, in order to maximized benefits in the shortest period of time, possibly leading to a decrease of meat quality. In contrast, Iberian pigs have been traditionally reared extensively in evergreen-oak forests in which animals are fed using natural resources, being that, a clear example of a ecologic-like, environmental friendly production system. Iberian dry-cured products such as hams and loins are highly appreciated by Spanish consumers because of their high sensory quality.

As a result of the permanent activity of slaughterhouses and meat factories, a large amount of by-products (back fat, boneless meat and some viscera) with high nutritional value are generated. In Spain, in the year 2000, 24,300 Tm of fat was obtained as a result of the slaughter of around 600,000 free-range reared Iberian pigs. This foodstuff is whether used in the local industry for the manufacture of low-quality products such as restructured meats or cured lards or removed, when the capacity of production is exceeded, using high cost processes, assuming sometimes environmental pollution.

Using adipose tissues, livers and muscles from Iberian pigs for the manufacture of novel cooked products such as liver pâté and frankfurters would increase the benefits of the local industry, offering to the consumer's demand a high quality product. Consumers could be influenced by the image of quality of the Iberian dry-cured products and will probably purchase and consume cooked products from Iberian pigs as high quality products though they were elaborated using low-cost materials.

There is no scientific information concerning the nutritional and technological properties of liver pâtés and frankfurters manufactured with tissues from Iberian pigs, even when some 'Iberian' pâtés and frankfurters are already being traded in the Spanish market.

On the other hand, substances with antioxidant activity are commonly used in muscle foods in order to inhibit the development of oxidative reactions during processing or storage. Synthetic phenolic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl, octyl, and

dodecyl gallates (PG, OG, DG), are easily available and largely used in different food products. Such synthetic compounds are frequently rejected by consumers and their use in foods has been linked to health risks generally believed to have a carcinogenic potential. Consequently, a high scientific effort has been exerted to select natural essential oils with antioxidant potential as alternatives to synthetic antioxidants. Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) are popular *Labiatae* herbs with verified potent antioxidant activity. The comparison between the effects of added natural (rosemary and sage essential oils) and synthetic antioxidants (BHT) on the oxidative stability of lipids and proteins from cooked products has never been carried out.

Two are the main purposes of the present thesis:

1. The manufacture and the nutritional and technological evaluation of liver pâtés and frankfurters manufactured using tissues from extensively reared Iberian pigs and intensively reared white pigs.

2. The evaluation of the effect of the addition of natural (sage and rosemary essential oils) and synthetic (BHT) antioxidants on the oxidative stability of lipids and proteins from liver pâtés and frankfurters.

Material and methods

I. MATERIALS

I.A. BIOLOGICAL MATERIAL

For the production of liver pâtés and frankfurters, tissues from extensively reared Iberian pigs and intensively reared white pigs were used.

Liver pâtés.

Seven Iberian pigs commonly produced in the South-West of Spain were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered at ~150 Kg live weight and an age of 12 months.

Seven white pigs (Large-white x Landrace) were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at 85 kg live weight and at the age of 7 months.

Iberian and white pigs were slaughtered in the same slaughterhouse one week apart during February 2003. After slaughter, adipose tissue, muscle *quadriceps femoris*, and livers were removed from the carcasses, vacuum packaged and stored at -80ºC until the day of their analysis and the manufacture of the pâté.

Frankfurters.

Seven Iberian pigs commonly produced in the South-West of Spain were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered at ~150 Kg live weight and an age of 12 months.

Seven white pigs were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at 85 kg live weight and at the age of 7 months.

Iberian and white pigs were slaughtered in the same slaughterhouse one week apart during February 2004. After slaughter, back fat and meat were

removed from the carcasses, vacuum packaged and stored at -80ºC until the day of their analysis and the manufacture of the frankfurters.

I.B. CHEMICALS

All reagents used for experiments were AAS grade and supplied by Panreac (Panreac Química, S.A., Barcelona, Spain), Sigma Chemicals (Sigma-Aldrich, Steinheim, Germany), Merck (Merck, Darmstadt, Germany) and Scharlau (Scharlau Chemie, S.A., Barcelona, Spain). Solvents of high-performance liquid chromatography (HPLC) grade were purchased from Panreac and Scharlau S.L. The gases used for gas chromatography (Helium, Nitrogen, Hydrogen, Air) and the evaporation of solvents (Nitrogen) were supplied by Air Liquide. Standards of fatty acids methyl esters and volatile compounds were purchased from Sigma (Sigma-Aldrich, Steinheim, Germany).

I.C. EQUIPMENT

Samples were kept frozen at -80ºC in a '-86 Econofreezer' (Forma Scientific). For the production of the cooked products the following machines were used:

-Foss Tecator Homogeniser, mod. 2094.

-Stuffer machine (Mainca, Barcelona, Spain).

-Scalding water cask (Mainca, Barcelona, Spain).

The distilled water was obtained from a water purification system (USF Elga, mod. Pure Lab Pro.). Water of Milli-Q quality was obtained with a water deionisation system (UHQ II, Elgastat) and used for the analysis of iron. For routine weighting a 'Selecta' balance (mod. 1409) (± 10 mg) was used. A precision scale (Kern, mod. 770) was used for accurate weighting (± 0.1 mg). Samples were homogenised using a 'Sorval Omnimixer' homogenisator. Samples were centrifuged in 'Epperdorf' centrifuges (either in mod. 5810-R or in mod. 5417-R). For the determination of moisture and the preparation of fatty acid methyl esters, a 'Selecta' drying-oven (mod. 210) was used. The ashes determination was carried out by using a 'Nabertherm' furnace (mod. D-2804). The evaporation of organic solvents was carried out using a 'Heidolph' rotary evaporator (mod. VV 2000) connected to a 'Savant' vacuum

pump (mod. SpeedVac Water Jet SWJ120). The water baths (Selecta, mod. 146) were used for TBA-RS analyses and the solid-phase microextractions (SPME) of volatile compounds. For the absorbance measurements, a 'Hitachi' spectrophotometer (mod. U-2000) was used. Fatty acids methyl esters (FAMEs) were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph (Avondale, PA, USA), equipped with a flame ionisation detector (FID). The derivatives were separated on a semi-capillary column (Hewlett Packard FFAP-TPA fused-silica column, 30m long, 0.53 mm internal diameter and 1.0 μm film thickness). Analysis of volatile compounds was carried out by using the solid-phase microextraction (SPME) coupled to gas chromatography (GC) and mass spectrometry (MS) using a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) and a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm id., 1.0mm film thickness).

II. METHODS

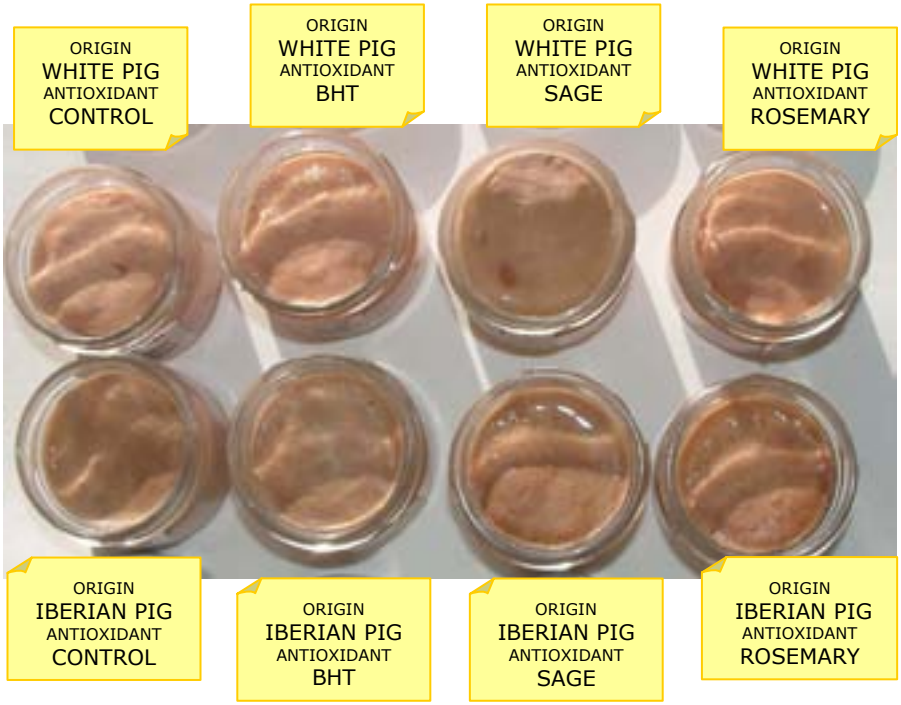
II.A. MANUFACTURE OF COOKED PRODUCTS

II.A.1. Manufacture of the liver pâté

The experimental pâtés were manufactured in a pilot plant. Depending on the origin of the raw material, two different types of liver pâtés were produced; pâtés from extensively reared Iberian pigs and those from intensively reared white pigs. For the manufacture of the pâtés, seven livers, muscles and adipose tissues from seven different animals were used for each of the groups (Iberian and white pigs). Depending on the addition of different antioxidants (rosemary essential oil, sage essential oil, and BHT) 4 groups of liver pâtés (including the control one) from both Iberian and white pigs were considered. The natural antioxidant extracts (Soria Natural S.L., Soria, Spain) were added at a level (0.1%) at which highest antioxidant activity would be exhibited (McCarthy *et al.*, 2001). BHT (Sigma-Aldrich, Steinheim, Germany) was

added up to the highest level allowed by the Spanish law for this kind of product (0.02%) (BOE, 2002). The same formulation was used for all pâtés except for the addition of the antioxidants. The ingredients were as follows per 100g of elaborated product: 28g liver, 40g adipose tissue, 5g muscle, 23g distilled water, 2g sodium caseinate, 2g sodium chloride. Sodium di- and tri-phosphates (0.3%) sodium ascorbate (0.05%) and sodium nitrite (0.03%) (ANVISA, Madrid, Spain) were also added. Following the aforementioned recipe, 1.5 kg of raw material was used for each group, to produce eight sets of experimental pâtés in eight independent production processes.

Photo 1. Liver pâtés from Iberian and white pigs with added antioxidants.



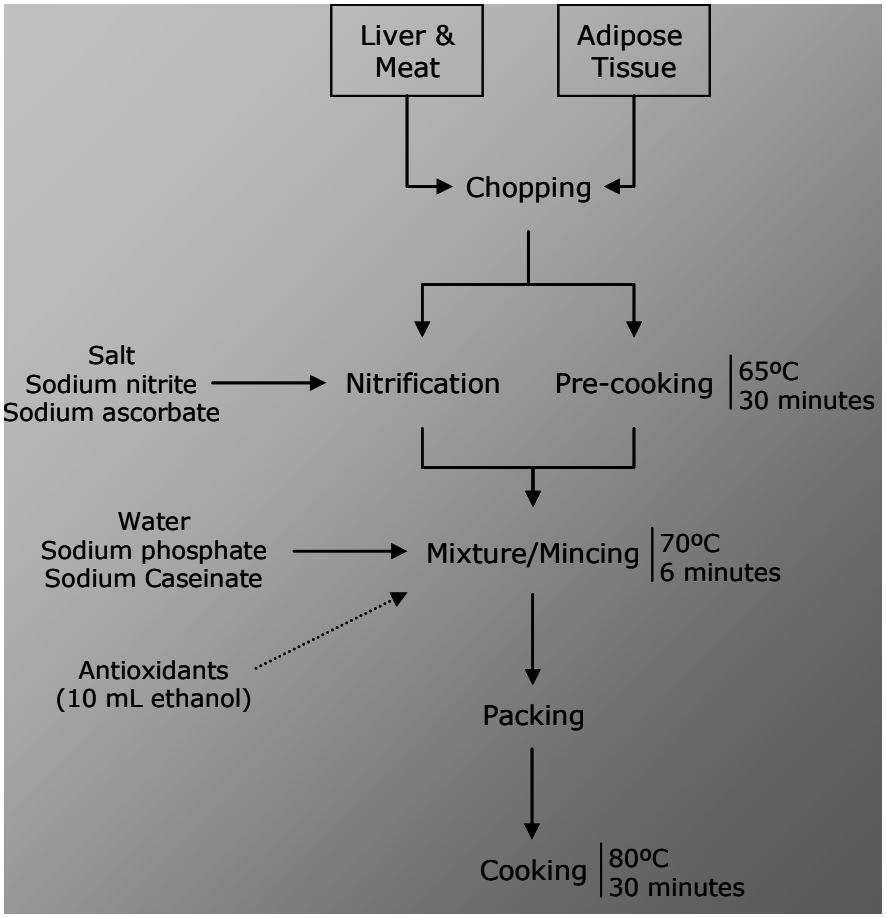
The day before to the manufacture of the liver pâté, the adipose tissue from Iberian and white pigs were chopped into small cubes (1.5 cm^3) and scalded in distilled water to an internal temperature of +65ºC during 30 min. Livers and muscles were also sliced into small cubes (1.5 cm^3) and mixed with the

sodium chloride, sodium nitrite and the sodium ascorbate in order to allow the nitrification of the samples. The cooked fat and the nitrification mixture were kept under refrigeration (+4ºC) in the darkness, before the manufacture of the liver pâtés (24 hours). The day of the elaboration, the sodium caseinate was dissolved in hot water (+75ºC) and then added to the scalded fat and mixed during mincing in a Foss Tecator Homogeniser (mod. 2094 Höganas, Sweden) for 3 minutes. After that, the nitrification mixture was added to the cutter bowl with the water and the sodium di- and tri-phosphates. The whole mixture was completely minced during 3 minutes until a homogenous raw batter was obtained. The antioxidants were previously dissolved in 10mL ethanol before being added to the mixture while mincing. 10 mL of ethanol without antioxidant were added to the control pâtés. Finally, the mixture was packed in a glass container (55 mm diameter; 55 mm height) and cooked by immersion in a hot water bath (+80ºC/30'). The packed liver pâtés were kept frozen (-80ºC) until required for analytical experiments.

II.A.2. Refrigeration of the liver pâté

Packed liver pâtés were refrigerated at +4ºC for 90 days in the darkness. Sampling was carried out at day 0 (day of manufacture), 30, 60 and 90 of refrigeration in order to perform the analyses.

Scheme 1. Diagram of liver pâtés processing.

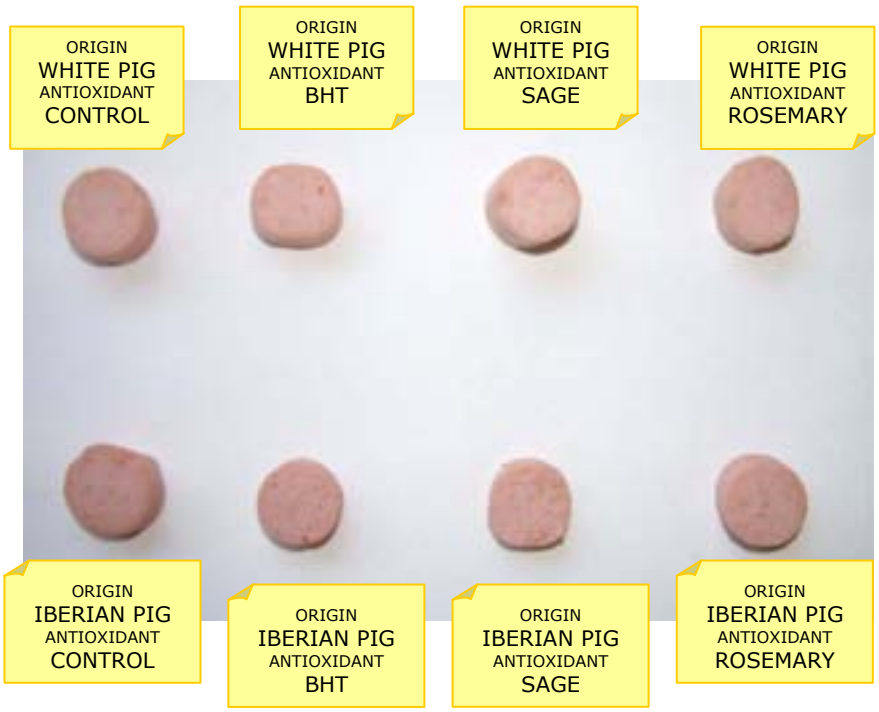


II.A.3. Manufacture of the frankfurters

The experimental frankfurters were manufactured in a pilot plant. Three different types of frankfurters were considered depending on the origin of the raw material: frankfurters from Iberian pigs (IF), frankfurters from white pigs (WF) and hybrid frankfurters (HF) elaborated with meat from white pigs and adipose tissue from Iberian pigs. Depending on the addition of rosemary essential oil (Soria Natural S.L., Soria, Spain) at different levels (0.015%, 0.03% and 0.06%) 4 different groups of frankfurters from both Iberian and white pigs (including the control ones) were considered. For the manufacture of the sausages, meat and adipose tissues from seven different animals were

used for each of the groups (Iberian and white pigs). The same formulation was used for all frankfurters. The ingredients were as follows per 100g of elaborated product: 50 meat, 10 adipose tissue, 37g distilled water, 2g sodium caseinate, 1g potato starch. Sodium chloride (2%), Sodium di- and tri-phosphates (0.5%) sodium ascorbate (0.05%) and sodium nitrite (0.03%) (ANVISA, Madrid, Spain) were also added. Following the aforementioned recipe, 1.3 kg of raw material was used to produce each of the nine sets of experimental frankfurters in nine independent production processes.

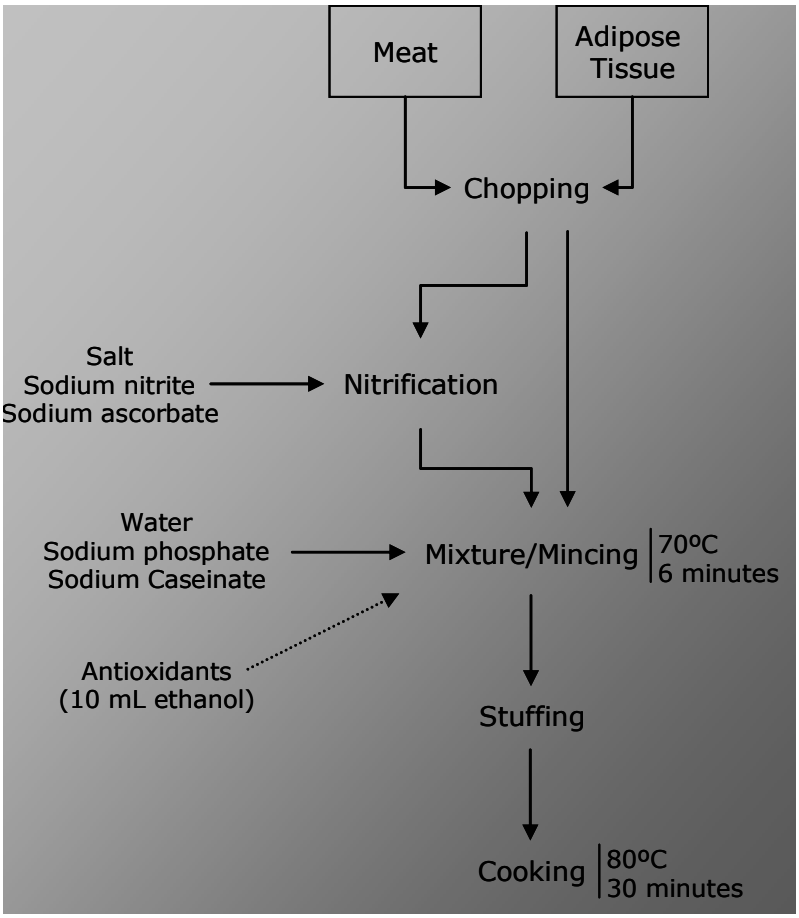
Photo 2. Sections of frankfurters from Iberian and white pigs with added antioxidants.



Firstly, the meat was chopped into small cubes (1 cm^3) and mixed with the sodium chloride, sodium nitrite and the sodium ascorbate in order to allow the nitrification of the samples 2 hours before the manufacture. Then, the meat was minced in a cutter (Foss Tecator Homogeniser, mod. 2094 Höganas, Sweden) for 2 minutes together with the starch and the 50% of the sodium caseinate which was previously dissolved in water (+75ºC). After that, the

adipose tissue was added together with the remaining dissolved sodium caseinate and minced for 4 more minutes until a homogenous raw batter was obtained. Before being added to the raw mixture while mincing, the rosemary essential oil was dissolved in 10 mL of ethanol. The mixture was finally stuffed into 18 mm diameter cellulose casings (Viscofan, Pamplona, Spain), handlinked at 10 cm intervals and cooked by immersion in a hot water bath (+80ºC/30'). The frankfurters were kept frozen (-80ºC) until required for analytical experiments.

Scheme 2. Diagram of frankfurters processing.

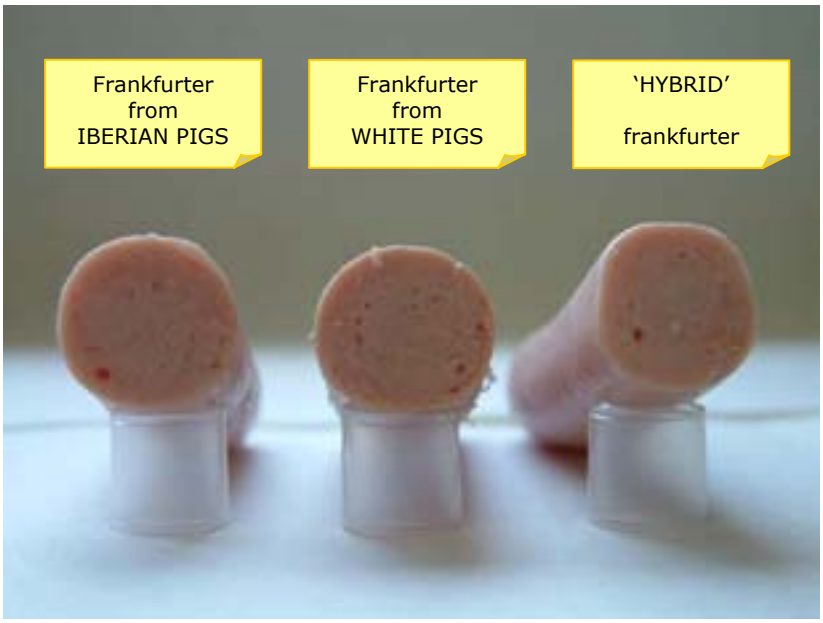


II.A.4. Refrigeration of the frankfurters

Frankfurters were over-wrapped in oxygen-permeable PVC films and refrigerated at +4ºC for 60 days in the darkness. Sampling was carried out at

days 0 (day of manufacture), 20, 40 and 60 of refrigeration in order to perform the analyses.

Photo 3. Frankfurters from Iberian and white pigs and 'hybrid' frankfurters.



II.B. ANALYTICAL METHODS

II.B.1. Moisture

Moisture contents were determined drying the samples in a drying-oven at +103ºC until a constant weight was obtained (AOAC, 2000a). Moisture was calculated as follows:

%Moisture= 100 x (wet weight-dry weight / wet weight).

II.B.2. Protein content

Protein content was determined in samples following the Kjeldhal method (AOAC, 2000b). This method involves three steps: digestion, distillation and direct titration. Firstly, 1 g of sample was digested in Kjeldhal digestion flasks with 20 mL of concentrated sulphuric acid and 15 g of Kjeldhal catalyst (Scharlau Chemie, Barcelona, Spain). The digestion flask was heated at

+370-400ºC for 30-60 minutes in a Kjeldhal digester. The flask was allowed to cool at room temperature and 100 mL of distilled water was added. The distillation was carried out by adding 100 mL of 30% sodium hydroxide and supplying steam to the Kjeldhal flask in a Büchi distillation unit (mod. K-314, Flawil, Switzerland). The ammonia was trapped in 100 mL of 2% boric acid solution with few drops of a mixed (methyl red plus methylene blue) indicator dye. 0.1 N HCl standard solution was used as the titration acid. The amount of nitrogen was calculated as follows:

% nitrogen= $[(0.1 \times V_{HCl}) \times 1400] / W_s$ (V_{HC}: Volume of standard HCl used in the titration step; W_s: Weight of the sample). The protein content was estimated based on the assumption that meat and liver proteins contain 16% nitrogen.

II.B.3. Fat content

A modified method from that described by Bligh & Dyer (1959) was used for extraction and quantification of total lipids from the samples. 5 grams of minced meat or liver (3 grams in the case of adipose tissue, liver pâtés or frankfurters) were placed in falcon-type cone plastic tubes, homogenised with 15 mL of chloroform:methanol (1:2) and centrifuged for 5 minutes at 2000 rpm. The supernatant was removed to other plastic tube and the pellet was homogenised with 10 mL of chloroform. The slurry was filtered through filter papers and the resulting liquid combined with the supernatant obtained from the first centrifugation. 5 mL of distilled water were added and the tubes were centrifuged for 5 minutes at 2000 rpm. The upper aqueous phase was carefully removed with Pasteur pipettes whereas the lower organic solution was filtered through sodium sulphate anhydrous into an Erlenmeyer flask. The remaining solvents were removed in a rotary evaporator coupled to a vacuum system. The amount of fat was calculated by difference of weight. In order to use the isolated fat for further analyses, the total lipids were dissolved in 10 mL of hexane and kept frozen at -80ºC until required.

II.B.4. Ash

The amount of ash was determined in 5 grams of sample by dry ashing in a 'Muffla' furnace at +550ºC until a constant weight was obtained (AOAC, 2000c).

II.B.5. Total iron

The total amount of iron in the samples was determined following the procedure described by Miller *et al*. (1994) with minor modifications. The sample (0.1-1 g depending on its iron content) was totally ashed in a 'Muffla' furnace at +550ºC. The ash was treated with 10 mL of an iron extraction solution (0.05% hydroxylamine monohydrochloride; 10% hydrochloric acid; 10% trichloracetic acid) and allowed to stand at room temperature for 10 minutes. 1 mL of the extracted iron solution was mixed with 3 mL of the chromogen solution (0.03% bathophenanthroline sulphonic acid in 3 M sodium acetate). Absorbance was measured at 540 nm. The solutions were prepared with milliQ water and the contact of the samples with materials or surfaces made with iron was carefully avoided. The standard curve of iron (powdered iron in concentrated hydrochloric acid) used for determining the amount of iron ranged from 1 µg/mL to 10 µg/mL. Blank samples (1mL of extraction solution + 3mL chromogen solution) were measured in the same conditions.

II.B.6. Non-heme iron

Non-heme iron (NHI) content was determined following the method of Rhee *et al*. (1987) with minor modifications. 1 gram of finely minced product was placed in a falcon-type cone plastic tube and 10 mL of iron extraction solution was added. Each tube was stirred for 1 minute and then allowed to stand at room temperature for 20 hours. During this period of time the tubes were stirred regularly. The slurry was filtered through filter paper into test tubes. 1 mL of the iron extraction solution was mixed with 3 mL of chromogen solution. Absorbance was measured at 540 nm. The solutions were prepared with milliQ water and the contact of the samples with materials or surfaces

made with iron was carefully avoided. The standard curve of iron (powdered iron in concentrated hydrochloric acid) used for determining the amount of iron ranged from 1 µg/mL to 10 µg/mL. Blank samples (1mL of extraction solution + 3mL chromogen solution) were measured in the same conditions.

II.B.7. Phenolic compounds content

The Folin Ciocalteu reagent was used for the quantification of total phenolics (TPH) as described by Turkmen *et al.* (*in press*) with minor modifications as follows: 0.5 gram of sample (1 gram for adipose tissue) was dispensed into a falcon-type cone plastic tube and homogenised with 10 mL of different solvents (water, methanol and 80% methanolic water) and centrifuged for 5 minutes at 3000 rpm and +4ºC. The supernatant was poured into a new plastic tube and the pellet homogenised with 5 mL of distilled water and filtered through filter paper. The supernatants were combined and the tubes centrifuged for 5 minutes (3000 rpm/ +4ºC) in order to precipitate solid residues. 1 mL aliquot was mixed with 5 mL of Folin Ciocalteu reagent (10% in distilled water) in test tubes. After 5 minutes, 4 mL of sodium carbonate (7.5% in distilled water) was added, the test tubes were screw-capped and the samples allowed to stand for 2 hours at room temperature in the darkness. Absorbance was measured at 740 nm and a mixture of distilled water (1 mL), Folin Ciocalteu reagent (5 mL) and sodium carbonate (4 mL) was used as blank. A standard curve with ethanolic gallic acid (ranged from 0.625×10^{-3} mg/mL to 0.02 mg/mL) was used for quantification. Results were expressed as mg of gallic acid equivalents (GAE) per gram of sample.

II.B.8. Tocopherol content

The amounts of tocopherol in tissues and cooked products were determined according to the method described by Rey *et al*. (1997). 0.8 g of tissue or cooked product were placed in plastic cone tubes and homogenised for 1 minute in 9.2 mL of 0.054 M phosphate buffer (pH: 7). While homogenisation, the plastic tubes were immersed in an ice bath to minimise the degradation of tocopherols. 15 mL of ethanol was added and the tubes stirred for 30

seconds. 3 mL of hexane was added, the tubes stirred again for 30 seconds and centrifuged for 5 minutes (2000 rpm/+4ºC). The organic phase was placed in test tubes. The last step was repeated twice with 2 mL of hexane and the supernatants obtained from centrifugations combined in the same test tube. The hexane was evaporated under nitrogen flow until approximately 0.5 mL of solvent remained in the tube. The hexane was dispensed in chromatography vials. The tube was cleaned twice with 0.5 mL of hexane and the solvents combined in the chromatography vial. The hexane was finally evaporated under nitrogen flow and 200 µL of ethanol added in the vial for high-performance liquid chromatography (HPLC). Analysis was carried out by reverse phase HPLC (HP 1050, Hewlett Packard, Waldbronn, Germany) using a PR 18 column (Hewlett Packard). The mobile phase was methanol:water (97:3) at flow rate of 2 mL/min and the ultraviolet detector fixed at 292 nm. The identification was carried out by comparing the retention time of the tocopherols extracted from the samples with those from standards supplied by Sigma (Sigma-Aldrich, Steinheim, Germany). The quantification was carried out with standard curves of α- and γ-tocopherols ranging from 0.05 to 2 µg/µL. Results were expressed as µg/g of sample.

II.B.9. Fatty acid profile

Fatty acid methyl esters (FAMEs) were prepared according to López-Bote *et al*. (1997). 100 µL of fat from the products were placed in screw-top test tubes and mixed with 1 mL of 0.5% sodium methylate. Test tubes were screw-capped and kept in an oven for 30 minutes at +80ºC and stirred regularly. After that, 1 mL of 5% sulphuric acid in methanol was added. After being stirred, the test tubes were kept again in the stove for 30 minutes. The test tubes were allowed to cool at room temperature. FAMEs were dissolved with 1 mL of hexane and kept in capped glass chromatography vials. When quantification of FAMEs was required, a hexane solution with 0.04% tridecanoic acid methyl ester as internal standard was used instead. FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph (Avondale, PA, USA) equipped with a flame ionisation detector (FID). The

derivatives were separated on a semi-capillary column (Hewlett Packard FFAP-TPA fused-silica column, 30m long, 0.53 mm internal diameter and 1.0 μm film thickness). The chromatography conditions were as follows:

Carrier gas (N_2) flow	1.8 mL min^{-1}
Injector temperature	230ºC
Column oven temperature (isothermal)	220ºC
Detector temperature	230ºC

Identification of FAMEs was based on retention times of reference compounds (Sigma-Aldrich, Steinheim, Germany). Fatty acid composition was expressed as percent of total fatty acid methyl esters. For fatty acid quantification, C13 was used as internal standard and results expressed as g fatty acids per 100 g of product.

II.B.10. Volatiles profile

The SPME fibre, coated with divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30 μm, was purchased from Supelco Co. (Bellefonte, PA). This coating phase was chosen because of the high reproducibility presented and the lower coefficients of variance obtained compared to others, such as the CAR/PDMS fibre (Machiels & Istasse, 2002). The SPME fibre was preconditioned prior analysis at +220 ºC during 45 min. The headspace sampling technique was used as follows: 1 g of product was placed in 2.5 mL vials. The fibre was exposed to the headspace of the solution while the sample equilibrated during 30 minutes immersed in water at either 60ºC (liver pâtés) or 50ºC (frankfurters). Based on preliminary studies, the sampling method was elected because in those conditions most of the analytes might have reached the equilibrium. Analyses were performed on a HP5890GC series II gas chromatograph (GC) (Hewlett-Packard, USA) coupled to a mass-selective detector (MS) (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek,

USA) (30 m x 0.25 mm id., 1.0mm film thickness). The GC/MS conditions were as follows:

Carrier gas (He) flow	1.6 mL min^{-1}
Inyector temperature (splitless)	220ºC
Column oven temperature (ramp)	40ºC 10 min. up to 250 ºC (7ºC min^{-1}) 250ºC 5 min.
GC/MS transfer line temperature	270ºC
Electron impact energy	70 eV
Multiplier voltage	1650 eV
Data collection rate	1 scan s^{-1}
Data collection range	m/z 40-300

n-Alkanes (Sigma R-8769) and standard compounds (Sigma-Aldrich, Steinheim, Germany) were run under the same conditions to calculate the Kovats index (KI) values for the compounds. Volatile compounds were tentatively identified by comparing their mass spectra with those contained in the Wiley and NIST libraries and by comparison of their KI with those reviewed by Kondjoyan & Berdagué (1996). Results from the volatiles analysis are provided in area units (AU).

II.B.11. Objective colour measurement

Instrumental colour (CIE L* a* b*; CIE, 1976) was automatically measured in triplicate on the surface of the raw material and elaborated products using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ, USA) with illuminant D_{65} and a 0º angle observer. Chroma (C) and Hue angle (hº) values were obtained by using the following equations: $C= (a^{*2} + b^{*2})^{0.5}$; Hº= (arctg b*/a*) x (360/6.28). Measurements were carried out at room temperature (~20ºC).

Photo 4. Measuring instrumental colour on the surface of liver pâtés.

II.B.12. Instrumental hardness of liver pâtés

The penetration test was performed with a Universal TA-XT2i texture analyser (Stable Micro Systems, Godalming, UK). Force in compression was measured with a 10-mm-diameter cylinder probe using a 5-kg load cell. After the probe touched the surface it proceeded to penetrate to a depth of 8 mm within the sample, measuring the force value as the hardness (N) of the sample. Force-distance deformation curves were recorded at a crosshead speed of 1.5 mm/s. Textural analyses were performed at room temperature (~20ºC).

Photo 5. Measuring instrumental texture on the surface of liver pâtés.

II.B.13. Texture profile analysis of frankfurters

The textural characteristics of the frankfurters were determined using a TA-XT2 TEXTURE ANALYSER (Stable Micro Systems Ltd., Surrey, England, UK). Uniform portions of 2 cm in length from the middle of the frankfurters were used as the test samples. The samples were compressed to 50% their original height at a crosshead speed of 5 mm/s through a two-cycle sequence. A 5 cm diameter probe was used in TPA measurements. Textural variables from force and area measurements were (Bourne, 1978):

- ✓ Hardness (N/cm^2)= maximum force required to compress the sample (peak force during the first compression cycle).
- ✓ Fracturability (N/cm^2)= the force during the first compression at which the material fracture.
- ✓ Adhesiveness (N s)= work necessary to pull the compressing plunger away from the sample.

- ✓ Springiness (cm)= height that the sample recovers during the time that elapses between the end of the first compression and the start of the second.
- ✓ Cohesiveness (dimensionless)= extent to which the sample could be deformed prior to rupture (A_1/A_2, A_1 being the total energy required to for the first compression and A_2 the total energy required for the second compression).
- ✓ Gumminess (N/cm^2)= the force needed to disintegrate a semisolid sample to a steady state of swallowing (hardness x cohesiveness).
- ✓ Chewiness (N s)= the work needed to chew a solid sample to a steady state of swallowing (gumminess x springiness).
- ✓ Resilience (dimensionless)= how well the product regains its original height , measured on the first withdrawal of the cylinder (area under the curve during the withdrawal of the first compression divided by the area of the first compression).

II.B.14. pH

The pH was measured directly using an Ingold pH-electrode connected to a Crison model 2001 pH-meter.

II.B.15. TBA-RS

Thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al*. (1996) with minor modifications as follows. 2.5 grams of product were dispensed in cone plastic tubes and homogenised with 7.5 mL of perchloric acid (3.86%), 0.5 mL of sulphanilamide (0.5% in 20% hydrochloric acid) and 0.25 mL of BHT (4.2% in ethanol). While homogenisation, the plastic tubes were immersed in an ice bath to minimise the development of oxidative reactions during extraction of TBA-RS. The slurry was filtered and centrifuged (2000 rpm/ 5 minutes) and 2 mL aliquots were mixed with 2 mL of thiobarbituric acid (0.02 M) in test tubes. The test tubes were placed in a hot water bath (+90ºC) for 30 minutes together with the tubes from the standard curve. After cooling, the absorbance was

measured at 532 nm. The standard curve was prepared using a 1,1,3,3-tetraethoxypropane (TEP) solution (0.2268 g) in 3.86% perchloric acid. The amount of TEP in the standard curve ranged from 0.028 mg TEP/mL to 1.134 mg TEP/mL.

II.B.16. Protein oxidation

Protein oxidation was assessed as the amount of carbonyls derived from protein oxidation per units of protein of product. The analysis was carried out following the method described by Oliver *et al*. (1987) with minor modifications (Mercier *et al*., 1998). The technique involves the quantification of carbonyls and protein from the same sample at the same time so that the results can be expressed as nmol of carbonyls per mg of protein. One gram of product was homogenised with 10 mL KCl for 30 seconds (0.15 M). From each sample, two aliquots containing approximately 0.7-1 mg of protein each (100 µL) were placed in 2 mL plastic eppendorfs. Protein was precipitated in both aliquots by 10% trichloracetic acid and centrifuged for 5 minutes at 5000 rpm. One pellet (A) was treated with 1 mL of 2 M HCl and the other (B) with an equal volume of 0.2% 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl. Both samples were incubated for 1 hour at room temperature and stirred regularly. The samples were precipitated with 1 mL of TCA and centrifuged for 5 minutes at 5000 rpm. Then, the remaining liquid was removed and the pellets washed twice with ethanol:ethyl acetate (1:1) and centrifuged at 10000 rpm for 5 minutes. The pellets were carefully drained and dissolved in 6 M guanidine HCl with 20 mM sodium phosphate buffer (pH: 6.5). When insoluble fragments were present, they were removed by centrifugation for 2 minutes at 5000 rpm. Absorbance at 280 nm was measured (eppendorf A) to determine the concentration of protein using BSA in guanidine as standard (concentrations from 0.075 to 1 mg/mL BSA). The results are expressed as mmol of carbonyls per mg of protein based on an average absorption of 21 mM^{-1} cm^{-1} (extinction coefficient) at 370 nm for protein hydrazones (eppendorf B).

II.C. DATA ANALYSIS

All experimental analyses were carried out in quindublicate within each group. The results of the experiments were used as variables and analysed using different statistical tests from the SPSS software (SPSS, 1997). In order to compare physico-chemical characteristics and oxidative stabilities of muscles, livers, adipose tissues, pâtés and frankfurters from Iberian and white pigs, T-Student tests for independent variables were carried out. The effect of the addition of the antioxidants on the products was evaluated using ANOVA tests. Statistical significance was predetermined at 0.05. Pearson correlation coefficients between parameters were also calculated.

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Chapters

CHAPTER I

Extensively reared Iberian pigs versus intensively reared white pigs for the manufacture of liver pâté[♣]

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I.1. Abstract

Physico-chemical characteristics and different quality traits from raw material (muscle *quadriceps femoris*, liver and adipose tissue) and the elaborated pâtés from extensively reared Iberian pigs and intensively reared white pigs, were evaluated. The differences found between muscles, livers and adipose tissues from Iberian and white pigs largely influenced the characteristics displayed by pâtés. Compared to pâtés from white pigs, pâtés from Iberian pigs presented a higher content of heme iron (27.5µg/g vs 11.5µg/g; $p<0.05$) and lower of non-heme iron (27.5µg/g vs 33.7µg/g; $p<0.05$). Pâtés from Iberian pigs exhibited a darker colour (L: 18.6 vs. 15.9, $p<0.05$) with less redness (a* values: 9.1 vs. 11.3; $p<0.05$) and yellowness (b* values: 13.1 vs. 14.8, $p<0.05$). Thus, pâtés from white pigs presented larger values of chrome (18.6 vs. 15.9, $p<0.05$) and smaller of hue (52.5 vs. 55.2, $p<0.05$) that those from Iberian pigs' pâtés. Concerning fatty acid composition, pâtés from white pigs presented higher proportions of SFA (37.9% vs. 32.8%, $p<0.05$) and PUFA (14.4% vs. 9.6%, $p<0.05$) than pâtés from Iberian pigs and lower percentages of oleic (53.4% vs. 43.6%, $p<0.05$) and total of MUFA (57.5% vs. 47.6%, $p<0.05$). Contrarily to pâtés from white pigs, pâtés from Iberian pigs presented a lower n-6/n-3 value (13.2 vs. 17.2; $p<0.05$).

I.2. Keywords: Liver pâté, Iberian pigs, extensive, white pigs, iron, instrumental colour, fatty acids

I.3. Introduction

Fifty years ago, industrial genotype pigs with high growth rates and producing lean carcasses were introduced in the Iberian Peninsula from foreign countries of Europe in order to substitute the foodstuff obtained from the rustic breed, traditionally free range reared in Spain: the Iberian pigs. In those years, meat and meat products from Iberian pigs were despised by Spanish consumers for the reason of their high fat content and the presence of some sanitary scandals such as the African Swine Fever (ASF) that led to the almost disappearance of this breed (Diéguez, 2001).

Nowadays, Iberian pig breed and its livestock farming is supported by the fact that the meat and meat products traditionally obtained from this breed (dry-cured products) are considered by Spanish consumers as high quality foodstuff, being always more appreciated than those from industrial genotype pigs (Cava *et al*., 2003; Ventanas *et al*., 2001). Recent studies have shown that meat from Iberian pigs have higher technological and nutritional value than that from industrial genotype pigs (Estévez *et al*., 2003a; Estévez *et al*., 2003b). Pigs from industrial genotypes are selected for high growth rates and traditionally reared intensively, under controlled conditions and fed with concentrated diets, in order to maximised benefits in the shortest period of time, possibly leading to a decrease of meat quality (Sundrum, 2001; Estévez *et al*., 2003b). In the other hand, Iberian pigs have been traditionally reared extensively in evergreen-oak forests named 'dehesas' in which animals are fed using natural resources such as grass and acorns, being that, a clear example of a ecologic-like, environmental friendly production system. These animals are slaughtered at high live weights (~150 kg) for the obtaining of dry-cured products and at lower live weights when meat for fresh consumption is required (Cava *et al*., 2003; Estévez *et al*., 2003a). The increase of tocopherol levels in tissues from Iberian pigs as a result of the intake of pasture (Cava *et al*., 2000), the high content of oleic acid from acorns (Cava *et al*., 1997) and the high content of iron in meat from Iberian pigs (Estévez *et al*., 2003a) are thought to be essential aspects of the high quality associated to meat products from Iberian pigs. In spite of that, there are considerable levels of by-products from Iberian pigs with a high nutritional value, such as back fat, boneless meat and some viscera that are whether used in the meat industry for the manufacture of low-quality products (i.e. restructured meats, hamburgers…), or removed using high cost processes, assuming sometimes environmental pollution (Silva *et al*., 2003).

Liver pâté is an emulsion-like fat product with an important gastronomic tradition, habitually considered as a high quality product (Le Ba & Zuber, 1996). Pork liver pâté contain large amounts of high bioavailable iron that can supply up to the 40% of the dairy requirements (Mataix & Aranceta, 2002).

Including a foodstuff with high iron content such as liver pâté in an equilibrated diet, could be a successful strategy to improve nutritional state of children and adolescents (Gibson, 1997). Using back fat, liver and meat from Iberian pigs for the manufacture of liver pâté would increase the benefits of the local industry, offering to the consumer's demand a high quality product. However, there is no scientific information of the characteristics displayed by liver pâté from Iberian pigs, even when some 'Iberian pâté' are already being traded in the Spanish market.

The aim of the present work is to study the physico-chemical characteristics of the raw material (muscle *quadriceps femoris*, liver and adipose tissue) and the manufactured product (liver pâté) from extensively reared Iberian pigs and intensively reared white pigs.

I.4. Material and Methods

Animals, feeds and sampling

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed selection schemes were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered at ~150 Kg live weight and an age of 12 months. Acorns (moisture: 46.10%, fat: 5.50%; protein: 4.31%) analysis showed the following fatty acid profile (expressed as percentage of total fatty acids analysed): palmitic acid (c16:0): 11.82%; stearic acid (c18:0): 0.56%; oleic acid (c18:1): 67.28%; linoleic acid (c18:2): 18.70%; linolenic acid (c18:3): 0.25%. The grass (moisture: 89.24%, fat: 6.26%; protein: 4.34%) fatty acid profile was as follows: c16:0: 13.95%; c18:0: 1.99%; c18:1: 5.24%; c18:2: 11.42%; c18:3: 57.80% (Table I.1.).

Seven white pigs were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at 85 kg live weight and at the age of 7 months. The analysis of the mixed diet (moisture: 10.42%, fat: 2.94%; protein: 18.28%) revealed the following fatty acid profile: c16:0: 19.86%; c18:0: 8.63%; c18:1:

32.84%; c18:2: 32.83%; c18:3: 2.45% (Table I.1.). Iberian pigs and white pigs were slaughtered at the same slaughterhouse one week apart.
After slaughter, adipose tissues, muscle *quadriceps femoris*, and livers were removed from the carcasses, vacuum packaged and stored at -+80ºC until the day of their analysis and the manufacture of the pâté.

Manufacture of the liver pâté
The experimental pâtés were manufactured in a pilot plant. The same formulation was used for all pâtés. The information concerning the recipe and the protocol followed for the production of the liver pâtés was described elsewhere (see main Material and Methods section). The packed liver pâtés were kept frozen (-80ºC) until required for analytical experiments.

Analytical methods
Compositional analysis of raw material and liver pâté
Moisture, total protein, total fat, and ash were determined using official methods (AOAC, 2000). The method of Bligh & Dyer (1959) was used for determining fat content of raw material and liver pâtés. Total iron was determined following the procedure described by Miller *et al*. (1994a). Non-heme iron (NHI) content was determined following the method described by Rhee *et al*. (1987). The amount of heme iron (HI) was calculated by difference between total and NHI.

Tocopherol determination
The levels of tocopherol in muscles, livers and adipose tissue were determined according to the method described by Rey *et al*. (1997).

Objective colour measurement
Instrumental colour (CIE L* a* b*; CIE, 1976) was measured in triplicate on the surface using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ). Chroma (C) and Hue angle (Hº) values were

obtained by using the following equations: $C= (a^{*2} + b^{*2})^{0.5}$; $H^{o}= \text{arctg } b^*/a^*x (360/6.28)$.

Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a semi-capillary column (Hewlett Packard FFAP-TPA fused-silica column, 30m long, 0.53 mm internal diameter and 1.0 µm film thickness). The injector and the detector temperature were held at +230ºC. Column oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma). Fatty acid composition was expressed as percent of total fatty acid methyl esters.

TBA-RS

Thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al*. (1996).

Data analysis

The results of the experiments were used as variables and analysed using a student's test for independent variables (SPSS, 1997) in order to compare physico-chemical characteristics of muscles, liver, adipose tissue and liver pâté from Iberian pigs and white pigs. Statistical significance was predetermined at 0.05.

I.5. Results and Discussion

General composition of muscle, liver and adipose tissue

Chemical composition of muscle *quadriceps femoris*, liver and back fat from Iberian pigs and white pigs is shown in Table I.2. The results obtained from

the analysis of the contents of moisture (72.9%-74.7%), fat (2.7%-2.6%) and protein (18.4%-17.8%) in the muscles agree with those obtained by other authors and ourselves in meat from Iberian pigs and white pigs (Cava *et al.*, 2003; Estévez *et al.*, 2003a; Serra *et al.*, 1998). No significant differences were found between groups which presented similar contents of moisture, fat and protein ($p>0.05$). According to previous papers (Estévez *et al.*, 2003a,b), the higher content of total iron in meat from Iberian pigs compared to that from white pigs (65.8 µg/g vs. 23.4 µg/g; $p<0.05$) was expected because of genetic traits, the higher age and weight at slaughter and the physical exercise performed by animals in extensively systems (Lawrie, 1998). No differences were found for non-heme iron (8.7 µg/g vs. 7.2 µg/g; $p>0.05$) between samples and therefore, differences for heme-iron content were large (57.1 µg/g vs. 16.20 µg/g; $p<0.05$). Meat from Iberian pigs has been described as an excellent source of high bioavailable iron, even though this fact could imply the promotion of oxidative processes in this meat after cooking and refrigeration (Estévez *et al.*, 2003b). Compared to muscle from white pigs, muscles from Iberian pigs presented a significantly higher content of tocopherols (6.18 mg/kg vs. 1.94 mg/kg), probably as a result of the high content of those substances in the grass (Cava *et al.*, 2000) with which Iberian pigs were fed.

Concerning chemical composition of liver, the results obtained for moisture (73.1%-73.5%), fat (2.6%-2.3%) and protein (13.9%-13.5%) showed similarity to those obtained by other authors (López-Bote & Rey, 2001; D' Arrigo *et al.*, 2002). No statistical differences ($p>0.05$) between breeds were found for any of these determinations. Livers from Iberian and white pigs presented high quantities of iron (Iberian: 152.6 µg/g; white: 145.0 µg/g; $p>0.05$), being larger that those presented by meat from different animal species and other meat products (Miller *et al.*, 1994b; Lombardi-Boccia *et al.*, 2002). The livers from white pigs presented higher contents of non-heme iron than the livers from Iberian pigs (68.8 µg/g vs. 51.9 µg/g). As commented for muscles, livers from Iberian pigs contained higher levels of tocopherols (7.9

mg/kg vs. 2.5 mg/kg; $p<0.05$) as a probable consequence of the intake of pasture.

In Table I.2. are shown the results obtained from the chemical analysis of the adipose tissue from Iberian and white pigs. As expected, fat was the principal component of adipose tissue from Iberian pigs and white pigs (76.3% and 75.1% respectively; $p>0.05$) while moisture (Iberian: 7.7%; white 9.7%; $p>0.05$) and protein (Iberian: 1.7%; white 3.8%; $p<0.05$) presented smaller extents. Except for protein content, no significant differences were found between adipose tissues from Iberian and white pigs ($p>0.05$). Large differences were found between samples when tocopherol content was analysed. According to results previously presented for muscle and liver, adipose tissues from Iberian pigs presented significantly higher tocopherol contents than those from white pigs (19.7 mg/kg vs. 1.2 mg/kg; $p<0.05$). Tocopherols are the most important natural antioxidants in meat and meat products and their protective activity against oxidation have been largely described in meat, liver and several meat products (Rey *et al*., 1997; Cava *et al*., 2000; López-Bote & Rey, 2001; Nilzén *et al*., 2001).

Colour characteristics of muscle, liver and adipose tissue

Table I.2. shows colour characteristics of muscle, liver and adipose tissue of Iberian pigs and white pigs. Compared to muscle *quadriceps femoris* from white pigs, muscles from Iberian pigs exhibited a redder colour (higher a* value) that resulted more intense (higher chrome value) and closer to the true red axis (lower hue value). In a previous work (Estévez *et al*., 2003a), we obtained similar results comparing colour characteristics between m. *longissimus dorsi* from Iberian and white pigs. In spite of the high differences between types of meat, little influence from meat is expected to appear in the manufactured product because of the small proportion of this (5%) in the pâté recipe.

The colour characteristics of the livers are quite similar between breeds since no statistical differences were found for most of colour parameters (Table

I.2.). Nevertheless, livers from Iberian pigs presented a higher b* value (7.6 vs. 5.4; $p<0.05$) and a higher hue angle value (26.9 vs. 18.8; $p<0.05$).
Except for L*, all the colour parameters of subcutaneous fat were different between breeds. Back fat from white pigs presented significantly higher redness (a* values: 5.9 vs. 2.9; $p<0.05$) and yellowness (b* values: 6.8 vs. 4.1; $p<0.05$) and, therefore, its colour was more intense (chrome values: 9.0 vs. 5.0; $p<0.05$) than that exhibited from Iberian pigs (Table I.2.).

Fatty acid composition of muscle, liver and adipose tissue

The tissues from Iberian and white pigs presented clear differences in their fatty acid composition (Table I.3.). Compared to meat from white pigs, meat from Iberian pigs presented a higher proportion of monounsaturated fatty acids (MUFA) such as palmitoleic (3.4% vs. 3.2%; $p<0.05$) and oleic (48.8% vs. 42.2%; $p<0.05$) acids and a lower of saturated fatty acids (SFA), such as palmitic (21.7% vs. 22.8%; $p<0.05$) and stearic (10.2% vs. 11.4%; $p<0.05$). Thus, meat from Iberian pigs presented a lower proportion of polyunsaturated fatty acids (PUFA), such as linoleic (9.6% vs. 13.0%; $p<0.05$) and araquidonic (1.8% vs. 2.0%; $p<0.05$) than that from white pigs. Although their percentages were extremely low, some n-3 PUFA, such as C20:5 and C22:6 presented higher percentages ($p<0.05$) in muscles from Iberian pigs than in those white pigs (0.11% vs. 0.07% and 0.37% vs. 0.08%, respectively)
Concerning fatty acid composition of the livers, the differences between breeds were similar to those described for muscles (Table I.3.), though no differences in percentages of polyunsaturated fatty acids were found (Iberian: 33.2%, white: 33.0%; $p>0.05$). Compared to livers from white pigs, those from Iberian pigs presented larger percentages of oleic acid (25.0% vs. 22.5%, $p<0.05$) and MUFA (26.4% vs. 24.3%, $p<0.05$) and smaller of stearic acid (27.6% vs. 22.2%; $p<0.05$) and SFA (40.5% vs. 42.6%; $p<0.05$). In livers, the proportion of long chain PUFA were relatively higher than in muscles. The percentages of some n-3 PUFA such as C22:5 and C22:6 were significantly higher ($p<0.05$) in livers from Iberian pigs (1.07% vs. 0.91% and

0.40% vs. 0.21% respectively) than in livers from white pigs. Contrarily, the latter showed larger percentages of C20:5 (1.21% vs. 0.45%; $p<0.05$).

The fatty acid profiles of adipose tissue are presented in Table I.3. Four fatty acids (palmitic, stearic, oleic and linoleic acids) comprised more than the 90% of the total fatty acids analysed. As expected, oleic acid was the most abundant (54.6%-43.9%) followed by palmitic (20.3%-23.3%), stearic (10.6%-13.3%) and linoleic acid (7.6%-11.9%). The fatty acid composition of adipose tissue presented large differences between Iberian and white pigs (Table I.3.). Compared to adipose tissue from Iberian pigs, adipose tissue from white pigs showed significant higher percentages of saturated fatty acids (38.7% vs. 32.5%; $p<0.05$) and polyunsaturated fatty acids (13.5% vs. 9.2%; $p<0.05$). On the contrary, Iberian pigs presented higher percentages of monounsaturated fatty acids (58.2% vs. 47.9%; $p<0.05$).

Differences in fatty acid composition of the animal tissues can be generally attributed to the differences in the fatty acid composition of the fat used in the feeding system (Rhee *et al.*, 1988; Miller *et al.*, 1990). Accordingly, raw material from Iberian pigs reflected the fatty acid composition of acorns (with high levels of oleic acid). Contrarily, tissues from white pigs reflected the general composition of the mixed diet, with relative high proportion of linoleic acid and PUFA. According to previous works, these results represent the general pattern of fatty acid composition of different tissues from Iberian pigs fed extensively with natural resources (Cava *et al.*, 1997; Ruiz *et al.*, 1998; Timón *et al.*, 2001) and white pigs fattened intensively with mixed diets (Flachowsky *et al.*, 1997; Serra *et al.*, 1998).

The livestock production system, the feed given to the animals and the genetic traits could have been largely influential on the different quality characteristics of the tissues from Iberian and white pigs. Furthermore, the large differences in the age and weight of the animals at slaughter might have influenced, since these factors surely affected the physiology and biochemical maturation of the tissues used for the manufacture of the pâtés.

General composition of porcine liver pâtés

The general composition of pork liver pâtés (moisture, fat, protein and iron contents) is shown in Table I.4. As expected, liver pâtés from Iberian and white pigs showed similar compositional characteristics, and no differences for moisture (48.4%-50.5%, $p>0.05$), fat (31.8%-33.4%, $p>0.05$), protein (10.0%-10.3%, $p>0.05$) and total iron (45.2 µg/g -50.6 µg/g, $p>0.05$) were detected. Agreeing with the results obtained, liver pâté is thought to be one of the best sources of iron for human diet. The levels of iron in liver pâtés is not only higher than even in other fortified foods (Kosse *et al.*, 2001), but, furthermore, its attractive appearance and pleasant taste, make of it the election option for the dairy intake of iron, specially in children and adolescents (Mataix & Lisbona, 2002). According to the results obtained from liver, pâtés from white pigs presented a significantly larger amount of NHI (33.7µg/g vs. 27.5µg/g) and smaller of HI (11.5µg/g vs. 27.5µg/g). Accurate knowledge of the chemical forms of iron is of great importance because of the strong differences displayed by HI and NHI in terms of availability -HI is thought to be more available- (Hunt & Roughead, 2000) and promotion of lipid oxidation –NHI is thought to be the most important oxidation promoter in meat systems- (Kanner *et al.*, 1991). Consequently, liver pâtés from Iberian pigs presented a better iron profile than liver pâtés from white pigs.

Colour characteristics of porcine liver pâtés

Cie L*, a*, b*, chrome and hue angle from liver pâtés are shown in Table I.4. Pâtés from Iberian pigs presented different colour characteristics to pâtés from white pigs, as suggested by the parameters measured. Compared to pâtés from white pigs, pâtés from Iberian pigs presented a darker colour (L: 18.6 vs. 15.9, $p<0.05$) with less redness (a* values: 9.1 vs. 11.3; $p<0.05$) and yellowness (b* values: 13.1 vs. 14.8, $p<0.05$). Thus, pâtés from white pigs presented larger values of chrome (18.6 vs. 15.9, $p<0.05$) and smaller of hue (52.5 vs. 55.2, $p<0.05$) that those from Iberian pigs' pâtés. No clear differences were observed between groups for the colour exhibited by livers, and the influence of meat colour on pâtés is not expected because of the low

proportion of meat in the recipe. Therefore, the colour characteristics of the adipose tissue could have been the main influence on the colour of pâtés. Actually, adipose tissue from white pigs presented higher a* and b* values than that from Iberian pigs. On the contrary, lard from Iberian pigs presented a higher L* score and liver pâtés from white pigs resulted paler than Iberian pigs' ones. The different colour characteristics of liver pâtés from Iberian pigs could identify them from other sort of pâtés, though the addition of natural or synthetic colorants in commercial pâtés would improve their colour characteristics.

Fatty acid composition and oxidative stability of porcine liver pâtés

The fatty acid composition of pork liver pâtés is shown in Table I.5. Large differences between pâtés from Iberian and white pigs were detected for most of the fatty acids analysed. Pâtés from white pigs presented higher proportions of palmitic (22.6% vs. 20.7%, $p<0.05$) stearic (13.4% vs. 10.6%) and total of SFA (37.9% vs. 32.8%, $p<0.05$) than pâtés from Iberian pigs. On the other hand, pâtés from Iberian pigs showed higher percentages of oleic (53.4% vs. 43.6%, $p<0.05$) and total of MUFA (57.5% vs. 47.6%, $p<0.05$) than pâtés from white pigs. The latter presented higher percentages of PUFA (14.4% vs. 9.6%, $p<0.05$) such as linoleic (12.2% vs. 7.7%, $p<0.05$) acid. As expected, fatty acid composition of pâtés reflected the fatty acid composition of the adipose tissue as long as the proportion of lard in the recipe was the highest of all ingredients. Therefore, the commented differences agree with the aforementioned for the adipose tissue and are mainly caused by the different fatty acid composition of the feeds given to the animals during the fattening period. Results obtained for liver pâtés from white pigs agree with those reported by other researchers in pâtés made with similar raw material (Ordóñez *et al.*, 2003). Compared with those, pâtés from Iberian pigs contained higher proportion of oleic acid that have been considered as one of the main characteristics of Iberian pig products and related to some of their high quality traits (Ruiz *et al.*, 1998). Focusing on nutritional and technological aspects, using raw material from extensively

reared Iberian pigs improved the lipid characteristics of the pâtés. Contrarily to MUFA, PUFA are very prone to oxidation, leading to the generation of unpleasant odours and reducing nutritional value of meat and fat products (Morrissey *et al.*, 1998). Thus, compared to SFA, MUFA are hypocholesterolemic, but, unlike PUFA, they do not decrease high-density lipoproteins (HDL) cholesterol which protects against coronary heart disease (Mattson & Grundy, 1985). The nutritional ratio between SFA hypercholesterolemic fatty acids (C12, c14, C16) and the unsaturated hypocholesterolemic ones (C18:1 n-9; C18:2 n-6) was also lower in pâtés from Iberian pigs (0.36 vs. 0.43; $p<0.05$). Concerning other minority fatty acids, the quantities of C20 and C22 n-3 PUFA in meat products have been largely taken into account because of the role played by the ratio n-6/n-3 in the development of coronary heart diseases (CHD) (Okuyama & Ikemoto, 1999). Increasing n-3 PUFA in meat and meat products has been one of the most common aims for food technologists. The content of these fatty acids in tissues of pigs reared outdoors with access to pasture, naturally increases because of the intake of grass with high content of n-3 PUFA (Nilzén *et al.*, 2001). The ratio n-6/n-3 was lower in pâtés from Iberian pigs (13.2 vs. 17.2; $p<0.05$) as a result of the higher content of C18:2 (n-6) in those from white pigs. However, the effect of increasing long chain n-3 PUFA in raw material and liver pâtés from extensively reared Iberian pigs was not generally detected. While in northern Europe the source of these essential fatty acids are mainly found in meat products (Enser *et al.*, 1995), in Mediterranean countries, such as Spain, the high consumption of fish and fishery products make of them the main source of n-3 fatty acids. In spite of that, Ordóñez *et al.* (2003) have recently developed pâtés with increasing amounts of n-3 fatty acids and tocopherols in order to improve n-6/n-3 ratios without producing an arise of oxidative deterioration and off-flavors. Using natural sources of n-3 fatty acids (fishery by-products), Aquerreta, Astiasarán, Mohino & Bello (2002) have found better n-6/n-3 ratios in pâtés elaborated with mackerel fish and tuna liver, than those obtained by the aforementioned researchers. In the present study, four fatty acids (palmitic, stearic, oleic and linoleic fatty

acids) comprised more than the 90% of the total fatty acids analysed, and therefore, any additional consideration of minority fatty acids is not justified.
As expected, pâtés from white pigs presented significant higher TBA numbers (0.83 mg MDA/ Kg pâté) than pâtés from Iberian pigs (0.35 mg MDA/ Kg pâté) ($p<0.05$) (Table I.4.). The oxidation stability of liver pâtés was closely related to their fatty acid composition, the chemical forms of iron and the content of tocopherols in the raw material used. The higher proportion of PUFA and the larger NHI content in the raw material and in the manufactured product, the higher TBA numbers in the liver pâtés. The high content of tocopherols in the raw material from Iberian pigs could have enhanced the oxidation stability of the pâté, improving its nutritional and technological properties.

I.6. Conclusions

Liver pâtés from Iberian and white pigs presented large differences mainly due to the differences found in the raw material. Using meat, liver and back fat from Iberian pigs for the manufacture of pâté results in a high quality product, because of its colour and compositional characteristics. Compared to pâtés from white pigs, pâtés from Iberian pigs presented a larger level of high bioavailable iron. Thus, the fat from Iberian 'pâtés' was rich in MUFA, scarce in hyperchiolesterolemic fatty acids and presented lower values of the ratio n-6/n-3 than pâtés from white pigs. The latter showed higher proportion of PUFA, and higher content of non-heme iron that could be predictable of a higher oxidative instability during later manipulation or storage.

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Table I.1. Compositional analysis of the finishing diets of Iberian pigs (acorns and grass) and white pigs (mixed diet).

	Grass	Acorn	Mixed diet
% Moisture	89.24	46.10	10.42
% Fat	6.26	5.05	2.94
% Protein	4.34	4.31	18.28
% Ashes	0.91	1.17	4.55
% FAMEs[1]			
c14:0	3.64	0.18	0.67
c16:0	13.95	11.82	19.86
c16:1 (n-7)	2.40	0.10	0.91
c18:0	1.99	0.56	8.63
c18:1 (n-9)	5.24	67.28	32.84
c18:2 (n-6)	11.42	18.70	32.83
c18:3 (n-3)	57.80	0.25	2.45
c20:0	2.40	0.25	0.42
c20:1 (n-9)	0.17	0.51	1.00
c20:2 (n-6)	0.03	0.09	0.21
c20:4 (n-6)	0.94	0.26	0.17

[1] FAMEs expressed as % of total fatty acids analysed.

Table I.2. Chemical composition and instrumental colour of meat, liver and back-fat from Iberian and white pigs.

	Meat			Liver			Adipose tissue		
	Iberian	white	p[1]	Iberian	white	p	Iberian	white	p
Moisture[2]	72.99±0.13	74.77±0.43	<0.001	73.05±0.06	73.46±0.09	<0.001	7.71±0.41	9.70±1.13	0.378
Fat[2]	2.74±0.31	2.62±0.08	0.427	2.58±0.24	2.30±0.30	0.146	76.32±0.84	75.14±1.98	0.273
Protein[2]	18.44±0.26	17.83±0.22	0.004	13.92±0.16	13.54±0.76	0.331	1.70±0.12	3.81±0.95	0.007
Total iron[3]	65.85±8.78	23.44±6.94	<0.001	152.57±28.92	145.01±52.06	0.786	tr[5]	tr	tr
Non-heme iron[3]	8.69±3.03	7.24±1.98	0.399	51.87±4.26	62.78±3.47	0.002	tr	tr	tr
Heme iron[3]	57.15±7.77	16.20±6.61	<0.001	100.69±37.47	82.23±29.40	0.492	tr	tr	tr
Tocopherol[4]	6.18±0.80	1.94±0.40	<0.001	7.93±0.90	2.49±0.60	<0.001	19.67±2.10	1.21±0.30	<0.001
L*	40.83±1.31	46.19±2.28	0.003	33.72±1.78	33.35±0.34	0.668	81.18±0.67	80.58±0.70	0.201
a*	18.49±2.92	14.06±3.35	0.057	15.75±0.82	14.91±0.82	0.130	2.98±0.33	5.89±0.53	<0.001
b*	6.72±1.98	7.77±1.13	0.340	5.36±1.25	7.59±1.32	0.026	4.11±0.25	6.80±0.28	<0.001
Chroma	19.69±3.41	16.08±3.44	0.134	16.68±0.68	16.76±0.91	0.881	5.08±0.25	9.00±0.52	<0.001
Hue	19.60±2.96	29.52±3.52	0.001	18.81±4.67	26.91±4.26	0.021	54.11±3.80	49.19±1.87	0.042

[1] Statistical significance in a Student test for independent variables.
[2] µg/g of raw material.
[3] mg/100g of raw material.
[4] mg/kg of raw material.
[5] traces.

Table I.3. Fatty acid composition (means ± standard deviation) of muscle, liver and adipose tissue from extensively reared Iberian pigs and intensively reared white pigs.

	Muscle			Liver			Adipose Tissue		
	Iberian	white	p[1]	Iberian	white	p	Iberian	white	p
C12:0[2]	0.04±0.00	0.06±0.00	0.001	0.04±0.02	0.01±0.00	0.117	0.03±0.00	0.04±0.01	0.111
C14:0	0.97±0.01	1.14±0.02	<0.001	0.65±0.09	0.26±0.06	<0.001	1.04±0.01	1.17±0.03	<0.001
C16:0	21.75±0.11	22.85±0.14	<0.001	13.50±7.38	14.35±0.21	0.809	20.32±0.06	23.38±0.28	<0.001
C17:0	0.18±0.00	0.54±0.00	<0.001	0.76±0.00	0.27±0.00	<0.001	0.26±0.00	0.52±0.04	<0.001
C18:0	10.25±0.08	11.39±0.12	<0.001	22.22±0.17	27.64±0.72	<0.001	10.69±0.02	13.32±0.53	<0.001
C20:0	0.12±0.06	0.14±0.06	0.702	0.05±0.00	0.07±0.00	<0.001	0.24±0.01	0.26±0.01	0.001
ΣSFA	33.33±0.08	36.13±0.20	<0.001	40.57±0.31	42.60±0.93	0.006	32.58±0.07	38.70±0.62	<0.001
C16:1 (n-7)	3.36±0.05	3.18±0.04	0.001	4.84±6.81	0.90±0.03	0.266	1.77±0.02	2.22±0.31	0.030
C17:1 (n-7)	0.22±0.00	0.47±0.00	<0.001	0.36±0.00	0.12±0.00	<0.001	0.23±0.01	0.45±0.05	0.001
C18:1 (n-9)	48.80±0.39	42.22±0.41	<0.001	24.05±0.31	22.58±0.40	<0.001	54.66±0.04	43.94±0.55	<0.001
C20:1 (n-9)	0.92±0.06	0.95±0.02	0.429	0.26±0.02	0.70±0.08	<0.001	1.54±0.00	1.30±0.28	0.128
C22:1 (n-9)	0.02±0.00	0.02±0.01	0.711	0.01±0.00	0.10±0.05	0.013	0.02±0.00	0.02±0.00	0.304
ΣMUFA	53.30±0.44	46.83±0.41	<0.001	26.46±0.35	24.30±0.45	<0.001	58.20±0.03	47.92±0.49	<0.001
C18:2 (n-6)	9.62±0.23	13.00±0.13	<0.001	13.68±0.35	11.29±0.32	<0.001	7.60±0.03	11.99±0.51	<0.001
C18:3 (n-6)	0.05±0.00	0.05±0.00	0.723	0.53±0.03	0.41±0.10	0.077	0.11±0.00	0.04±0.00	0.021
C18:3 (n-3)	0.40±0.01	0.51±0.01	<0.001	0.40±0.01	0.30±0.13	0.175	0.61±0.02	0.54±0.07	0.100
C20:2 (n-6)	0.34±0.00	0.49±0.00	<0.001	0.24±0.01	0.39±0.01	<0.001	0.53±0.00	0.48±0.05	0.127
C20:3 (n-3)	0.06±0.00	0.09±0.00	<0.001	0.77±0.01	0.42±0.02	<0.001	0.03±0.00	0.04±0.01	0.180
C20:3 (n-6)	0.20±0.01	0.26±0.01	<0.001	0.41±0.01	0.58±0.04	0.001	0.02±0.00	0.03±0.01	0.086
C20:4 (n-6)	1.83±0.13	2.04±0.11	0.021	15.28±0.18	17.16±1.60	0.058	0.10±0.00	0.18±0.07	0.073
C20:5 (n-3)	0.11±0.01	0.07±0.01	0.002	0.45±0.01	1.21±0.11	<0.001	0.03±0.00	0.02±0.00	0.386
C22:2 (n-6)	0.03±0.00	0.05±0.00	0.001	0.04±0.00	0.04±0.01	0.598	0.05±0.00	0.04±0.01	0.345
C22:4 (n-6)	0.23±0.21	0.01±0.00	0.081	0.01±0.00	0.07±0.02	<0.001	0.01±0.00	0.04±0.01	0.008
C22:5 (n-3)	0.28±0.02	0.37±0.01	<0.001	1.07±0.03	0.91±0.02	<0.001	0.10±0.04	0.11±0.00	0.577

	Muscle			Liver			Adipose Tissue		
	Iberian	white	p[1]	Iberian	white	p	Iberian	white	p
C22:6 (n-3)	0.37±0.08	0.08±0.01	0.001	0.40±0.01	0.21±0.05	0.001	0.02±0.01	0.02±0.00	0.279
ΣPUFA	13.52±0.65	17.03±0.27	<0.001	33.26±0.62	33.00±1.33	0.706	9.22±0.08	13.52±0.44	<0.001
nutritional ratio[3]	0.39±0.00	0.44±0.00	<0.001	0.46±0.01	0.43±0.00	<0.001	0.34±0.00	0.44±0.01	<0.001
n-6/n-3	7.07±1.35	11.75±0.08	0.001	4.71±0.06	3.94±0.20	0.001	9.67±0.58	16.64±4.1	0.018

[1] Statistical significance in a student test for independent variables.
[2] Fatty acids expressed as percentages of total fatty acids analysed.
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: polyunsaturated fatty acids;[3] Nutritional ratio: (C12:0 + C14:0 + C16:0) / (C18:1 + C18:2).

Table I.4. Chemical composition, instrumental colour and TBA numbers from liver pâtés from extensively reared Iberian and intensively reared white pigs.

	Iberian	White	p
Moisture	48.42±0.19	50.51±0.62	0.001
Fat	33.37±1.81	31.78±1.73	0.193
Protein	10.34±0.24	10.04±0.71	0.412
Total iron	50.59±18.11	45.19±13.07	0.416
Heme iron	27.25±3.10	11.50±3.99	0.010
Non-heme iron	27.35±8.33	33.69±1.84	0.001
L*	61.45±0.33	66.52±0.93	<0.001
a*	9.13±0.47	11.32±0.29	<0.001
b*	13.10±0.35	14.75±0.19	<0.001
Chroma	15.97±0.38	18.60±0.22	<0.001
Hue	55.16±1.57	52.52±0.83	0.016
TBA (mg MDA/Kg pâté)	0.35±0.10	0.83±0.02	0.020

See footnotes of Table I.2.

Table I.5. Fatty acid composition (means ± standard deviation) of liver pâtés from extensively reared Iberian and intensively reared white pigs.

	Iberian	White	p[1]
C12:0[2]	0.04±0.00	0.05±0.00	0.048
C14:0	1.12±0.01	1.11±0.01	0.111
C16:0	20.69±0.08	22.65±0.05	<0.001
C17:0	0.25±0.00	0.56±0.00	<0.001
C18:0	10.58±0.00	13.40±0.03	<0.001
C20:0	0.20±0.01	0.22±0.07	0.543
ΣSFA	32.87±0.09	37.98±0.12	<0.001
C16:1 (n-7)	2.00±0.01	2.44±0.00	<0.001
C17:1 (n-7)	0.25±0.00	0.48±0.00	<0.001
C18:1 (n-9)	53.43±0.07	43.57±0.07	<0.001
C20:1 (n-9)	1.83±0.02	1.08±0.04	<0.001
C22:1 (n-9)	0.02±0.00	0.02±0.00	0.147
ΣMUFA	57.52±0.06	47.58±.010	<0.001
C18:2 (n-6)	7.71±0.11	12.23±0.04	<0.001
C18:3 (n-6)	0.03±0.00	0.04±0.00	<0.001
C18:3 (n-3)	0.49±0.01	0.61±0.01	<0.001
C20:2 (n-6)	0.57±0.02	0.45±0.23	0.296
C20:3 (n-3)	0.03±0.00	0.06±0.00	<0.001
C20:3 (n-6)	0.08±0.00	0.11±0.00	<0.001
C20:4 (n-6)	0.56±0.00	0.70±0.03	<0.001
C20:5 (n-3)	0.05±0.00	0.05±0.02	0.875
C22:2 (n-6)	0.04±0.01	0.06±0.01	0.001
C22:4 (n-6)	0.01±0.00	0.04±0.01	0.001
C22:5 (n-3)	0.06±0.00	0.02±0.00	<0.001
C22:6 (n-3)	0.01±0.00	0.03±0.00	<0.001
ΣPUFA	9.63±0.29	14.40±0.37	<0.001
nutritional ratio[3]	0.36±0.00	0.43±0.00	<0.001
n-6/n-3	13.22±0.07	17.22±0.70	<0.001

See footnotes of Table I.3.

CHAPTER II

Lipid and protein oxidation, release of iron from heme molecule and colour deterioration during refrigerated storage of liver pâté[♣]

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II.1. Abstract

In the present work, lipid and protein oxidation, increase of non-heme iron (NHI) content and colour changes occurred during refrigerated storage (90 days/+4ºC) of liver pâtés from Iberian and white pigs, were studied. Iberian pigs were reared outdoors and fed on natural resources (grass, acorns) while white pigs were intensively reared and fed on a mixed diet. Lipid and protein oxidation were respectively measured by determining TBA reactive substances (TBA-RS) and protein carbonyls groups. Pâtés from Iberian pigs presented higher oxidative stability since presented at all stages of storage, lower amounts of TBA-RS and carbonyls compared to those obtained from white pigs ($p<0.05$). NHI increased during refrigerated storage of liver pâtés, being those from white pigs that presented a higher amount of NHI at all stages of storage ($p<0.05$). During refrigerated storage, L*-values tended to increase while the evolution of a* and b* depended on the group and did not seem to be related to oxidative processes.

II.2. Keywords: Pâté, Iberian pigs, white pigs, refrigeration, oxidation stability, non-heme iron, instrumental colour.

II.3. Introduction

Regardless of microbial spoilage, oxidation process is the major factor reducing quality and acceptability of meat and fat products (Morrissey *et al.*, 1998). Lipid oxidation is a complex process whereby polyunsaturated fatty acids are degraded via formation of free radicals, causing flavour, texture, colour and nutritional deterioration of foodstuffs (Gray, 1978). Proteins from animal tissues are also targets for oxygen radicals attack *in vivo* and in foods (Stadtman, 1986; Oliver *et al.*, 1987; Mercier *et al.*, 1998; Mercier *et al.*, 2004), but much less is known about oxidative degradation of proteins and the repercussions on quality of meat and fat products. Concerning oxidation promoters in animal foodstuff, iron is thought to have high catalytic activity (Rhee *et al.*, 1987a). Non-heme iron (NHI) is considered the most important oxidation promoter in meat systems and, therefore, knowledge of the

proximal proportion between the chemical forms of iron is of great importance (Kanner *et al*., 1991). An increase in the amount of NHI as a result of thermal processes on meat systems has been described (Schricker *et al*., 1982; Lombardi-Boccia *et al*., 2002). For Miller *et al*. (1994a,b), cooking is not as important as the subsequent refrigeration of cooked meats for the release of NHI from myoglobin. Anyhow, the increase of NHI in meats and fishes has been considered to be a reflection of the decrease of heme iron (HI) as a consequence of the breakdown of heme molecule during cooking or storage (Gómez-Basauri & Regenstein, 1992a,b; Miller *et al.*, 1994a,b) and has been eventually linked to oxidative deterioration of the porphyrin ring of myoglobin (Schricker & Miller, 1983).

On the other hand, colour of meat products is an important quality attribute that influences consumer acceptance, and a brown-gray colour is preferred for cooked products (Cornforth, 1994). Colour changes in cooked products during refrigerated storage have been linked to oxidation phenomena, and several factors such as the characteristics and amount of fat, the packaging method and the presence of antioxidants have been reported as influential factors (Jo *et al*., 1999; Jo *et al*., 2000).

Liver pâté is a traditional product with increasing demand by European consumers in the last 15 years (Rosmini *et al*. 1996). Liver pâtés exhibits high amounts of fat and iron, and therefore, oxidative deterioration of liver pâtés during refrigeration is expected. Derived from their physico-chemical analysis, liver pâtés from extensively reared Iberian pigs present higher nutritional and sensory characteristics than those from intensively reared white pigs (Estévez *et al*., in press, a). The differences between pâtés from Iberian and white pigs in terms of their fatty acid composition and antioxidative status are expected to influence on their oxidative deterioration during refrigerated storage. There is a large lack of knowledge concerning the occurrence of lipid and protein oxidation on liver pâtés. Moreover, the relationships between oxidative processes and the release of iron from myoglobin, and the effect of these chemical changes on colour characteristics of cooked products has not been previously described. The aim of the present work were to study the physico-

chemical changes of liver pâtés from Iberian and white pigs during refrigerated storage as assessed by lipid and protein oxidation, increase in the amount of NHI and colour deterioration. Relationships between the different parameters measured were also established.

II.4. Material and Methods

Animals and sampling

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed selection schemes were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered at ~150 Kg and an age of 12 months. Seven white pigs (Large-white x Landrace) were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at ~85 kg live weight and at the age of 7 months. Iberian pigs and white pigs were slaughtered at the same slaughterhouse one week apart. After slaughter, back fat, muscle *quadriceps femoris*, and liver were removed from carcasses, vacuum packaged and stored at -80ºC until the manufacture of the pâté.

Experimental Design

For the manufacture of the pâtés, seven livers, muscles and adipose tissues from seven different animals were used for each of the breeds (Iberian and white pigs). The procedure for the manufacture of the pâtés was described elsewhere (Estévez *et al*., 2004). Liver pâtés were packed in glass containers prior to thermal treatment (+80ºC/30'). After the containers were allowed to cool at room temperature, they were stored in the darkness at +4ºC during 90 days since the day of the manufacture (day 0). Liver pâtés were analysed at days 0, 30, 60 and 90 for lipid and protein oxidation, concentration of NHI and instrumental colour. After being accomplished each of the refrigeration stages, instrumental colour was measured on the surface of liver pâtés and then, they were stored at -80ºC until analytical experiments.

Analytical methods

Compositional analysis of liver pâtés

Moisture, total protein, and ash were determined using official methods (AOAC, 2000). The method of Bligh & Dyer (1959) was used for determining fat content.

Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The characteristics and conditions of the separation of FAMEs using gas chromatography were reported in a previous paper (Estévez *et al*., 2004). Identification of FAMEs was based on retention times of reference compounds (Sigma). Fatty acid composition was expressed as percent of total fatty acid methyl esters.

Protein oxidation measurement

Protein oxidation as measured by the total carbonyl content was assessed following the method described by Oliver *et al*. (1987). Protein concentration was calculated by spectrophotometry using BSA as standard.

TBA-RS measurement

Malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al*. (1996).

Iron analysis

NHI concentration was measured following the methods described by Schricker *et al*. (1982) and by Rhee *et al*. (1987b).

Objective colour measurement

Instrumental colour (CIE L* a* b*; CIE, 1976) was measured in triplicate on the surface of liver pâtés using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ). Chroma (C), Hue angle (Hº) values were obtained by using the following equations: $C= (a^{*2} + b^{*2})^{0.5}$; Hº= arctg b*/a* x (360/6.28). A numerical total colour difference (ΔE) between pâtés at day 0 and day 90 of storage was calculated by: $\Delta E_{0\text{-}90}= [(L_{90}\text{-}L_0)^2+(a_{90}\text{-}a_0)2+(b_{90}\text{-}b_0)2)]^{1/2}$.

Data analysis

The results of the experiments were used as variables and analysed by using a Student's *t*-test for independent variables (SPSS, 1997) in order to compare pâtés from Iberian and white pigs. The effect of refrigerated storage on liver pâtés was assessed by using an Analysis of Variance (ANOVA) from SPSS software. Statistical significance was considered as follows: $p>0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

II.5. Results and Discussion

General composition of liver pâtés

Chemical composition of liver pâtés (moisture, fat, protein and ash) is shown in Table II.1. Liver pâtés from Iberian and white pigs showed no differences for moisture (48.4%-50.5%, $p>0.05$), fat (31.8%-33.4%, $p>0.05$), protein (10.0%-10.3%, $p>0.05$) or ash contents (2.7%-2.8%; $p>0.05$). Fatty acid profiles of liver pâtés were significantly different between groups (Table II.1). Compared to pâtés from white pigs, pâtés from Iberian pigs presented a larger proportion of monounsaturated fatty acids (MUFA) and smaller of saturated (SFA) and polyunsaturated fatty acids (PUFA). Liver pâtés reflected the fatty acid composition of the raw material (fat, liver and meat) used for their manufacture, as it was profusely discussed in a previous paper (Estévez *et al.*, in press, a).

Lipid oxidation during refrigerated storage of liver pâtés

TBA-RS of liver pâtés from Iberian and white pigs gradually increased during 90 days of refrigerated storage at +4ºC (Figure II.1.). Significant changes ($p<0.05$) were detected for MDA content between day 0 and day 90 for liver pâtés from Iberian (from 0.36 mg MDA/Kg pâté to 1.00 mg MDA/Kg pâté) and white pigs (from 0.83 mg MDA/Kg pâté to 1.69 mg MDA/Kg pâté). Pâtés from white pigs presented significantly higher TBA-RS numbers than pâtés from Iberian pigs at all the stages of refrigerated storage, being detected the largest difference at day 90 (white: 1.69 mg MDA/Kg pâté; Iberian: 1.00 mg MDA/Kg pâté; $p<0.001$). Accordingly, the rate of TBA-RS increase during storage was higher in pâtés from white pigs compared to pâtés from Iberian pigs (Δ TBA-RS 'Iberian': 0.64 mg MDA/Kg pâté; Δ TBA-RS 'white': 0.86 mg MDA/Kg pâté). These results agree with those obtained in previous works devoted to the study of the oxidation stability of raw and cooked meats from Iberian and white pigs (Estévez *et al*., 2003; Estévez *et al*., in press, b) and could be partly explained by the equilibrium between prooxidant and antioxidant factors in the pâtés. Pâtés from white pigs presented a higher proportion of PUFA and lower of MUFA than pâtés from Iberian pigs that makes the former more prone to oxidation than the latter, as long as PUFA are more likely to be oxidised than MUFA or SFA. Moreover, in a previous work (Estévez *et al*. in press, a) we reported a significantly higher amount of tocopherols in the raw material used for the manufacture of pâtés (fat, liver and muscles) from Iberian pigs when compared to those from white pigs. The relationship between the nutritional background (pasture- and mixed diet finishing) and the fatty acid profile and oxidation stability of liver, pork and their based products is profusely documented (Cava *et al*., 2000; López-Bote & Rey, 2001; Nilzén *et al*., 2001). The intake of pasture by animals increases in their tissues the level of tocopherols, enhancing their oxidation stability (Cava *et al*., 2000; Nilzén *et al*., 2001). On the other hand, the pasture-grazing has been considered to increase the levels of *n-3* PUFA in meats as a result of the intake of grass (Nilzén *et al*., 2001; Mercier *et al*., 2004) that could be regarded as a prooxidant factor. This general pattern was neither

detected in this study, nor in a previous one (Estévez *et al*., in press, a). Despite of minority *n-3* fatty acids, muscles, back-fat and liver tissues from Iberian pigs presented large amounts of oleic acid and MUFA as a result of the intake of acorns during the last stage of their fattening period that has been linked to several quality traits (Cava *et al*., 1997; Ruiz *et al*., 1998). In the particular case of Iberian pigs, feeding on natural resources might enhance the oxidation stability of liver pâtés as a result of the presence of high levels of tocopherol and MUFA in the tissues used for their manufacture.

Protein oxidation during refrigerated storage of liver pâtés

Refrigerated storage had a significant effect on carbonyl content in liver pâtés (Figure II.2.). The amount of carbonyls significantly increased ($p<0.05$) from 2.88 nM carbonyls/mg protein to 13.64 nM carbonyls/mg protein and from 5.58 nM carbonyls/mg protein to 22.49 nM carbonyls/mg protein for pâtés from Iberian and white pigs, respectively. The accumulation of carbonyls mainly occurred from day 60 to day 90 of storage, being detected the highest amount of carbonyls at the end of the refrigerated storage. Compared to pâtés from Iberian pigs, pâtés from white pigs presented a higher amount of carbonyls at all days of study, being noticed the highest difference at day 90 (Iberian: 13.64 nM carbonyls/mg protein, white: 22.49 nM carbonyls/mg protein; $p<0.05$). These results agree with those obtained for lipid oxidation and indicated the possible linkage between lipid and protein oxidation. In fact, a statistically significant correlation was found (R^2: 0.71; $p<0.01$) between lipid and protein oxidation (Table II.3.). Similar results have been reported previously on beef (Mercier *et al*., 1995), turkey (Mercier *et al*., 1997) and microsomes 'in vitro' systems (Batifulier *et al*., 2002) when lipid and protein oxidation were assessed using TBA-RS and carbonyls quantification, respectively. In this sense, the possible protective effect of tocopherols in pâtés from Iberian pigs and the large differences in the fatty acid composition between pâtés from Iberian and white pigs could explain the higher oxidative instability of proteins in the latter. Though a loss of protein functionality associated to protein oxidation has been described (Stadtman, 1990), scarce

information is available on the impact of protein oxidation on meat products quality.

Release of iron from heme during refrigerated storage of liver pâtés

NHI content increased significantly during refrigerated storage (Figure II.3.). The amount of NHI increased from 27.35 µg/g pâté to 53.92 µg/g pâté and from 33.69 µg/g pâté to 73.01 µg/g pâté from day 0 to day 90 in samples from Iberian and white pigs, respectively. Pâtés from white pigs presented at all days of analysis, significantly higher amounts of NHI than pâtés from Iberian pigs ($p<0.05$). Results suggest that some disruption of the porphyrin ring could have occurred during storage that led to the release of iron. For Gómez-Basauri & Regenstein (1992a) and Miller *et al*. (1994a) the increase of NHI during refrigeration of meat is a reflection of the degradation of heme iron. Damage in the porphyrin ring during cooking or storage has been suggested to cause the breakdown of heme molecule and the release of iron from globin (Gómez-Basauri & Regenstein, 1992a; Miller *et al*. 1994a) yet, no conclusive theory has been reported. Results from this work may suggest a possible relationship between protein oxidation and the release of iron from heme. In fact, the release of iron mainly occurred in the last stage of storage (from day 60 to day 90), when the sharply increase of protein oxidation was detected. Miller *et al*. (1994a,b) found statistically significant correlations between TBA-RS and NHI content in cooked meat and ground and refrigerated pork. Results from this work are in good agreement since NHI significantly correlated to TBA-RS (R^2: 0.67; $p<0.01$), and to a higher extent (R^2: 0.86; $p<0.01$) to protein oxidation. The damage of the globin molecule may affect the stability of the heme molecule, leading to the subsequent release of iron.

The increase of NHI could have some decisive consequences affecting to both nutritional and technological properties of liver pâtés. The degradation of heme iron would reduce the nutritional value of the pâtés in terms of bioavailability of iron, since HI is more available than NHI (Hunt & Roughead, 2000). Thus, iron gains enhanced ability of promoting oxidation processes

when it is released from heme molecule (Kanner *et al.*, 1991), and therefore, pâtés with increasing amounts of NHI might also increase their oxidative susceptibility.

Evolution of instrumental colour during refrigerated storage of liver pâtés

At day 0, pâtés from Iberian and white pigs presented largely different colour characteristics (Table II.2.). Compared to pâtés from white pigs, pâtés from Iberian pigs showed a darker colour (L*: 61.45 *vs*. 64.54, $p<0.05$) with less redness (a* values: 9.13 *vs*. 11.32; $p<0.05$) and yellowness (b* values: 13.10 *vs*. 14.75, $p<0.05$). Thus, pâtés from white pigs presented larger values of chroma (18.60 *vs*. 15.97, $p<0.05$) and smaller of hue (52.52 *vs*. 55.16, $p<0.05$) that those from Iberian pigs' pâtés. Colour characteristics of liver pâtés are principally derived from the colour displayed by the fats, livers and muscles used for their manufacture (Estévez *et al.*, in press, a).

Storage significantly affected major instrumental colour parameters (Table II.2.). The lightness of the samples significantly increased during refrigerated storage from 61.45 to 64.54 in pâtés from Iberian pigs and from 66.52 to 67.19 in pâtés from white pigs. The increase of L* values was higher in pâtés from Iberian pigs (ΔL^*-value: 3.07) than those measured in pâtés from white pigs (ΔL^*-value: 0.67). The evolution of a*- and b*- values depended on the group studied. Pâtés from white pigs significantly increased their redness and yellowness during storage, reaching the higher values at day 60 (a*- and b*-values: 12.68 and 15.07, respectively). In contrast, a*- and b*- values from pâtés from Iberian pigs tended to decrease during refrigeration from 9.13 to 8.43 ($p>0.05$) and from 13.10 to 12.14 ($p<0.05$), respectively. The evolution of chroma in pâtés from Iberian and white pigs was similar to that described for a*- and b*-values. Hue angle did not vary during refrigerated storage of liver pâtés. The evolution of colour parameters reported in the present work are in agreement to results obtained from others authors studying colour stability of cooked sausage and liver pâté during refrigerated storage (Jo *et al.*, 1999, 2000; Perlo *et al.*, 1995). Nevertheless, in the aforementioned works, cooked products were refrigerated during a shorter period of time

(under 10 days) and therefore, results from those papers and from the present work are not totally comparable. Tarladgis (1962) reported that the compound responsible of the brownish grey colour of cooked meats is a ferric-porphyrin coordination complex of the denatured globin molecule. The modification of this structure, assessed in the present work as the degradation of heme molecule and release of iron, might affect the colour displayed by pâtés. In spite of the good correlations found between oxidative processes and colour parameters (Table II.3.), colour changes reported in the present work seemed not to be directly related to lipid or protein oxidation since pâtés with the higher oxidative stability (from Iberian pigs) suffered higher colour changes when compared to pâtés from white pigs. In fact, total colour change as measured by the numerical colour difference between day 0 and day 90 ($\Delta E_{0\text{-}90}$), was significantly higher in pâtés from Iberian pigs than in those from white pigs (3.35±1.25 *vs*. 0.97±0.29; $p<0.05$). In spite of the large colour changes detected, differences between groups remained unchanged after 90 days of storage and, therefore, pâtés from Iberian pigs would keep the particular colour that might difference them from pâtés elaborated with raw material from white pigs (Estévez *et al*., in press). Compared to refrigerated pâtés from Iberian pigs, those from white pigs were paler (L*: 64.52 *vs*. 67.19, $p<0.05$) and exhibited a more reddish (a* values: 8.43 *vs*. 11.89; $p<0.05$) and yellowish (b* values: 12.14 *vs*. 14.89, $p<0.05$) colour.

II.6. Conclusions

According to this study, lipid oxidation, protein oxidation and the increase of NHI during refrigerated storage of liver pâtés could be closely related. Colour changes seem not to be linked to oxidative processes, or at least, are affected by other circumstances not considered in the present work. Free-range rearing and feeding Iberian pigs on natural resources enhances oxidative stability of liver pâtés as a result of the high content of MUFA and tocopherols in their tissues. Compared to pâtés from intensively reared white pigs, pâtés from extensively reared Iberian pigs present higher aptitude to be stored

under refrigeration conditions. Further research on the relationships among lipid and protein oxidation and the stability of myoglobin on cooked products would be of interest.

II.7. Acknowledgements

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Table II.1. General composition (mean ± standard deviation) of liver pâtés from Iberian pigs and white pigs.

	Iberian	white	p[2]
Moisture[1]	48.42±1.37	50.51±0.62	ns
Protein[1]	10.34±0.24	10.04±0.70	ns
Ash[1]	2.69±0.09	2.78±0.21	ns
Fat[1]	33.37±1.81	31.82±0.57	ns
Fatty acids[3]			
SFA	32.87±0.09	37.98±0.12	***
MUFA	57.52±0.06	47.58±0.10	***
PUFA	9.63±0.11	14.40±0.25	***

[1] g/100g pâté.
[2] Statistical significance.
[3] Percentage of total methyl esters analysed.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
Ns: non significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Table II.2. Evolution of colour parameters L*, a*, b*, chroma and hue measured on liver pâtés from Iberian and white pigs during 90 days under refrigerated storage.

		Storage (days)				
		0	30	60	90	SEM[1]
L*	Iberian	61.45^{b}	63.31^{a}	63.99^{a}	64.52^{a}	0.31
	white	66.52^{b}	66.32^{b}	66.87^{ab}	67.19^{a}	0.38
	SEM	0.87	0.62	0.45	0.44	-
a*	Iberian	9.13	9.33	9.29	8.43	0.14
	white	11.32^{b}	11.53^{ab}	12.68^{a}	11.89^{ab}	0.18
	SEM	0.38	0.40	0.63	0.61	-
b*	Iberian	13.10^{a}	12.33^{b}	12.73^{ab}	12.14^{b}	0.11
	white	14.75^{ab}	14.55^{b}	15.07^{a}	14.89^{ab}	0.07
	SEM	0.29	0.38	0.41	0.44	-
Chroma	Iberian	15.97^{a}	15.46^{ab}	15.76^{a}	14.79^{b}	0.14
	white	18.60^{b}	18.57^{b}	19.70^{a}	19.11^{ab}	0.10
	SEM	0.45	0.53	0.70	0.71	-
Hue	Iberian	55.16	52.92	53.91	55.29	0.41
	white	52.52	51.67	50.01	51.20	0.63
	SEM	0.58	0.52	0.80	0.90	-

[1] SEM: standard error of the mean.
Means within a row with different superscripts are different ($p<0.05$).
All pairs of means from Iberian and white pigs for each parameter, within a day, resulted different ($p<0.05$).

Table II.3. Pearson's correlation coefficients (R^2).[1]

	R^2
TBA-RS vs. Pox[2]	0.71**
TBA-RS vs. NHI	0.67**
Pox vs. NHI	0.86**
TBA-RS vs. L*	0.74**
TBA-RS vs. a*	0.66**
Pox vs. L*	0.39*
Pox vs. a*	0.25
NHI vs. L*	0.36*
NHI vs. a*	0.17

[1]n= 10 liver pâtés for correlation coefficients taken from measurements throughout the storage period.
[2]Protein oxidation as assessed by total carbonyl content.

Figure II.1. Evolution of lipid oxidation of liver pâtés from Iberian and white pigs under refrigerated storage.

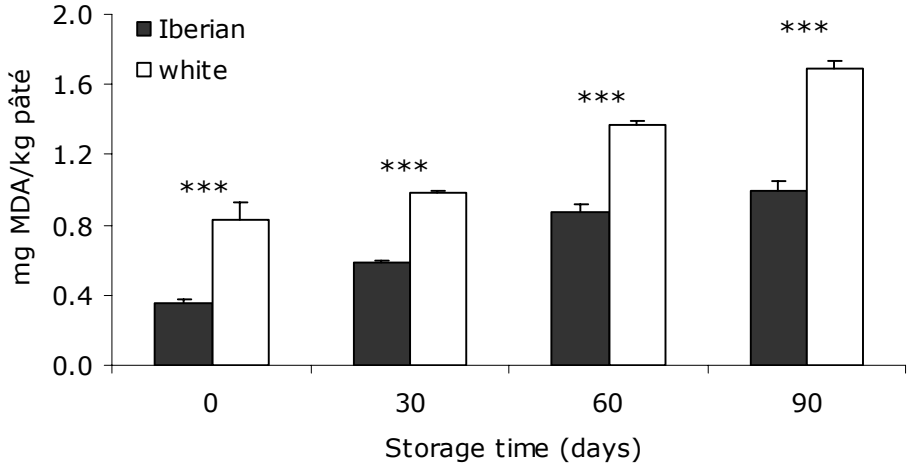


Figure II.2. Evolution of protein oxidation of liver pâtés from Iberian and white pigs under refrigerated storage.

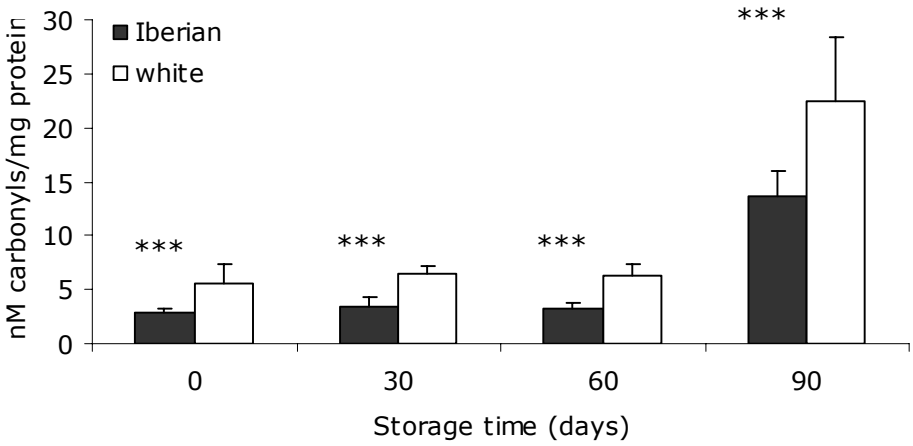
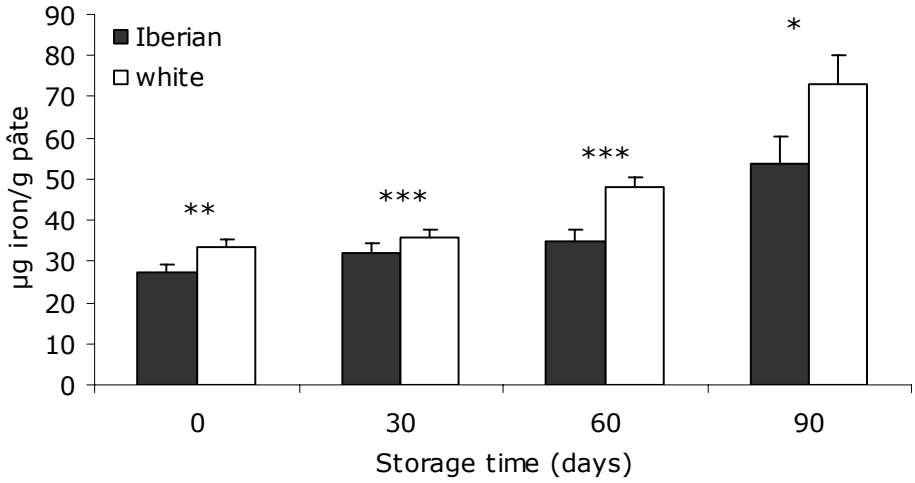


Figure II.3. Evolution of non-heme iron content of liver pâtés from Iberian and white pigs under refrigerated storage.



CHAPTER III

PUFA degradation, TBA-RS and lipid-derived volatiles in refrigerated stored liver pâté: effect of natural and synthetic antioxidants[♣]

[♣] Accepted for publication in *LWT- Food Science and Technology* (14 July, 2005)

III.1. Abstract

The antioxidant effect of two plant extracts (sage and rosemary essential oils) and one synthetic antioxidant (BHT) on refrigerated stored liver pâté (+4ºC/90 days) was evaluated. Pâtés with no added antioxidants were used as controls. Liver pâtés were analysed for the amount of PUFA, TBA-RS numbers, and lipid-derived volatiles at days 0, 30, 60 and 90 of refrigerated storage. The amount of PUFA gradually decreased during refrigerated storage of porcine liver pâtés as a likely consequence of the development of oxidative reactions with this increase being significantly higher ($p<0.05$) in control and BHT pâtés than in those with added essential oils. The oxidative degradation of PUFA caused a significant increase of TBA-RS during refrigerated storage of liver pâtés with this increase being significantly higher in control pâtés than in the treated counterparts. At days 60 and 90, TBA-RS numbers in treated pâtés were significantly smaller ($p<0.05$) than in the control counterparts.

Headspace solid-phase microextraction (HS-SPME) successfully isolated recognised indicators of lipid decomposition (i.e. hexanal, nonanal, hexan-1-ol, oct-1-en-3-ol, deca-(*E,Z*)-2,4-dienal) from liver pâtés whereas some others volatiles analysed are typical contributors to the overall off-flavour of oxidised liver (i.e. hept-(*Z*)-4-enal, non-(E)-2-enal, nona-(*E,E*)-2,4-dienal). The addition of antioxidants significantly ($p<0.05$) reduced the total amount of lipid-derived volatiles isolated from liver pâtés HS. Plant extracts inhibited oxidative deterioration of liver pâtés to a higher extent than BHT did.

III.2. Keywords: Liver pâté, PUFA, TBA-RS, lipid-derived volatiles, natural antioxidants, BHT.

III.3. Introduction

Apart from microbial spoilage, lipid oxidation is the primary process by which nutritional and sensory quality traits decline in muscle and fat foods (Gray *et al.*, 1996). Lipid oxidation undergoes degradation of polyunsaturated fatty acids (PUFA) and generation of residual products, such as malondialdehyde (MDA) and lipid-derived volatiles leading to sensory and nutritional

deterioration of meat (Kanner *et al*., 1991). Oxidative reactions in foodstuffs are enhanced after cooking and refrigerated storage through the increase of their oxidative instability due to the degradation of natural antioxidants and the release of free fatty acids and iron from the heme molecule (Kingston *et al*., 1998; Kristensen & Purslow, 2001; Estévez & Cava, 2004). Particularly remarkable is the rapid onset of 'rancid' off-notes that develop in cooked meats during refrigerated storage as a result of the generation of some volatile compounds from PUFA degradation that is known as warmed-over flavour (WOF) (Tims & Watts, 1958).

Liver pâté is a product with an important gastronomic tradition and is generally considered an added-value product with high nutritional and sensory quality (Le Ba & Zuber, 1996; Russell *et al*., 2003). For the manufacture of porcine liver pâté, ingredients (mainly liver and fat from animals) are finely minced and are given a thermal treatment after which the raw batter is placed in terrines (Estévez *et al*., 2004a). After manufacture, packed liver pâtés are stored under refrigerated conditions, during which, the development of oxidative reactions reduces liver pâté's quality (Estévez & Cava, 2004; Russell *et al*., 2003; Fernández-López *et al*., 2004). Liver pâté is a product with a high oxidative instability as a consequence of (i) its chemical composition and (ii) the technologies applied for its manufacture. Liver pâté have high amounts of fat (around 35%) and non-heme iron (around 30µg/g pâté) (Estévez *et al*., 2004a) with the latter being considered the most important prooxidant in meat systems (Kanner, 1994). Furthermore, mincing and macerating animal tissues increases oxidative instability by causing interactions between free fatty acids and oxygen in the presence of catalysts such as heat and metalloproteins (Kanner *et al*., 1991; Morrissey *et al*., 1998). Moreover, high temperatures during cooking reduce activation energy for the development of oxidative reactions and cause the breakdown of preformed hydroperoxides (Kanner, 1994). On the other hand, pâté matrix may be low in natural antioxidants, because these are often lost during processing, justifying the addition of exogenous antioxidants in order to inhibit the development of the aforementioned oxidative reactions (Madhavi *et al*.,

1996). In the last years many researchers have evaluated the antioxidant properties of extracts from different plants and vegetables (Ichikawa *et al*., 2003; Chen *et al*., 2002; Ibanez *et al*., 2003). Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officianalis*) are popular *Labiatae* herbs with a verified potent antioxidant activity (Dorman *et al*., 2003). Nevertheless, the protective activity of these plant extracts against lipid oxidation on a product with the oxidative instability of liver pâté remains unknown.

The aims of the present work were to investigate the effect of the addition of natural antioxidants (sage and rosemary essential oils) on the lipid oxidative stability of refrigerated stored pâtés and compare this effect with displayed by a synthetic antioxidant (BHT). In addition, the analysis of the oxidative stability of a foodstuff taking into account at the same time the degradation of PUFA and the generation of residual products such as malondialdehyde (MDA) and volatile compounds is an interesting challenge since they are commonly studied separately.

III.4. Material and Methods

Animals and sampling

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed selection schemes were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered around the weight of 150 Kg and an age of 12 months. After slaughter, back fat, muscle *quadriceps femoris*, and liver were removed from carcasses, vacuum packaged and stored at -80ºC until the day of the manufacture of pâtés.

Manufacture of the liver pâté

The experimental pâtés (spread type) were manufactured in a pilot plant. The same experimental formula was used for all pâtés, except for the addition of the different antioxidants. In the basic formulation the ingredients were as follows per 100g of manufactured product: 28 g liver, 40 g subcutaneous fat,

5 g meat, 23 g distilled water, 2 g sodium caseinate, and 2 g sodium chloride. Sodium di- and tri-phosphates (0.3%), sodium ascorbate (0.05%), and sodium nitrite (0.03%) (all from ANVISA, Madrid, Spain) were also added. Depending on the experimental batch, different antioxidants were added to the aforementioned formula: rosemary extract ('ROSE') (0.1%), sage extract ('SAGE') (0.1%), and BHT ('BHT') (0.02%). The natural antioxidant extracts (Soria Natural S.L., Soria, Spain) are considered as GRAS (Generally Recognised as Safe) and were added at a level (0.1%) at which highest antioxidant activity would be exhibited (McCarthy *et al*., 2001). BHT (Sigma-Aldrich, Steinheim, Germany) was added up to the highest level allowed by the Spanish law for this kind of product (0.02%) (BOE, 2002). Control pâtés ('CON') containing no added antioxidants were also prepared. The protocol followed for the manufacture of liver pâtés has been explained elsewhere (Estévez *et al*., 2004a). Following the aforementioned recipe, 1.5 kg of raw material was used for each group, to produce the experimental pâtés. A composite mixture of fat, liver and meat from seven animals was used to prepare the 4 types of pate. The day before to the manufacture, the fat was chopped into small cubes (1.5 cm^3) and scalded in distilled water to an internal temperature of +65ºC during 30 min. Liver and muscle were also sliced in small cubes (1.5 cm^3) and mixed with the sodium chloride, the sodium nitrite and the sodium ascorbate in order to allow the nitrification of the tissues. The cooked fat and the nitrified mixture were kept under refrigeration (+4ºC) in the darkness, before the manufacture of the liver pâtés (24 hours). The day of the production, the sodium caseinate was totally dissolved in hot water (+75ºC) and then added to the scalded fat and mixed during mincing in a cutter (Foss Tecator Homogeniser, mod. 2094) during 3 minutes. After that, the nitrified mixture was added to the cutter bowl, together with the water and the sodium di- and tri-phosphates. The whole mixture was completely minced during 3 minutes until a homogenous raw batter was obtained. Finally, the mixture was packed in glass containers (~50g of pâté per container; 5 containers per group) and given a thermal treatment (+80ºC/30 min.). After the containers were allowed to cool at room

temperature, they were stored in the dark at +4ºC for 90 days, with the day of manufacture being day 0. Liver pâtés were analysed at days 0, 30, 60 and 90 for PUFA content, TBA-RS numbers and lipid-derived volatiles extracted from their headspace (HS) using the solid-phase microextraction (SPME) technique. After each of the refrigeration periods the samples were stored at -80ºC until the other analytical experiments were conducted.

Analytical methods

Compositional analysis of liver pâté

Moisture, total protein and ash were determined using official methods (AOAC, 2000). The method of Bligh and Dyer (Bligh & Dyer, 1959) was used for determining fat content of liver pâtés.

Fatty acid composition and PUFA quantification

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a FFAP-TPA fused-silica column (Hewlett Packard 30m long, 0.53 mm internal diameter and 1.0 µm film thickness). The injector and the detector temperature were held at +230ºC. Oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma). Fatty acid composition was expressed as percent of total fatty acid methyl esters. The quantification of PUFA (sum of C18:2, C18:3 and C20:4) was carried out by using C13 as an internal standard. Results are expressed as g PUFA $100g^{-1}$ pâté. The percent protection of PUFA was calculated at day 90 as $[(T_{90} - C_{90})/T_{90})] \times 100$, where T_{90} is the amount of PUFA in the treated pâté at day 90 and C_{90} is the amount of PUFA in control pâtés at day 90.

TBA-RS numbers

Thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al*. (1996). Results are expressed as mg malondialdehyde (MDA)/ kg pâté. The percent inhibition against lipid oxidation was calculated at day 90 as $[(C_{90} - T_{90})/C_{90})] \times 100$, where T_{90} is the amount of MDA in the treated pâté at day 90 and C_{90} is the amount of MDA in control pâtés at day 90.

Lipid-derived volatiles analysis

The SPME fibre, coated with a divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30µm, was preconditioned prior analysis at +220ºC during 45 min. The HS sampling was performed following a method previously described (Estévez *et al*., 2004b). 1 g of pâté was placed in 2.5 mL vials and the SPME fibre was exposed to the headspace of the pâté while the sample equilibrated during 30 minutes immersed in water at +60ºC. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm id., 1.0mm film thickness). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40 ^{o}C. The SPME fibre was desorbed and maintained in the injection port at 220 ^{o}C during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at +40ºC and then raised at the rate of +7ºC min^{-1} to +250 ^{o}C, and held for 5 min. n-Alkanes (Sigma R-8769) were run under the same conditions to calculate the Kovats index (KI) values for the compounds. The GC/MS transfer line temperature was +270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Volatile compounds were tentatively identified by comparing their mass spectra with those contained in the Wiley and NIST libraries and by comparison of their KI with those reviewed by Kondjoyan and

Berdagué (1996). Results from the volatiles analysis are provided in area units (AU).

Data Analysis

Means and deviations from 5 measurements within a batch were obtained from all analytical experiments. Results from the experiments were used as variables and analysed by using an Analysis of Variance (ANOVA) from SPSS software in order to assess the effect of the addition of the antioxidants on the oxidative stability of liver pâtés. The study of the effect of refrigerated storage on the oxidative deterioration of liver pâtés was carried out by using a t-student test for dependant variables. When statistically significant differences were found, Tukey test's were performed. Statistical significance was set at $p<0.05$.

III.5. Results and Discussion

General composition of liver pâtés

No significant differences between groups were detected for the chemical composition since all pâtés presented similar moisture, fat, protein and ash contents (Table III.1.). 'SAGE' and 'ROSE' pâtés showed smaller percentages of stearic and saturated fatty acids (SFA) ($p<0.05$) compared to the 'CON' ones. Compared to the other pâtés, 'BHT' ones had smaller proportion of MUFA. Regardless of the fact that the addition of antioxidants had a significant effect on the fatty acid profiles of pâtés the differences between groups were slightly marked and did not show a clear pattern.

PUFA degradation

As expected, liver pâtés had similar amounts of PUFA at day 0. The amount of PUFA gradually decreased during refrigerated storage of liver pâtés as a likely consequence of the development of oxidative reactions (Figure III.1.). The study of the oxidative deterioration of a fat product as assessed by PUFA degradation is of high importance because PUFA are preferentially affected by

oxidative reactions due to the presence of double bounds in the hydrocarbon chain (Gray *et al.*, 1998). Furthermore, PUFA are mainly placed on the phospholipids located in cellular membranes where the oxidative reactions commence because of the proximity to cellular prooxidants such as metalloproteins (Morrissey *et al.*, 1998). The decrease rate of PUFA content as measured by the slopes of the calculated trend lines ('CON': -0.053; 'BHT': -0.059; 'SAGE': -0.049; 'ROSE': -0.045) revealed that the rate of PUFA loss was higher in 'CON' and 'BHT' pâtés than in those with added plant extracts. Accordingly, 'CON' and 'BHT' liver pâtés lost 38.76% and 38.38% of the initial PUFA content whereas 'SAGE' and 'ROSE' pâtés lost a smaller content of PUFA (34.85% and 31.55%, respectively). The addition of antioxidants in pâtés probably protected PUFA from oxidative degradation since at days 30 and 60 pâtés with added antioxidants presented a significantly higher amount of PUFA than 'CON' pâtés. At day 90, a similar trend was observed but ANOVA did not detect significant differences among groups. The percent protection of PUFA by the added antioxidants at day 90 was similar amongst groups ('BHT': 7.92%; 'SAGE': 7.16%; 'ROSE': 6.91%).

TBA-RS

Results from the analysis of the oxidative deterioration of lipids from pâtés during refrigerated storage as measured by TBA-RS numbers are shown in Figure III.2. The oxidative degradation of PUFA caused a gradual increase of TBA-RS during refrigerated storage of liver pâtés. TBA-RS numbers significantly ($p<0.05$) increased after 90 days of refrigerated storage in 'CON', 'BHT' 'SAGE', and 'ROSE' pâtés, with this increase being significantly higher in 'CON' pâtés than in the treated counterparts (Δ TBA-RS 'CON': 0.64, 'BHT': 0.41, 'SAGE': 0.14, 'ROSE': 0.11; $p<0.05$). Though similar in all batches at days 0 and 30, TBA-RS numbers in treated pâtés were significantly smaller ($p<0.05$) than in the control counterparts at days 60 and 90. Compared to those with added sage and rosemary essential oils, 'BHT' pâtés had significantly higher TBA-RS numbers at days 60 and 90. Considering the percent inhibition against lipid oxidation ('BHT': 27.95%; 'SAGE': 48.22%;

'ROSE': 52.50%), the natural antioxidants were more effective protectors as they inhibited the generation of MDA better than the BHT.

Plant essential oils have been successfully introduced to inhibit oxidative deterioration of meat and fat products, being this deterioration generally referred to the accumulation of lipid oxidation derived products and to the generation of lipid-derived volatiles in meat products (McCarthy *et al*., 2001; Yu *et al*., 2002*;* Ahn *et al*., 2002). Chen *et al*. (1999), Formanek *et al*. (2001) and McCarthy *et al*. (2001) reported the high effectiveness of antioxidants from natural resources against oxidative reactions that showed similar activity to those from synthetic origin such as BHT. Sebranek *et al*. (2004) reported similar antioxidant activities of rosemary extracts and synthetic ones (BHT/BHA) regarding MDA generation in refrigerated sausages. Results from the present study agree with those obtained by the aforementioned authors, denoting even the possibility of replacing synthetic antioxidants such as BHT with natural extracts with antioxidant activity obtained from plants.

Lipid-derived volatiles

From the total of volatile compounds isolated from the headspace (HS) of the experimental pâtés, eighteen lipid-derived volatiles are shown in Table III.2. Hexanal, hexan-1-ol, nonanal and non-(*Z*)-2-enal were the most abundant lipid-derived volatiles in pâtés HS. Some others such as hept-(*E*)-4-enal, non-(*Z*)-2-enal and 2,4-alkadienals are given high importance despite of their small amounts because of their low thresholds and impact odours (Frankel, 1984). The off-flavours produced as a consequence of the thermal treatment of lipid-rich foods such as liver pâté, are mainly derived from the autooxidation of lipids (Estévez *et al*., 2004b; Im *et al*., 2004). In fact, the major lipid-derived volatile compounds detected in the present study, have been described as indicators of lipid decomposition and contributors to the overall off-flavours of oxidised liver (Im *et al*., 2004). Hexanal, for instance, has been considered as an indicator of lipid oxidation and has been profusely taken into account to assess oxidative deterioration of muscle foods (Shahidi & Pegg, 1993). On the other hand, hept-(*Z*)-4-enal has been linked to 'fishy'

and unpleasant flavours while some alkadienals such as nona-2,4-dienal has been associated with the oxidative deterioration of PUFA and rancid odours (Lee *et al.*, 2003; Im *et al.*, 2004). The oxidation of PUFA undergoes the formation of some other volatile compounds such as non-(*Z*)-2-enal related to 'cardboard-like' odour and deca-(*E,Z*)-2,4-dienal) associated to rancid and warmed-over flavours (Lee *et al.*, 2003; Im *et al.*, 2004).

The generation of lipid-derived volatiles significantly ($p<0.05$) increased in all batches from day 0 to day 90 as the development of oxidative reactions progressed during storage of pâtés (Figure III.3.), with this increase being significantly higher in 'CON' pâtés than in the treated counterparts ('CON': 35.97; 'BHT': 12.48; 'SAGE': 4.19; 'ROSE': 6.36; $p<0.05$). This increase was particularly remarkable for straight-chain saturated aldehydes such as hexanal, octanal, nonanal and unsaturated aldehydes such as but-2-enal, hept-(*Z*)-4-enal and non-(*Z*)-2-enal (Table III.2.). Some other volatiles such as dec-(*E*)-2-enal and deca-(*E,Z*)-2,4-dienal were generated during refrigerated storage since they were not detected at day 0.

The addition of antioxidants significantly reduced the total amount of lipid-derived volatiles isolated from liver pâtés HS. At day 90, 'CON' pâtés (49.54 AU) had a significantly higher total amount of lipid-derived volatiles than 'SAGE' and 'ROSE' pâtés (9.50 AU and 11.80 AU, respectively) while 'BHT' pâtés presented an intermediate content (20.20 AU). At day 90, treated pâtés presented, compared to control pâtés, significantly smaller amounts of hexanal, hexan-1-ol, oct-1-en-3-ol, 2-pentyl-furan, nonanal, but-2-enal, nona-(*E,E*)-2,4-dienal, non-(*E*)-2-enal, dec-(*E*)-2-enal, and deca-(*E,Z*)-2,4-dienal.

The development of oxidative reactions during refrigerated storage of liver pâtés could have worsen the aroma characteristics of liver pâtés since most volatiles generated during refrigerated storage are closely related to WOF and rancid aromatic notes (Frankel, 1984; Im *et al.*, 2004). In this sense, the addition of antioxidants might have reduced this deterioration through the inhibition of some lipid-derived volatiles generation. Furthermore, the addition of plant extracts greatly influences on the aromatic profile of the products in

which they are added since some volatile components of this extracts are terpenes which might contribute to add specific aromatic notes (Estévez *et al*., 2004b). The addition of BHT, however, was not so efficient than the addition of sage and rosemary essential oils since compared to 'SAGE' and 'ROSE' pâtés, 'BHT' pâtés presented significantly higher amounts of several volatiles such as but-2-enal, hept-(*Z*)-4-enal and nona-(*E,E*)-2,4-enal closely related to lipid oxidation and off-flavours in liver products (Im *et al*., 2004). These results are consistent with those obtained from the TBA-RS analysis and agree with those previously reported (McCarthy *et al*., 2001; Chen *et al*., 1999; Yu *et al*., 2002; Ahn *et al*., 2002).

III.6. Conclusions

The analysis of PUFA content together with the detection of TBA-RS and lipid-derived volatiles using SPME were interesting techniques to accurately assesses oxidative deterioration of refrigerated storage liver pâtés. The addition of exogenous antioxidants on liver pâtés improved the oxidative stability of lipids, reducing the degradation of PUFA and inhibiting the generation of residual components such as MDA and lipid-derived volatiles. According to the results from the present work, sage and rosemary essential oils protected liver pâtés from oxidation processes and could be used as alternative options to synthetic antioxidants such as BHT.

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Table III.1. General composition of liver pâtés with added BHT and sage and rosemary essential oils[1].

	CON	BHT	SAGE	ROSE	sem[2]
Moisture[3]	4.84	4.91	4.92	4.93	0.25
Fat[3]	3.34	3.20	3.34	3.26	0.12
Protein[3]	1.03	0.98	1.00	0.99	0.30
Ash[3]	0.27	0.28	0.28	0.29	0.04
Fatty acids[4]					
c14:0	1.12^{b}	1.14^{ab}	1.13^{b}	1.15^{a}	0.00
c16:0	20.69^{ab}	20.80^{a}	20.61^{b}	20.62^{b}	0.02
c18:0	10.58^{a}	10.58^{a}	10.50^{b}	10.42^{c}	0.01
∑ SFA	32.87^{ab}	32.99^{a}	32.72^{bc}	32.69^{c}	0.03
c16:1	2.00^{a}	2.00^{a}	1.98^{ab}	1.96^{b}	0.00
c18:1	53.43^{bc}	53.38^{c}	53.59^{a}	53.54^{ab}	0.03
c20:1	1.83^{bc}	1.79^{c}	1.86^{ab}	1.90^{a}	0.00
∑ MUFA	57.52^{ab}	57.42^{b}	57.62^{a}	57.65^{a}	0.03
c18:2	7.71	7.69	7.82	7.75	0.02
c18:3	0.49	0.49	0.51	0.50	0.01
c20:4	0.56^{ab}	0.55^{b}	0.57^{a}	0.53^{c}	0.00
∑ PUFA	9.63	9.57	9.65	9.64	0.03

[1] Values with a different letter (a-c) within a row are significantly different ($p<0.05$).
[2] Standard error of the mean.
[3] g kg^{-1} pâté.
[4] Percentage of total methyl esters determined.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table III.2. Lipid-derived volatiles (AUx10^6) isolated from the HS of liver pâtés before (day 0) and after 90 days (day 90) of refrigerated storage[1].

	Day 0					Day 90				
	CON	BHT	SAGE	ROSE	sem[2]	CON	BHT	SAGE	ROSE	sem[2]
hexanal	2.26^{a}	0.25^{b}	0.29^{b}	0.31^{b}	0.21	4.16^{a}	0.60^{b}	0.34^{b}	0.33^{b}	0.38
hexan-1-ol	1.29^{a}	1.08^{a}	0.48^{b}	0.57^{b}	0.09	1.70^{a}	0.61^{b}	0.16^{b}	0.18^{b}	0.16
heptanal	0.51^{a}	0.36^{b}	0.11^{c}	0.46^{ab}	0.04	0.98^{a}	0.67^{ab}	0.52^{ab}	0.35^{b}	0.09
oct-1-en-3-ol	0.52^{a}	0.26^{b}	0.43^{ab}	0.58^{a}	0.03	0.83^{a}	0.43^{b}	0.46^{b}	0.48^{b}	0.04
2-pentyl-furan	0.33^{b}	0.16^{c}	0.45^{a}	0.10^{c}	0.03	0.51^{a}	0.21^{b}	0.13^{b}	0.25^{b}	0.04
octanal	0.65	0.42	0.38	0.44	0.04	1.48^{a}	0.83^{ab}	0.52^{b}	0.70^{ab}	0.12
2-ethyl-hexan-1-ol	0.52^{b}	0.53^{ab}	0.56^{ab}	0.84^{a}	0.05	12.06^{a}	6.44^{b}	1.21^{c}	1.70^{c}	1.03
hexa-2,4-dien-1-ol	0.25^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.03	1.30^{a}	0.48^{ab}	0.36^{b}	0.62^{ab}	0.13
2,5-dihydro-furan	0.37^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.04	0.35	0.19	0.16	0.13	0.03
octan-1-ol	0.50	0.48	0.48	0.43	0.05	0.89^{a}	0.72^{ab}	0.62^{b}	0.70^{ab}	0.08
nonanal	4.05^{a}	2.82^{ab}	0.89^{b}	0.85^{b}	0.42	15.77^{a}	4.68^{b}	1.35^{b}	2.32^{b}	1.51
hept-(*Z*)-4-enal	0.42	0.51	0.63	0.40	0.04	1.03^{a}	0.85^{a}	0.51^{b}	0.47^{b}	0.06
but-2-enal	0.32^{ab}	0.39^{a}	0.30^{ab}	0.26^{b}	0.01	1.39^{a}	0.52^{b}	0.51^{b}	0.18^{c}	0.11
nona-(*E,E*)-2,4-dienal	0.51^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.05	1.57^{a}	1.12^{b}	0.72^{c}	0.65^{c}	0.09
non-(*Z*)-2-enal	0.79^{a}	0.11^{b}	0.00^{c}	0.00^{c}	0.08	0.98	1.26	0.84	0.36	0.23
non-(*E*)-2-enal	0.29^{b}	0.34^{b}	1.09^{a}	0.19^{c}	0.19	2.15^{a}	0.59^{b}	0.61^{b}	0.00^{c}	0.23
dec-(*E*)-2-enal	0.00	0.00	0.00	0.00	0.00	0.74^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.07
deca-(*E,Z*)-2,4-dienal	0.00	0.00	0.00	0.00	0.00	1.65^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.17

[1] Within a day, values with a different letter (a-c) were significantly different ($p<0.05$).
[2] Standard error of the mean.

Figure III.1. Evolution of PUFA amounts during refrigerated storage of porcine liver pâtés. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

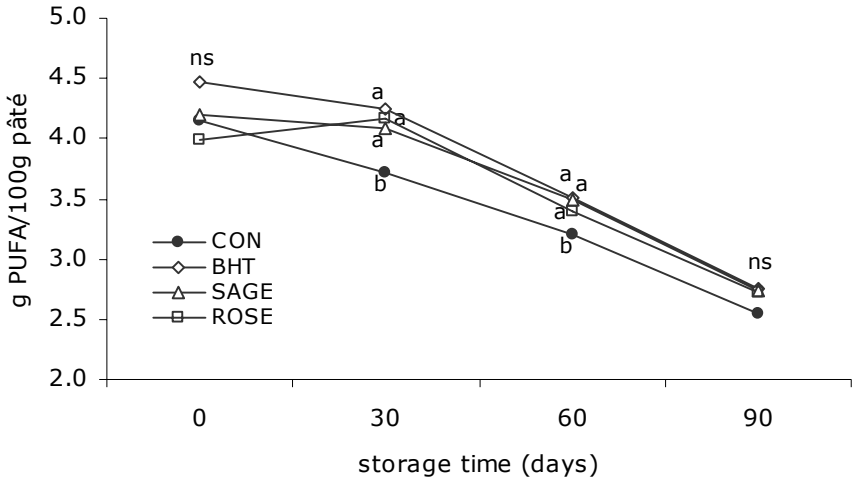


Figure III.2. Evolution of TBA-RS numbers during refrigerated storage of porcine liver pâtés. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

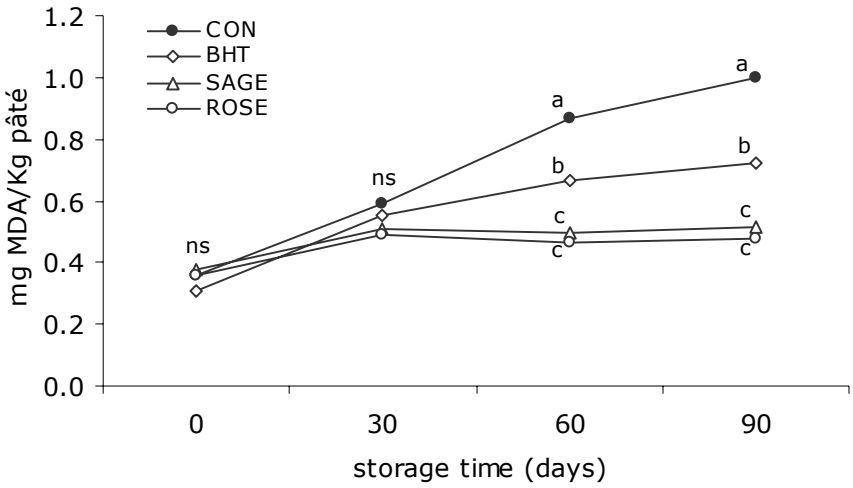
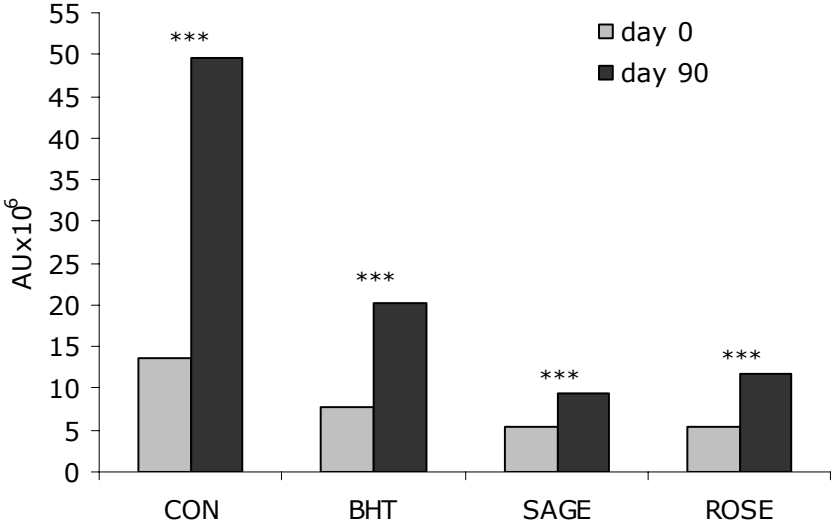


Figure III.3. Total lipid-derived volatiles isolated from the HS of porcine liver pâtés at day 0 (before refrigeration) and day 90 of refrigerated storage. (Statistical significance was considered as follows: $p>0.05$ (ns), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***)).



CHAPTER IV

Protein oxidation and colour and texture changes in refrigerated stored liver pâté: effect of natural and synthetic antioxidants[*]

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IV.1. Abstract

The antioxidant effect of two plant essential oils (sage and rosemary) and one synthetic antioxidant (BHT) on refrigerated stored liver pâté (+4ºC/90 days) was evaluated. Pâtés with no added antioxidants were used as controls. Liver pâtés were analysed for protein oxidation, modification of heme (HI) and non-heme iron (NHI) concentrations, and colour and texture characteristics at days 0, 30, 60 and 90 of refrigerated storage. The amount of carbonyls from protein oxidation significantly ($p<0.05$) increased during refrigerated storage, and this increase was significantly higher in control pâtés than in the treated counterparts. Antioxidants successfully protected heme molecule from degradation and significantly inhibited the increase of NHI in refrigerated stored liver pâtés. Colour changes seemed not to be directly related to oxidation processes since pâtés with added antioxidants suffered higher colour modifications than the controls ones. The addition of rosemary essential oil significantly reduced hardness of liver pâtés. Sage and rosemary essential oils exhibited similar antioxidant properties than the pure antioxidant (BHT) denoting their suitability as alternative options to synthetic antioxidants.

IV.2. Keywords: Liver pâté, oxidation, heme iron, non-heme iron, colour, texture, natural antioxidants.

IV.3. Introduction

Regardless of microbial spoilage, lipid oxidation is the major factor reducing quality and acceptability of meat and fat products (Morrissey *et al.*, 1998). Lipid oxidation involves degradation of polyunsaturated fatty acids and generation of free radicals, leading to the deterioration of proteins, the oxidation of heme pigments, and the generation of rancid odours (Kanner, 1994). Though recent studies of protein oxidation in biomedical sciences have shed light on the mechanisms by which extracellular and membrane proteins can be affected by ROS leading to adverse biological effects (Butterfield & Stadtman, 1997; Chevion *et al.*, 2000), hardly any work devoted to the study of protein oxidation in muscle foods has been carried out. Recent studies on

model and food systems have pointed out that the oxidative damage of proteins leads to alterations in gelation, emulsification, viscosity, solubility and hydratation (Wang *et al*., 1997; Wang & Xiong, 1998). Little is known, however, about protein oxidation in muscle foods concerning the precise chemical mechanism of protein oxidation, the characterisation of the protein oxidation products, the adverse effects on meat quality and the effectiveness of different antioxidant strategies against protein oxidation.Concerning oxidation promoters in animal food products, iron is thought to have high catalytic activity (Rhee *et al*., 1987a). The knowledge of the approximate proportion between the different chemical forms of iron is of a great importance because of the strong differences displayed by heme iron (HI) and nonheme iron (NHI) in terms of promotion of lipid oxidation and bioavailability. NHI is considered the most important oxidation promoter in meat systems (Kanner *et al*., 1991) whereas HI is thought to be more bioavailable (Hunt & Roughead, 2000).

Liver pâté is a traditional product with increasing demand by Spanish, French and Danish consumers in the last 15 years (Perlo *et al*., 1995; Fernández-López *et al*., 2004). Pâté contains high levels of fat and iron which considerably increases its oxidative instability (Russell *et al*., 2003; Estévez *et al*., 2004). The development of lipid and protein oxidation during refrigerated storage of liver pâtés leads to the degradation of the heme molecule and the release of iron, increasing even more the oxidative instability of the product (Estévez & Cava, 2004). As other meat systems, the pâté matrix is relatively poor in natural antioxidants, which justifies the addition of exogenous antioxidants (Madhavi *et al*., 1995) in order to inhibit the development of oxidative reactions. Synthetic phenolic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl, octyl, and dodecyl gallates (PG, OG, DG), are easily available and largely used in different food products (Pinho *et al*., 2000). Using such synthetic compounds has been linked to health risks generally believed to have a carcinogenic potential (Clayson *et al*., 1986). Consequently, a high scientific effort has been exerted to select natural essential oils with antioxidant potential as

alternative options to synthetic antioxidants. Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) are popular *Labiatae* herbs with verified potent antioxidant activity. Carnosic acid and other diphenolic abietane diterpenes are the major phenolic constituents of these plants (Tada, 2000). The effectiveness of sage and rosemary essential oils as antioxidants has been demonstrated in a large variety of foodstuffs including refrigerated beef (Djenane *et al*., 2003), frozen pork patties (McCarthy *et al*., 2001), seed oils (Abdalla & Roozen, 1999) and deep-fat-fried potatoes (Chen Man & Tan, 1999). Nevertheless, the protective effect of these substances against protein oxidation in meat and liver products is unknown.

The objectives of the present work were to investigate the effect of the addition of natural antioxidants (sage and rosemary essential oils) on refrigerated stored pâtés and compare this effect with that displayed by a synthetic antioxidant (BHT).

IV.4. Material and Methods

Raw material

Seven Iberian pigs produced in south-western Spain were free-range-reared and fed on natural resources (grass and acorns). The animals were slaughtered when weighing around 150 kg and at an age of 12 months. After slaughter, subcutaneous fat, *quadriceps femoris* muscles, and livers were removed from the carcasses, freed from connective tissue, and stored vacuum-packaged at -80ºC until the day of the manufacture of pâtés.

Manufacture of the liver pâté

The experimental pâtés (spread type) were manufactured in a pilot plant. The same experimental recipe was used for all pâtés, except for the addition of the different antioxidants. In the basic formulation the ingredients were as follows per 100g of manufactured product: 28 g liver, 40 g subcutaneous fat, 5 g muscle, 23 g distilled water, 2 g sodium caseinate, and 2 g sodium chloride. The concentrations of all added additives and antioxidants were

calculated in the basis of total ingredients. Sodium di- and tri-phosphates (0.3%), sodium ascorbate (0.05%), and sodium nitrite (0.03%) (all from ANVISA additives, Madrid, Spain) were included. Depending on the experimental batch, different antioxidants were added to the aforementioned formula: rosemary extract ('ROSE') (0.1%), sage extract ('SAGE') (0.1%), and BHT ('BHT') (0.02%). The essential oils (Soria Natural S.L., Soria, Spain) are considered as GRAS (Generally Recognised as Safe) and were added at a level (0.1%) at which the highest antioxidant activity would be exhibited (McCarthy *et al.*, 2001). BHT (Sigma-Aldrich, Steinheim, Germany) was added up to the highest level allowed by the Spanish law for this kind of product (0.02%) (BOE, 2002). Control pâtés ('CON') containing no added antioxidants were also prepared. The protocol followed for the manufacture of liver pâtés has been explained elsewhere (Estévez *et al.*, 2004). Following the aforementioned recipe, 1.5 kg of raw material was used to produce the experimental pâtés from the four groups, with those being elaborated independently in four production processes. The day before to the manufacture of the liver pâté, the subcutaneous fat was chopped into small cubes (1.5 cm^3) and scalded in distilled water to an internal temperature of +65ºC. Liver and muscle were also sliced into small cubes (1.5 cm^3) and mixed with the sodium chloride, sodium nitrite and the sodium ascorbate in order to allow the nitrification of the samples. The scalded fat and the nitrification mixture were separately kept under refrigeration (+4ºC) in the darkness, before the manufacture of the liver pâtés (24 hours). The day of the production, the sodium caseinate was totally dissolved in hot water (+75ºC) and then added to the scalded fat and mixed during mincing in a Foss Tecator Homogeniser (mod. 2094) during 3 minutes. After that, the nitrification mixture was added to the cutter bowl, together with the water, the sodium di- and tri-phosphates and the antioxidants previously dissolved in 10 mL ethanol. For the production of the control batch, 10 mL of ethanol with no antioxidant was added. The whole mixture was completely minced during 3 minutes until a homogenous raw batter was obtained. Finally, the mixture was packed in glass containers (~50g of pâté per container; 5 containers per

group) and heated in a hot water bath (+80ºC/30 min.). After cooling at room temperature, pâtés were stored at 4 ºC for 90 days in the dark. Liver pâtés were analysed at days 0, 30, 60, and 90 for protein oxidation, concentrations of HI and NHI, and instrumental colour and texture. At sampling times, instrumental colour and texture were measured on the surface of liver pâtés and then the samples were stored at -80ºC until the other analytical experiments were conducted.

Analytical methods

Compositional analysis of liver pâté

Moisture, total protein, and ash were determined using AOAC methods (AOAC, 2000a, b, c). The method of Bligh & Dyer (1959) was used for the extraction and quantification of the fat from liver pâtés.

Iron analysis

Total iron was determined by spectrophotometry according to Miller *et al*. (1994). NHI content was determined by spectrophotometry following the method described by Rhee *et al*. (1987b). The amount of heme iron (HI) was calculated by difference between total and NHI. The amounts of iron were expressed as µg iron/g pâté.

Protein oxidation measurement

Protein oxidation as measured by the total carbonyl content was assessed following the 2,4-dinitrophenylhydrazine (DNPH) coupling method described by Oliver *et al* (1987). DNP hydrazones were quantified by measuring absorbance values at 370 nm. Protein concentration was determined by spectrophotometry at 280 nm using bovine serum albumin (BSA) as standard. The amount of carbonyls was expressed as nM carbonyls/ mg protein. The percent inhibition against protein oxidation was calculated at day 90 as $[(C_{90} - T_{90})/C_{90})] \times 100$, where T_{90} is the amount of carbonyls in the treated pâté at day 90 and C_{90} is the amount of carbonyls in control pâtés at day 90.

Instrumental colour measurement

Instrumental colour (Cie, 1976) was automatically measured in triplicate on the surface of pâté (high: 4.5 cm; diameter: 4.5 cm) by a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ, USA) at room temperature (~20ºC) with illuminant D_{65} and a 0º angle observer. A numerical total colour difference (ΔE) between pâtés at day 0 and day 90 of storage was calculated by: $\Delta E_{0\text{-}90} = [(L_{90}\text{-}L_0)^2+(a_{90}\text{-}a_0)^2+(b_{90}\text{-}b_0)^2)]^{1/2}$ (Yudd & Wyszecki, 1975).

Instrumental texture measurement

The penetration test was performed with a Universal TA-XT2i texture analyser (Stable Micro Systems, Godalming, UK). Force in compression was measured with a 10-mm-diameter cylinder probe using a 5-kg load cell. After the probe touched the surface it then proceeded to penetrate to a depth of 8 mm within the sample, measuring the force value as the hardness (N) of the sample. Force-distance deformation curves were recorded at a crosshead speed of 1.5 mm/s. Textural analyses were performed at room temperature (~20ºC).

Data analysis

Means and deviations from 5 measurements within each batch were obtained from all analytical experiments. Results from the experiments were used as variables and analysed by using an Analysis of Variance (ANOVA) from SPSS software in order to assess the effect of the addition of antioxidants and the effect of refrigerated storage on liver pâtés. When statistically significant differences were found, Tukey test's were performed. Statistical significance was set at $p<0.05$.

IV.5. Results and Discussion

General composition of liver pâtés

No significant differences among groups were detected for the chemical composition since all pâtés contained similar amounts of moisture (4.8-4.9 g

kg^{-1} pâté), fat (3.2-3.3 g kg^{-1} pâté), protein (0.98-1.03 g kg^{-1} pâté), and ash (0.27-0.29 g kg^{-1} pâté). Results from this work concerning the chemical composition of these pâtés are in agreement with those obtained by other authors recently studying different types of commercial pâtés (Echarte *et al*., 2004).

Protein oxidation during refrigerated storage of liver pâtés

Results from the analysis of the oxidative deterioration of proteins from liver pâtés during refrigerated storage are shown in Figure IV.1. The amount of carbonyls from protein oxidation significantly ($p<0.05$) increased during refrigerated storage in 'CON', 'BHT', 'SAGE' and 'ROSE' pâtés; with this increase being significantly higher in 'CON' pâtés than in the treated counterparts (Δ carbonyls 'CON': 10.76, 'BHT': 1.20, 'SAGE': 2.35, 'ROSE': 3.93; $p<0.05$). Results suggest that protein oxidation mainly occurred from day 60 to day 90, with the highest amount of carbonyls being detected at the end of the refrigerated storage. At day 90, pâtés with added antioxidants had significantly smaller amounts of carbonyls than 'CON' pâtés. The percent inhibition of protein oxidation was similar in pâtés with added essential oils ('ROSE': 51.28%; 'SAGE': 59.66%) and larger in those with added BHT (75.18%). Plant essential oils have been successfully introduced to inhibit oxidative deterioration of meat and fat products, and to reduce the accumulation of lipid oxidation-derived products and to the generation of lipid-derived volatiles (Chen *et al*., 1999; Yu *et al*., 2000; McCarthy *et al*., 2001; Ahn *et al*., 2002). Proteins from foods are also targets for oxygen radicals attack, (Oliver *et al*., 1987; Mercier, *et al*., 1995; Mercier *et al*., 1998; Batifoulier *et al*., 2002; Estévez & Cava, 2004) but little is known about oxidative degradation of proteins in foods. Results from the present study suggest that the addition of plant essential oils and BHT to liver pâtés inhibited the development of protein oxidation during their refrigerated storage as previously reported for lipid oxidation (Yu *et al*., 2000; McCarthy *et al*., 2001; Ahn *et al*., 2002; Estévez & Cava, 2004). In this sense, Mercier *et al*. (1995), Batifoulier *et al*. (2002) and ourselves (Estévez & Cava, 2004)

reported a possible linkage between lipid and protein oxidation based on the significantly correlation coefficients found between both processes. Were free radicals from lipid oxidation to damage proteins, phenolic compounds from plant essential oils might scavenge reactive oxygen substances (ROS) and inhibit protein degradation during cooking or storage of meat and fat products such as liver pâté. In this work, sage essential oil exhibited similar antioxidant activity than BHT. Rosemary essential oil also significantly inhibited protein oxidative reactions in liver pâtés.

Closely associated to the development of oxidative reactions in meats and cooked products, the breakdown of the heme molecule and the subsequent release of iron from the porphyrin ring have been reported to occur as a consequence of the high temperatures reached during cooking (Schricker *et al*., 1982; Lombardi-Boccia *et al*., 2002). Moreover, Miller *et al*. 1994a, b) and ourselves (Estévez & Cava, 2004) established relationships between a gradual increase of NHI and the development of oxidative deterioration during refrigerated storage of meat and meat products. In the present work, the amount of HI significantly decreased in liver pâtés during storage (Figure IV.2a.) and, as a likely reflection of this fact, the amount of NHI steadily increased in the four sets of pâté (Figure IV.2b.). The decrease of HI content was significantly smaller in pâtés with added antioxidants than in the control counterparts. Significant differences were detected among groups at days 60 and 90, in which pâtés with added antioxidants showed, in general, higher amounts of HI, compared to the control. Consistently, the increase of NHI during refrigerated storage was significantly larger in 'CON' pâtés when compared to 'BHT', 'SAGE' and 'ROSE' pâtés. Pâtés with added antioxidants had significantly decreasing amounts of NHI at days 60 and 90. These results could be partly explained by a likely protective effect of antioxidants on the heme molecule through the inhibition of protein oxidation, reducing the release of iron. The increase of NHI affects both nutritional and physico-chemical properties of liver pâtés. The degradation of heme iron could decrease the nutritional value of the pâtés in terms of iron bioavailability, since HI is more available than NHI (Hunt & Roughead, 2001). Thus, iron

achieves enhanced ability of promoting oxidation processes when it is released from heme molecule (Kanner *et al.*, 1991) and, therefore, pâtés with increasing amounts of NHI might also increase their oxidative instability.

Evolution of instrumentally measured colour during refrigerated storage of liver pâtés

Liver pâtés exhibited different colour characteristics depending on the addition of antioxidants (Table IV.1.). At day 0, 'ROSE' pâtés were redder than 'CON' pâtés, and no differences in L*- and b*-values were found. 'BHT' pâtés showed higher a*- and b*-values than control pâtés.

Colour characteristics of liver pâtés significantly changed during refrigerated storage. These modifications in instrumentally colour measurements can be considered as noticeable visual changes since the total colour difference ($\Delta E_{0\text{-}90}$) values were higher than 2 (Francis & Clydesdale, 1975). Lightness (L*-values) gradually increased over time in 'CON', 'BHT', 'SAGE', and 'ROSE' pâtés. At day 90, higher L*-values were measured in pâtés with added antioxidants than in 'CON' ones. The evolution of redness (a*-values) depended on the group studied since pâtés with added antioxidants tended to display less redness over time and a*-values from the control counterpart did not change significantly during refrigerated storage. In general, the evolution of yellowness did not follow a defined trend as long as b*-values increased and decreased variably throughout the refrigerated storage. At day 90, no significant differences ($p<0.05$) were detected among pâtés, neither for a* nor for b*-values. Results are consistent with those reported in previous studies devoted to the study of colour changes during refrigeration of liver pâtés and other cooked products (Perlo *et al.*, 1995; Fernández-Ginés *et al.*, 2003; Fernández-López *et al.*, 2004; Estévez & Cava, 2004). Tarladgis (1962) reported that the compound responsible for the brownish grey colour of cooked meats is a ferric-porphyrin coordination complex of the denatured globin molecule. The modification of this structure, suggested in the present work by the degradation of heme molecule and release of iron, might affect the colour displayed by pâtés. In fact, changes in L* and a* parameters

measured on refrigerated stored bologna sausages and liver pâtés have recently been associated to nitrosopigments degradation (Fernández-López *et al.*, 2004; Fernández-Ginés *et al.*, 2003). Nevertheless, colour changes reported in the present work seemed not to be directly related to oxidation processes since pâtés with the higher oxidative stability (those with added antioxidants) suffered more colour changes when compared to 'CON' pâtés. In fact, total colour change between day 0 and day 90 as measured by $\Delta E_{0\text{-}90}$, was significantly higher in treated pâtés than in the 'CON' ones ('CON': 3.38; 'BHT': 5.45; 'SAGE': 5.34; 'ROSE': 4.49; $p<0.05$). These results agree with those reported in a previous study in which colour and oxidative changes occurring during refrigerated storage of different types of pâtés were assessed (Estévez & Cava; 2004). Some compositional or physical changes not directly related to oxidative processes and not considered in the present study could have affected colour traits of liver pâtés much more than protein oxidation did.

Evolution of instrumentally measured texture during refrigerated storage of liver pâtés

Textural properties of pâtés were affected by refrigerated storage (Figure IV.3.). After 90 days of refrigeration, hardness significantly increased 105%, 112%, 109%, and 85% in 'CON', 'BHT', 'SAGE', and 'ROSE' pâtés. These results are in agreement with those obtained by other authors on ostrich liver pâté (Fernández-López *et al.*, 2004). Several could be the reasons explaining the hardness increase, including the polymerisation of lipids, proteins and/or these, and the drying of liver pâtés during storage. However, the chemical composition of the liver pâtés was identical at day 0 and day 90 of storage (data not shown) reflecting that no drying process happened. Hardness increase during refrigerated storage of liver pâté and other food emulsions has been previously described and related to the process of emulsion destabilisation due to water and fat separation from the protein matrix (Fernández-López *et al.*, 2004). The addition of BHT and sage essential oil significantly affected the texture characteristics of liver pâté, reducing

hardness at day 30 of refrigerated storage. At day 60 and day 90, 'ROSE' pâtés showed significant lower values of hardness than those measured in 'CON', 'BHT', and 'SAGE' pâtés. To form a stable emulsion, proteins must surround the finely chopped fat particles before cooking and, therefore, protein functionality is essential to yield stable products (Smith, 1988). The oxidative damage of proteins has an impact in protein solubility, leading to the aggregation and complex formation due to cross links (Karel & Schaich, 1975) which could explain the increase of hardness in liver pâtés. In fact, significant correlations were found between cooking yield and oxidative deterioration of proteins while studying the oxidative stability of liver emulsions (Estévez *et al.*, in press). Results from the present study suggest that essential oils of sage and rosemary might have reduced the hardness of pâtés by increasing emulsion stability through their protective role on proteins against oxidation. These results are in agreement with those reported in previous studies in which the addition of plant extracts enhanced stability of meat and fat emulsions (Abdalla & Roozen, 1999; Nenadis *et al.*, 2003; Cava *et al.*, 2004).

IV.6. Conclusion

The addition of exogenous antioxidants on liver pâtés was demonstrated to enhance oxidation stability of proteins, significantly reducing the increase of non-heme iron during refrigerated storage. At different days of storage, the added antioxidants also improved pâté texture characteristics. Sage and rosemary essential oils protected liver pâtés from oxidation processes and could be used as alternative options to synthetic antioxidants such as BHT. Nevertheless, further analyses concerning the activity of essential oils on the oxidative stability of proteins from foods will be needed to support the present results and shed light on the protein oxidation mechanisms. The effect of protein oxidation on some essential quality characteristics of foods such as colour and other sensory traits should be elucidated in future works.

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Table IV.1. Evolution of color parameters L*, a*, b*, and the numerical total color difference between day 0 and day 90 (ΔE) measured on liver pâtés with added BHT and sage and rosemary essential oils during refrigerated storage.[1,2]

		CON	BHT	SAGE	ROSE	sem[3]
L*	Day 0	61.45^{z}	61.29^{y}	61.10^{z}	61.87^{z}	0.18
	30	63.31^{y}	62.79^{y}	62.68^{yz}	63.39^{y}	0.12
	60	63.99^{xy}	64.77^{x}	63.91^{y}	64.22^{y}	0.15
	90	$64.52^{b,x}$	$66.33^{a,x}$	$66.16^{a,x}$	$65.88^{ab,x}$	0.07
	sem[4]	0.29	0.49	0.44	0.35	-
a*	Day 0	9.13^{b}	$9.89^{ab,x}$	$9.68^{ab,xy}$	$10.09^{a,xy}$	0.13
	30	9.33^{b}	$9.81^{b,x}$	$10.08^{ab,x}$	$10.85^{a,x}$	0.16
	60	9.29^{ab}	$8.81^{b,y}$	$9.33^{ab,y}$	$9.75^{a,y}$	0.13
	90	8.43	8.23^{y}	8.45^{z}	8.53^{z}	0.11
	sem[4]	0.14	0.18	0.16	0.22	-
b*	Day 0	$13.10^{a,x}$	$12.11^{b,x}$	$13.11^{a,x}$	$13.22^{a,x}$	0.13
	30	$12.33^{ab,y}$	$11.69^{b,y}$	$12.41^{a,y}$	$12.60^{a,xy}$	0.11
	60	$12.73^{ab,xy}$	$12.25^{b,x}$	$13.10^{a,x}$	$13.05^{a,x}$	0.10
	90	12.14^{y}	12.06^{xy}	12.09^{y}	12.36^{y}	0.24
	sem[4]	0.11	0.07	0.13	0.11	-
ΔE		3.38^{b}	5.45^{a}	5.34^{a}	4.49^{a}	0.09

[1] Values with a different letter (a-b) within a row of the same storage day are significantly different ($p<0.05$).
[2] Values with a different letter (x-z) within a column of the same antioxidant are significantly different ($p<0.05$).
[3] Standard error of the mean within the same storage day (n=20).
[4] Standard error of the mean within the same antioxidant group (n=20).

Figure IV.1. Progression of protein oxidation during refrigerated storage of liver pâtés with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

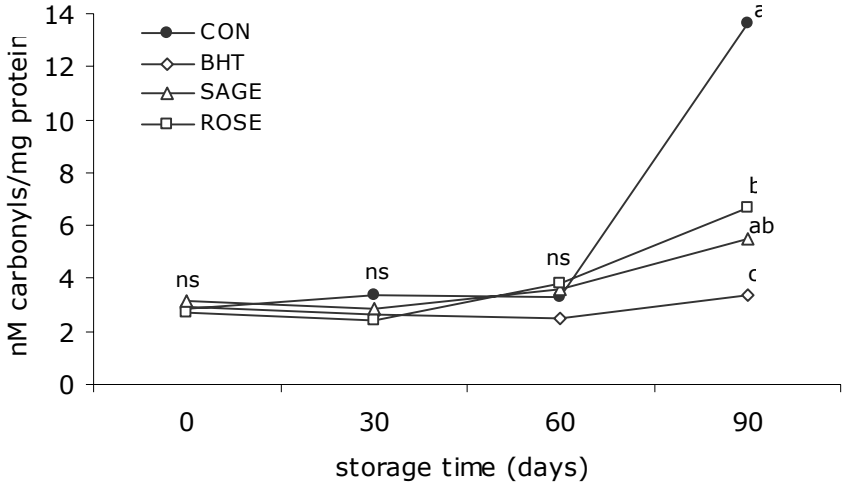


Figure IV.2. Evolution of heme- (a) and non-heme iron (b) contents during refrigerated storage of liver pâtés with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

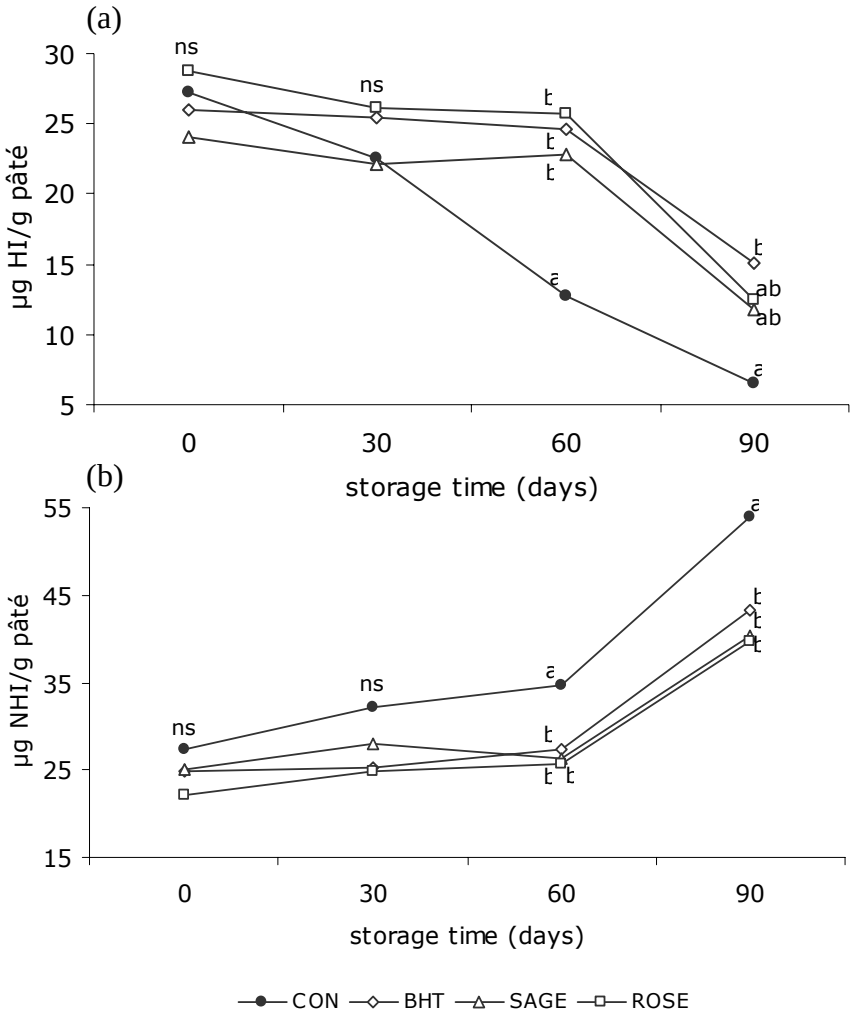
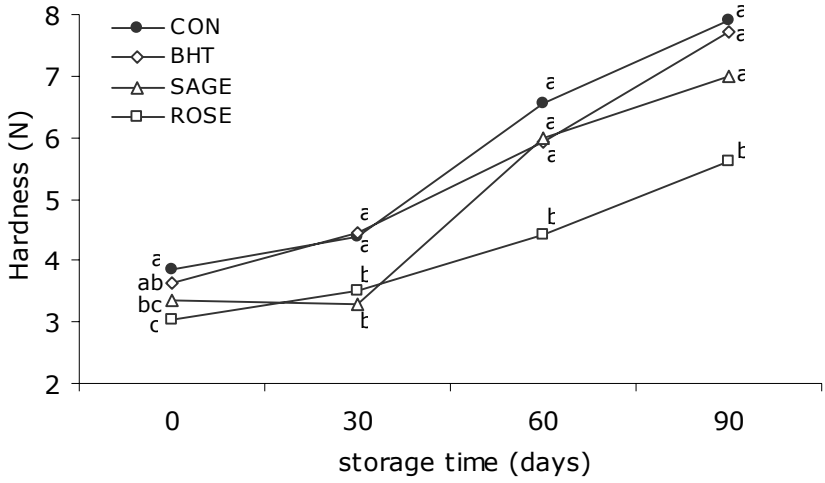


Figure IV.3. Hardness measurements during refrigerated storage of liver pâtés with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).



CHAPTER V

Analysis of volatiles in porcine liver pâtés with added sage and rosemary essential oils by using SPME-GC-MS[♣]

[♣] Published in the *Journal of Agricultural and Food Chemistry*, *52*, 5168-5174. (2004)

V.1. Abstract

The effect of the addition of two natural antioxidant extracts (sage and rosemary essential oils) and one synthetic (BHT) on the generation of volatile compounds in liver pâtés from Iberian and white pigs was analysed using SPME-GC-MS. Lipid-derived volatiles such as aldehydes (hexanal, octanal, nonanal, hept-(*Z*)-4-enal, oct-(*E*)-2-enal, non-(*Z*)-2-enal, dec-(*E*)-2-enal, deca-(*E,Z*)-2,4-dienal) and alcohols (pentan-1-ol, hexan-1-ol, oct-1-en-3-ol) were the most abundant compounds in the headspace (HS) of porcine liver pâtés. Pâtés from different pig breeds presented different volatiles profiles due to their different oxidation susceptibility as a probable result of their fatty acid profiles and tocopherols content. Regardless of the origin of the pâtés, the addition of BHT successfully reduced the amount of volatiles derived from PUFA oxidation. Added essential oils showed a different effect on the generation of volatiles whether they were added in pâtés from Iberian or white pigs since they inhibited lipid oxidation in the former and enhanced oxidative instability in the latter. SPME successfully allowed the isolation and analysis of 41 volatile terpenes from pâtés with added sage and rosemary essential oils including α-pinene, β-myrcene, 1-limonene, (*E*)-caryophyllene, linalool, camphor and 1,8-cineole, that might contribute to the aroma characteristics of liver pâtés.

V.2. Keywords: Liver pâtés, fatty acids, lipid-derived volatiles, volatile terpenes, rosemary, sage, BHT.

V.3. Introduction

The study of volatiles in meat and meat products has reached high importance because of the interesting diversity of information given by this type of analysis. For example, the study of the aroma characteristics of a foodstuff as analysed by its volatiles profile allows the achievement of objective and valuable information (Mottram, 1998). Many researchers have established close relationships between volatiles profiles and the aroma characteristics of different meat products, shedding light on the mechanisms

of generation of volatile compounds (Mottram, 1998; Vergnais *et al*., 1998; Elmore *et al*., 1999; Chevance & Farmer, 1999a; Chevance & Farmer, 1999b). Besides, the deterioration of meat and meat products during storage or manipulation can be also evaluated analysing volatiles generated as a result of enzymatic, microbial or biochemical alteration phenomena (Vergnais *et al*., 1998; Vinauskiene *et al*., 2002; Estévez *et al*., 2003). Oxidation of lipids is considered one of the most important causes of quality degradation in meat and fat products (Morrissey *et al*., 1998). Nevertheless, the degradation of lipids during meat cooking and the manufacture of meat and fat products is considered to be necessary in order to achieve a desirable and specific aroma, since lipid derived volatiles, such as aldehydes, ketones and alcohols are important odour active compounds due to their low molecular weight and low thresholds values (Mottram, 1998).

Liver pâté is a traditional fat product with an increasing demand in European countries such as France, Denmark and Spain (Rosmini *et al*., 1996). This product is highly prone to oxidation due to its high fat content, the presence of large amounts of iron and the relatively low occurrence of natural antioxidants that justifies the addition of exogenous substances with antioxidant activity (Madhavi *et al*., 1996; Estévez *et al*., 2004). Although synthetic antioxidants with phenolic structures, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl, octyl and dodecyl gallates (PG, OG, DG), are easy available and largely used in the food industry, the presence of such synthetic compounds in foods has been linked to health risks generally referred to carcinogenic potential (Clayson *et al*., 1986). Consequently, alternative substances with proved antioxidant activity such as sage (*Salvia officinalis*) and rosemary (*Rosmarinus officianalis*) extracts have been successfully introduced to control oxidative deterioration in several types of foodstuffs (Wong *et al*., 1995; Chen *et al*., 1999; McCarthy *et al*., 2001; Yu *et al*., 2002). On the other hand, the origin of the raw material (back fat, liver and meat) used for the manufacture of liver pâtés determines the physicochemical characteristics and oxidative status exhibited by the elaborated products (Estévez *et al*., 2004). In this sense, liver pâtés

from extensively reared Iberian pigs are fairly different to those manufactured with raw material from intensively reared white pigs (Estévez *et al*., 2004). These dissimilarities could have a direct influence on the generation of volatiles since several factors such as the fatty acid composition and non-heme iron (NHI) and tocopherol contents are closely related to the intensity of the oxidation phenomena and the characteristics of the oxidation products (Elmore *et al*., 1999*,* Estévez *et al*., 2003*,* Morrissey *et al*., 1998).

The purposes of the current work were to analyse the volatile compounds generated in the headspace of liver pâtés as affected by natural (sage and rosemary essential oils) and synthetic (BHT) added antioxidants as well as to evaluate the differences between liver pâtés manufactured with raw material from extensively reared Iberian pigs and those manufactured with raw material from intensively reared white pigs regarding their volatiles profiles.

V.4. Material and Methods

Animals, feeds and sampling

Raw material (back fat, liver and muscle *quadriceps femoris*) from two different origins were considered for the manufacture of liver pâtés. Raw material from free-range reared Iberian pigs (n=7) were obtained from the carcasses after being slaughtered at ~ 150 kg live weight. Raw material from white pigs (n=7) (Large-White x Landrace) were obtained after being slaughtered at 85 kg live weight. Iberian pigs were extensively reared under traditional schemes and fed with natural resources (acorns and grass), while white pigs were reared in an intensive livestock farm and fed a mixed diet (Estévez *et al*., 2004). Acorns (moisture: 46.10%, fat: 5.50%; protein: 4.31%) analysis showed the following fatty acid profile (expressed as percentage of total fatty acids analysed): palmitic acid (c16:0): 11.82%; stearic acid (c18:0): 0.56%; oleic acid (c18:1): 67.28%; linoleic acid (c18:2): 18.70%; linolenic acid (c18:3): 0.25%. The grass (moisture: 89.24%, fat: 6.26%; protein: 4.34%) fatty acid profile was as follows: c16:0: 13.95%; c18:0: 1.99%; c18:1: 5.24%; c18:2: 11.42%; c18:3: 57.80%. The analysis

of the mixed diet (moisture: 10.42%, fat: 2.94%; protein: 18.28%) revealed the following fatty acid profile: c16:0: 19.86%; c18:0: 8.63%; c18:1: 32.84%; c18:2: 32.83%; c18:3: 2.45%. After slaughter, livers, muscles and back fat were vacuum packaged and kept frozen (-80ºC) until the manufacture of the liver pâtés (less than 3 weeks).

Manufacture of liver pâtés

The experimental pâtés were manufactured in a pilot plant. The same formulation was used for pâtés from Iberian and white pigs. The ingredients were as follows per 100 g of elaborated product: 28 g liver, 40 g back fat, 5 g muscle, 23 g distilled water, 2 g sodium caseinate, 2 g sodium chloride. Sodium di- and tri-phosphates (0.3%) sodium ascorbate (0.05%) and sodium nitrite (0.03%) (ANVISA, Madrid, Spain) were also added. Three groups of pâtés from both, Iberian and white pigs, were considered depending on the addition of rosemary essential oil (1000 ppm), sage essential oil (1000 ppm) and BHT (200 ppm) dissolved in 10 mL of ethanol. Control pâtés containing no added antioxidants but 10 mL of ethanol, were also prepared. According to previous studies (McCarthy *et al*., 2001), rosemary and sage oils (Soria Natural, Soria, Spain) exhibit at the aforementioned level, their largest antioxidant activity. BHT (Sigma-Aldrich, Steinheim, Germany) was included up to the highest level permitted by Spanish law in this type of fat products. The protocol followed for the manufacture of liver pâtés was profusely explained elsewhere (Estévez *et al*., 2004). The raw mixture of fat, liver and muscle was packed in a glass container and given the thermal treatment (+80ºC/30 min). The packed liver pâtés (n=5 for each group) were kept frozen (-80ºC) until required for analytical experiments (less than 1 month).

Proximate Composition of Liver Pâtés

Moisture (AOAC, 2000a), total protein (AOAC, 2000b) and ash (AOAC, 2000c) were determined using official methods. The method of Bligh and Dyer (1959) was used for determining fat content of liver pâtés. Total iron was determined following the procedure described by Miller *et al*. (1994).

Fatty Acid Profiles of Liver Pâtés

Fatty acid methyl esters (FAMEs) were prepared by acidic sterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionization detector (FID). The derivatives were separated on a FFAP-TPA fused-silica column (Hewlett Packard, USA) (30 mm long, 0.53 mm internal diameter and 1.0 μm film thickness). The injector and the detector temperature were held at +230ºC. Column oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma-Aldrich, Steinheim, Germany). Fatty acid composition was expressed as percent of total FAMEs.

Analysis of Volatiles from Liver Pâtés

The SPME fibre, coated with divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30 μm, was purchased from Supelco Co. (Bellefonte, PA). This coating phase was chosen because of the high reproducibility presented and the lower coefficients of variance obtained compared to others, such as the CAR/PDMS fibre (Machiels & Istasse, 2002). The SPME fibre was preconditioned prior analysis at +220ºC during 45 min. The headspace sampling technique was used as follows: 1 g of pâté was placed in 2.5 mL vials. The fibre was exposed to the headspace of the solution while the sample equilibrated during 30 minutes immersed in water at +60ºC. Based on preliminary studies, the sampling method was elected because in those conditions most of the analytes might have reached the equilibrium. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm id., 1.0mm film thickness). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40 ^{o}C. The SPME fibre was desorbed and maintained in the injection port at 220 ^{o}C during the whole chromatography run. The injector port was in the splitless

mode. The temperature program was isothermal for 10 min at +40ºC and then raised at the rate of +7ºC min^{-1} to +250ºC, and held for 5 min. n-Alkanes (Sigma R-8769) were run under the same conditions to calculate the Kovats index (KI) values for the compounds. The GC/MS transfer line temperature was +270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Volatile compounds were tentatively identified by comparing their mass spectra with those contained in the Wiley and NIST libraries and by comparison of their KI with those reviewed by Kondjoyan and Berdagué (Kondjoyan & Berdagué, 1996). Results from the volatiles analysis are provided in area units (AU).

Data Analysis

The effects of pâté origin (Iberian or white pigs) and addition of antioxidants (BHT, sage and rosemary essential oils) on proximate composition and fatty acid profiles of liver pâtés were analysed using an Analysis of Variance (ANOVA) for a four (antioxidants) x two (origins) together with the interaction following the generalised linear model (GLM) procedure of SPSS software (11.0 version). Chromatographic areas of all tentatively identified peaks were used as variables. In order to determinate the effect of the pâté origin and the four different added antioxidants on the generation of volatiles, an ANOVA for a four (antioxidants) x two (origins) together with the interaction was used. Tukey's tests were used when ANOVA found significance differences between treatments. Significance was defined at $p<0.05$.

V.5. Results and Discussion

Proximate and fatty acid composition of liver pâtés

No significant differences between groups were detected for the proximate composition (Table V.1.) since all pâtés presented similar contents of moisture, fat, protein, ash and iron. Notable differences among types of pâté

were detected in the analysis of the fatty acid profile. Regardless of the addition of antioxidants, pâtés from white pigs presented larger percentages of palmitic, stearic, and total of saturated fatty acids (SFA) than pâtés from Iberian pigs ($p<0.05$). On the other hand, pâtés from Iberian pigs showed a higher proportion of oleic and total of monounsaturated fatty acids (MUFA) than pâtés from white pigs ($p<0.05$). The latter presented higher percentages of polyunsaturated fatty acids (PUFA) such as linoleic acid ($p<0.05$). As profusely discussed in a previous paper (Estévez *et al.*, 2004), the differences in the fatty acid profiles between 'Iberian' and 'white' pâtés are mainly caused by the different fatty acid composition of the feeds given to the animals during the fattening period. Results obtained in the present work are in good agreement with those reported by other researchers in pâtés made with similar raw material (Ordóñez *et al.*, 2003). Added natural extracts of sage and rosemary had a significant effect on most fatty acids from pâtés from white pigs but, in this case, the differences between groups were less pronounced and did not show a clear pattern. The addition of BHT did not affect the fatty acid profiles of liver pâtés (Table V.1.).

Focusing on the generation of volatiles, PUFA are, contrarily to MUFA, very prone to oxidation, leading to the generation of residual substances and unpleasant odours in meat and fat products (Morrissey *et al.*, 1998). Thus, considerable attention has been given to fatty acids in relation to the generation of volatiles as long as the pathways for the formation of volatile compounds from lipid oxidation are considered to be fairly specific for each fatty acid (Chen & Ho, 1998).

Analysis of volatiles from liver pâtés

Tables V.2. and V.3. summarise GC-MS data obtained from the analysis of volatile compounds from liver pâtés. From the total of volatile compounds detected in the extracts, 93 of them were tentatively identified (good match of MS and/or coincidence of KI). In order to perform an appropriate discussion of the results, essential oils-derived terpenes are separately presented in a

table from other volatile compounds mainly derived from the liver pâtés ingredients (muscle, liver and adipose tissue).

Analysis of volatiles related to ingredients

Table V.2. shows volatile compounds derived from the main ingredients of liver pâtés (liver, muscle and adipose tissue) categorised into 9 classes. Lipid derived volatiles such as aldehydes (hexanal, octanal, nonanal, hept-(*Z*)-4-enal, oct-(*E*)-2-enal, non-(*Z*)-2-enal, dec-(*E*)-2-enal, deca-(*E,Z*)-2,4-dienal) and alcohols (pentan-1-ol, hexan-1-ol, oct-1-en-3-ol) were the most abundant compounds in the HS of liver pâté. A relatively high amount of esters were also detected while ketones, furans and hydrocarbons were minority. Results from the present study agree with those obtained in previous works focused on the study of HS volatiles from cooked pork, oxidised liver and canned liver sausage (Estévez *et al*., 2003*,* Ruiz *et al*., 2001*,* Im *et al*., 2001). The off-flavours produced as a consequence of the thermal treatment of lipid-rich foods such as liver pâté, are mainly derived from the autoxidation of lipids (Labuza, 1971). Moreover, liver pâté exhibits relatively high levels of non-heme iron derived from the liver tissue (Estévez *et al*., 2004) that could promote oxidation phenomena in the manufactured product. In fact, some of the volatile compounds detected in the present study, have been described as indicators of lipid decomposition and contributors to the overall off-flavour of oxidised liver (Im *et al*., 2004). Large importance has been given to hept-(*Z*)-4-enal due to its low threshold level (0.04 ppb) and has been linked to fishy and unpleasant flavours (McGill *et al*., 1977; Im *et al*., 2004). Some alkadienals such as hepta-(*E,E*)-2,4-dienal or nona-2,4-dienal have been associated with the oxidative deterioration of PUFA and have been linked to unpleasant characters in cooked liver, with 'fishy' notes the former, and 'rancid' odours the latter (Lee *et al*., 2001; Im *et al*., 2004). The oxidation of unsaturated fatty acids undergoes the formation of some other volatile compounds such as non-(*Z*)-2-enal related to 'cardboard' like odour and deca-(*E,Z*)-2,4-dienal) and deca-(*E,E*)-2,4-dienal associated to 'rancid' and 'warmed-over' flavours (Lee *et al*., 2001; Im *et al*., 2004).

Regardless of the addition of antioxidants, liver pâtés from white pigs ('control' group) showed, compared to those from Iberian pigs, a higher number of lipid derived volatiles since pentan-2-one, but-3-en-2-one, pentanal, hepta-(*E,E*)-2,4-dienal, hexa-2,4-dienal, deca-(*E,Z*)-2,4-dienal and deca-(*E,E*)-2,4-dienal were not detected in the HS of 'Iberian' pâtés. Furthermore, pâtés from white pigs presented significantly ($p<0.05$) higher chromatographic areas of certain compounds closely related to lipid oxidation and off-flavours such as heptan-1-ol (0.67 AU vs. 0.17 AU), oct-3-en-1-ol (1.86 AU vs. 0.52 AU), octan-1-ol (1.60 AU vs. 0.50 AU), hex-(*E*)-2-en-1-ol (1.03 AU vs. 0.59 AU), heptanal (2.14 AU vs. 0.51 AU), buten-2-enal (2.97 AU vs. 0.62 AU), octanal (3.25 AU vs. 0.65 AU), nonanal (9.95 AU vs. 4.05 AU), oct-(*E*)-2-enal (3.32 AU vs. 0.65 AU), nona-2,4-dienal (2.19 AU vs. 0.51 AU), non-(*Z*)-2-enal (4.74 AU vs. 0.79 AU) and dec-(E)-2-enal (4.65 AU vs. 0.29 AU). Differences between types of pâté are remarkably high on hexanal (white: 21.77 AU, Iberian: 2.66 AU; $p<0.05$) that has been widely used on meat products as indicator of lipid oxidation (Mottram, 1998; Elmore *et al.*, 1999; Estévez *et al.*, 2003). These results are in agreement with those obtained in a previous work in which oxidation stability of liver pâtés as measured by TBA-RS was studied (Estévez *et al.*, 2004). A higher proportion of MUFA and lower of PUFA in pâtés from Iberian pigs and the presence of significantly ($p<0.05$) higher amounts of tocopherols in muscles, livers and adipose tissues from Iberian pigs, compared to those from white pigs (Estévez *et al.*, 2004), could partly explain the results obtained in this study.

On the other hand, the large differences between types of pâtés in terms of fatty acid profiles could affect the aromatic characteristics of pâtés as long as the oxidative decomposition of oleic acid leads to the formation of volatile compounds associated to pleasant notes, described as 'floral' and 'sweet' (Specht & Baltes, 1994), while the aromatic notes of linoleic and PUFA-derived volatiles have been described as intense 'grass-like' and related to rancidity in cooked meat and porcine liver (Shahidi & Pegg, 1933; Im *et al.*, 2004). Consistently with results from fatty acid profiles, the ratio between oleic-derived volatiles (octanal, nonanal and octan-1-ol) and linoleic-derived

volatiles (hexanal, oct-(*E*)-2-enal, and non-(*Z*)-2-enal) resulted significantly higher in pâtés from Iberian pigs (Iberian: 1.41, white: 0.50; $p<0.05$) suggesting a more pleasant aromatic profile in the latter. The high content of oleic acid and its oxidation-derived aldehydes in meat products from Iberian pigs has been related to essential quality traits (Ruiz *et al.*, 1999; Cava *et al.*, 2000; Estévez *et al.*, 2004).

The addition of BHT and essential oils of sage and rosemary on liver pâtés had a significant effect on the generation of most volatiles (Table V.2.). Regardless of the origin of the pâté, the addition of BHT successfully inhibited the development of oxidative deterioration since the presence of major lipid-derived volatiles in the HS of pâtés decreased when this antioxidant was added. The antioxidant effect of BHT on meat products has been largely reported in the scientific literature (Chen *et al.*, 1999; McCarthy *et al.*, 2001). In the present work, the addition of BHT had the most evident effect on pâtés with higher oxidative instability (those from white pigs). Compared to the 'control' group, pâtés from white pigs with added BHT presented significantly ($p<0.05$) smaller amounts of oct-3-en-1-ol (1.86 AU vs. 0.52 AU), octan-1-ol (1.60 AU vs. 1.06 AU), pentan-2-one (0.70 AU vs. 0.11 AU), pentanal (4.16 AU vs. 1.48 AU), hexanal (21.77 AU vs. 2.26 AU), heptanal (2.14 AU vs. 0.99 AU), buten-2-enal (2.97 AU vs. 0.98 AU), octanal (3.25 AU vs. 0.59 AU), nonanal (9.95 AU vs. 3.02 AU), oct-(*E*)-2-enal (3.32 AU vs. 0.40 AU), non-(*Z*)-2-enal (4.74 AU vs. 0.67 AU), nona-2,4-dienal (2.19 AU vs. 0.47 AU), dec-(*E*)-2-enal (4.65 AU vs. 0.38 AU) , deca-(*E,E*)-2,4-dienal (1.43 AU vs. 0.35 AU) and deca-(*E,Z*)-2,4-dienal (3.67 AU vs. 0.40 AU), among others.

On the other hand, the addition of sage and rosemary essential oils had a significant effect on the generation of major volatile compounds but this effect was different depending on whether they were added on 'Iberian' or 'white' pâtés. In fact, the interaction between 'origin of pâté' and 'antioxidant' resulted significant for most volatiles (Table V.2.). In agreement with previous research on several meats and meat products (Wong *et al.*, 1995, Chen *et al.*, 1999) the addition of sage and rosemary oils had an antioxidant effect on pâtés from Iberian pigs as long as smaller amounts of hexanal, nonanal and

other lipid-derived volatiles were detected in the HS of treated pâtés when compared to the 'control' counterparts. In contrast, the addition of sage and rosemary essential oils in pâtés from white pigs had an opposite behaviour, significantly increasing ($p<0.05$) the formation of volatiles generated from PUFA and associated to 'fishy' and unpleasant flavours in liver products (Im *et al.*, 2004) such as hept-(*Z*)-4-enal ('control': 1.41 AU, 'sage': 11.88 AU, 'rosemary': 11.51 AU), pent-4-enal ('control': 0.27 AU, 'sage': 5.03 AU, 'rosemary': 2.04 AU), hexa-2,4-dienal ('control': 0.61 AU, 'sage': 4.90 AU, 'rosemary': 1.98 AU), nona-2,4-dienal ('control': 2.19 AU, 'sage': 3.99 AU, 'rosemary': 3.66 AU) and deca-(*E,Z*)-2,4-dienal ('control': 3.67 AU, 'sage': 8.64 AU, 'rosemary': 3.48 AU). Although the antioxidant activity of plant phenolics extracts are generally recognised (Huang *et al.*, 1996), the pro-oxidant properties of these substances have also been described, being able to generate reactive oxygen species and damage lipids, proteins and other cellular components (Aruoma *et al.*, 1992; Yen *et al.*, 1997). Results from the present work suggest that the activity of essential oils of sage and rosemary are dependent on the compositional characteristics of the food matrix. In fact, the activity of plant phenolics on food systems has been considered to be influenced by the presence of other active substances in the food matrix (Yen *et al.*, 1997; McCarthy *et al.*, 2001). Food systems, and particularly liver pâtés, are very complex in the number and the type of chemicals in the mixture, and a particular combination of these compounds might behave differently from the individual components. In this sense, Wong *et al.* (1995) and Fang & Wada (1993) reported possible interactions between phenolic compounds from sage and rosemary essential oils and tocopherols, resulting in different activities depending on the individual amounts of these substances in the food system. Significant differences ($p<0.05$) were found between Iberian and white pigs regarding tocopherol content in muscles (6.18 vs. 1.94 mg/kg muscle), livers (7.93 vs. 3.49 mg/kg liver) and adipose tissues (19.67 vs. 1.21 mg/kg adipose tissue) used for the manufacture of liver pâtés (Estévez *et al.*, 2004). The presence of a certain amount of an endogenous antioxidant (tocopherol) in the raw material and manufactured product might

influence on the activity of exogenous active extracts, leading to antioxidant or pro-oxidant effects.

Analysis of terpenes from sage and rosemary extracts

SPME allowed the isolation and analysis of 41 terpenes derived from sage and rosemary essential oils (Table V.3.). Monoterpenes hydrocarbons such as α-pinene, camphene, β-myrcene and 1-limonene, sesquiterpenes hydrocarbons such as α-cubenene and (*E*)-caryophyllene and oxygen-derivative terpenes such as alcohols (linalool, endo-borneol, terpinene-4-ol), esters (linalyl acetate, linalyl propionate), carbonyls (camphor), and ethers (1,8-cineole) were the most abundant. Most of these compounds have been previously reported as volatile components of sage and rosemary essential oils and isolated in the HS of several spiced foods (Chevance & Farmer, 1999; Ibáñez *et al*., 1999; Paleari *et al*., 2004). As expected, no differences were detected between 'white' and 'Iberian' pâtés as long as the same formulation was used for all of them. Although most volatile terpenes were detected in both groups, significant differences were detected between sage and rosemary essential oils regarding their terpenes profile. Compared to that from pâtés with added sage extract, HS from pâtés with added rosemary presented significantly ($p<0.05$) higher amounts of α-pinene, β-2-pinene and 1,8-cineole, while the former showed higher amounts of γ-terpinene, β-terpinene, 1-limonene, alloo-cimene, and most abundant terpenoids such as linalool and their esterified derivatives, p-menth-3-en-1-ol, terpinene-4-ol, p-cymen-8-ol and camphor, among others. However, the main differences between essential oils were detected for sesquiterpene hydrocarbons since pâtés with added sage extract showed relatively high amounts of certain compounds such as α-cubenene, farnesol, α-ylangene, α-gurjunene and junipene, which were not detected in pâtés with added rosemary extract. Other compounds such as α-copaene and α-elemene were only detected in pâtés with added rosemary extract. Several of the volatile terpenes detected are recognised odorants and are commonly used in the food industry as flavour and fragrance ingredients (Ibáñez *et al*., 1999). Volatile terpenes such as α-pinene, 1,8-cineole and

linalool have been related to 'spices, pine needles', 'medicinal, cough syrup' and 'flowers, carnation' odours, respectively, and have been reported as contributors to the aroma of spiced cooked sausages (Chevance & Farmer, 1999). In absence of olfactometry or sensory assessment of pâtés, the contribution of these compounds to the overall aroma of pâtés remains unknown, and therefore, the attitude of consumers towards pâtés with odour notes referred to such aromatic herbs would be a future work of interest. On the other hand, using deodorised extracts of these plants would be also an interesting option in order to achieve antioxidant effects in meat and fat products without including unexpected aroma components (Dorman *et al.*, 2003).

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Table V.1. Proximate and fatty acid composition of liver pâtés from white and Iberian pigs with added BHT and sage and rosemary essential oils.

	CONTROL		BHT		SAGE		ROSEMARY			p-value[1]		
	white	Iberian	white	Iberian	white	Iberian	white	Iberian	sem[2]	A	O	AxO
Moisture[3]	50.51	48.42	50.45	49.1	50.74	49.15	49.69	49.25	0.21	0.774	0.090	0.461
Fat[3]	31.82	33.37	32.56	32.01	32.99	33.38	33.7	32.57	0.11	0.858	0.214	0.532
Protein[3]	10.04	10.34	10.23	9.81	10.68	10.00	10.36	9.90	0.23	0.391	0.977	0.197
Ash[3]	2.78	2.69	2.77	2.76	2.65	2.76	2.85	2.93	0.03	0.102	0.790	0.557
Total iron[4]	45.19	50.59	47.27	50.82	49.99	49.04	52.65	50.93	1.46	0.838	0.620	0.821
Fatty Acids[5]												
c14:0	1.11^{d}	1.12^{cd}	1.12^{cd}	1.14^{bc}	1.17^{a}	1.13^{cd}	1.19^{a}	1.15^{b}	0.00	<0.001	0.001	<0.001
c16:0	22.65^{b}	20.69^{cd}	22.63^{b}	20.80^{c}	23.26^{a}	20.61^{d}	23.27^{a}	20.62^{d}	0.19	<0.001	<0.001	<0.001
c18:0	13.40^{b}	10.58^{c}	13.44^{b}	10.58^{c}	13.91^{a}	10.50^{cd}	13.88^{a}	10.42^{d}	0.25	<0.001	<0.001	<0.001
Σ SFA	37.98^{b}	32.87^{cd}	38.09^{b}	32.99^{c}	39.11^{a}	32.72^{d}	39.12^{a}	32.69^{d}	0.46	<0.001	<0.001	<0.001
c16:1	2.44^{a}	2.00^{d}	2.42^{ab}	2.00^{d}	2.39^{c}	1.98^{de}	2.40^{bc}	1.96^{e}	0.03	<0.001	<0.001	0.026
c18:1	43.57^{c}	53.43^{ab}	42.96^{d}	53.38^{b}	42.10^{f}	53.59^{a}	42.39^{e}	53.54^{ab}	0.86	<0.001	<0.001	<0.001
c20:1	1.08^{d}	1.83^{bc}	1.04^{d}	1.79^{c}	0.98^{e}	1.86^{ab}	0.98^{e}	1.90^{a}	0.07	0.004	<0.001	<0.001
Σ MUFA	47.58^{c}	57.52^{ab}	46.92^{d}	57.42^{b}	45.96^{f}	57.62^{a}	46.24^{e}	57.65^{a}	0.87	<0.001	<0.001	<0.001
c18:2	12.23^{b}	7.71^{cd}	12.54^{a}	7.69^{d}	12.57^{a}	7.82^{c}	12.45^{a}	7.75^{cd}	0.38	<0.001	<0.001	<0.001
c18:3	0.61^{a}	0.49^{b}	0.63^{a}	0.49^{b}	0.62^{a}	0.51^{b}	0.63^{a}	0.50^{b}	0.01	0.097	<0.001	0.315
c20:4	0.70^{a}	0.56^{b}	0.78^{a}	0.55^{b}	0.75^{a}	0.57^{b}	0.74^{a}	0.53^{b}	0.02	<0.001	<0.001	0.093
Σ PUFA	14.40^{b}	9.63^{c}	14.99^{a}	9.57^{c}	14.90^{a}	9.65^{c}	14.90^{a}	9.64^{c}	0.42	<0.001	<0.001	<0.001

[1] P-values for the studied factors; A: antioxidant, O: pâté origin, AxO: interaction antioxidant x pâté origin.
[2] Standard error of the mean.
[3] g/100g pâté.
[4] µg iron/g pâté.
[5] Results are expressed as means in percent of methyl esters from total analysed.
SFA: Saturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids.
[a,b,c,d,e,f] In the same line, means with different superscript resulted statistically different.

Table V.2. Volatile compounds detected in headspace of liver pâtés from white and Iberian pigs with added BHT and sage and rosemary essential oils[1].

	CONTROL		BHT		SAGE		ROSEMARY			p-value[2]			
Compds	white	Iberian	white	Iberian	white	Iberian	white	Iberian	SEM	A	O	AxO	MI[3]
alcohols													
pentan-1-ol	2.85^{a}	1.50^{abc}	1.10^{abc}	0.90^{bc}	2.64^{ab}	0.14^{c}	1.68^{abc}	0.20^{c}	0.20	0.015	<0.001	0.060	MS,KI
hexan-1-ol	2.31^{bc}	1.29^{bc}	2.96^{b}	1.08^{bc}	7.50^{a}	0.48^{c}	1.50^{bc}	0.57^{c}	0.38	<0.001	<0.001	<0.001	MS,KI
heptan-1-ol	0.67^{b}	0.17^{de}	0.43^{bcd}	0.17^{de}	1.08^{a}	0.40^{cd}	0.66^{bc}	0.00^{e}	0.06	<0.001	<0.001	0.004	MS,KI
oct-1-en-3-ol	1.86^{ab}	0.52^{d}	0.59^{cd}	0.26^{d}	2.33^{a}	0.43^{d}	1.32^{bc}	0.58^{cd}	0.13	<0.001	<0.001	<0.001	MS,KI
2-ethyl-hexan-1-ol	0.83^{b}	0.52^{b}	0.59^{b}	0.53^{b}	1.52^{a}	0.56^{b}	1.11^{ab}	0.84^{ab}	0.07	0.007	0.001	0.003	MS,KI
hexa-2,4-dien-1-ol	0.49^{a}	0.25^{b}	0.00^{c}	0.00^{c}	0.41^{ab}	0.00^{c}	0.38^{ab}	0.00^{c}	0.03	<0.001	<0.001	<0.001	MS
octan-1-ol	1.60^{a}	0.50^{cd}	1.06^{b}	0.48^{cd}	0.90^{bc}	0.80^{bcd}	0.45^{d}	0.43^{d}	0.07	<0.001	<0.001	<0.001	MS,KI
2,5-dimethyl-ciclohexanol	1.46^{a}	0.70^{b}	0.92^{b}	0.48^{bc}	1.51^{a}	0.00^{c}	1.74^{a}	0.00^{c}	0.11	0.010	<0.001	<0.001	MS
3-methyl-butan-1-ol (nitrate)	0.44^{b}	0.21^{c}	0.31^{bc}	0.28^{bc}	0.71^{a}	0.00^{d}	0.00^{d}	0.00^{d}	0.04	<0.001	<0.001	<0.001	MS
nonan-1-ol	0.76^{ab}	0.56^{b}	0.60^{b}	0.00^{c}	0.94^{a}	0.00^{c}	0.89^{a}	0.00^{c}	0.06	<0.001	<0.001	<0.001	MS
hex-(*E*)-2-en-1-ol	1.03^{a}	0.59^{bc}	0.60^{bc}	0.48^{c}	0.89^{ab}	0.00^{d}	1.06^{a}	0.00^{d}	0.07	<0.001	<0.001	<0.001	MS
acids													
acetic acid	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.82^{a}	0.00^{b}	0.64^{a}	0.00^{b}	0.06	<0.001	<0.001	<0.001	MS,KI
esters													
acetic acid ethyl ester	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.51^{a}	0.00^{b}	0.51^{a}	0.00^{b}	0.04	<0.001	<0.001	<0.001	MS,KI
hexanoic acid ethyl ester	1.86^{abc}	2.00^{ab}	1.95^{ab}	0.91^{cd}	2.28^{a}	0.67^{d}	1.26^{bcd}	0.46^{d}	0.12	<0.001	<0.001	0.003	MS,KI
heptanoic acid ethyl ester	0.73^{ab}	0.60^{bc}	0.75^{ab}	0.32^{c}	0.95^{a}	0.40^{c}	0.61^{bc}	0.50^{bc}	0.04	0.090	<0.001	0.003	MS,KI
octanoic acid ethyl ester	6.37^{a}	6.92^{a}	7.18^{a}	5.88^{ab}	3.03^{bc}	2.59^{c}	1.90^{c}	1.84^{c}	0.41	<0.001	0.506	0.560	MS,KI
nonanoic acid ethyl ester	3.27^{a}	1.06^{b}	3.16^{a}	3.45^{a}	2.97^{a}	1.30^{b}	1.19^{b}	0.79^{b}	0.20	<0.001	<0.001	0.001	MS,KI
decanoic acid ethyl ester	10.60^{b}	6.18^{cd}	6.87^{c}	14.27^{a}	4.89^{cd}	4.68^{cd}	3.73^{d}	4.41^{cd}	0.59	<0.001	0.081	<0.001	MS
dodecanoic acid ethyl ester	3.15^{a}	1.00^{b}	1.24^{b}	4.35^{a}	1.30^{b}	1.64^{b}	0.71^{b}	1.08^{b}	0.21	<0.001	0.052	<0.001	MS
tetradecanoic acid ethyl ester	3.29^{ab}	0.89^{c}	1.13^{c}	4.97^{a}	1.52^{bc}	1.63^{bc}	1.01^{c}	0.71^{c}	0.26	<0.001	0.305	<0.001	MS
hexadecanoic acid ethyl ester	3.80^{ab}	1.15^{c}	1.34^{c}	5.10^{a}	1.27^{c}	1.72^{bc}	1.28^{c}	1.08^{c}	0.27	<0.001	0.303	<0.001	MS
ketones													

	CONTROL		BHT		SAGE		ROSEMARY			p-value[2]			
Compds	white	Iberian	white	Iberian	white	Iberian	white	Iberian	SEM	A	O	AxO	MI[3]
cicloex-2-en-1-one	0.57^{c}	0.54^{c}	0.00^{c}	0.58^{c}	3.27^{b}	0.25^{c}	4.30^{a}	0.00^{c}	0.25	<0.001	<0.001	<0.001	MS,KI
pentan-2-one	0.70^{b}	0.00^{c}	0.11^{c}	0.00^{c}	1.27^{a}	0.00^{c}	0.75^{b}	0.00^{c}	0.08	<0.001	<0.001	<0.001	MS
but-3-en-2-one	1.11^{a}	0.00^{b}	0.00^{b}	0.00^{b}	1.22^{a}	0.00^{b}	1.08^{a}	0.00^{b}	0.09	<0.001	<0.001	<0.001	MS,KI
aldehydes													
pentanal	4.16^{a}	0.00^{c}	1.48^{bc}	0.00^{c}	4.76^{a}	0.00^{c}	3.10^{ab}	0.00^{c}	0.36	0.038	<0.001	0.038	MS
hexanal	21.77^{a}	2.26^{b}	2.68^{c}	0.25^{c}	21.19^{a}	0.29^{c}	16.96^{b}	0.31^{c}	1.52	<0.001	<0.001	<0.001	MS,KI
3-methyl-tio-propanal	1.15^{a}	1.16^{a}	1.38^{a}	0.88^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.10	<0.001	0.003	<0.001	MS
heptanal	2.14^{a}	0.51^{bc}	0.99^{b}	0.36^{bc}	2.20^{a}	0.11^{c}	1.13^{b}	0.46^{bc}	0.13	0.002	<0.001	<0.001	MS,KI
buten-2-enal	2.97^{a}	0.62^{bc}	0.98^{b}	0.53^{bc}	0.80^{bc}	0.12^{c}	0.70^{bc}	0.28^{c}	0.14	<0.001	<0.001	<0.001	MS,KI
benzaldehyde	3.60^{a}	1.19^{cd}	2.94^{ab}	3.05^{ab}	2.11^{bc}	0.48^{d}	2.22^{bc}	0.56^{d}	0.19	<0.001	<0.001	<0.001	MS,KI
octanal	3.25^{b}	0.65^{c}	0.59^{c}	0.42^{c}	5.28^{a}	0.38^{c}	4.42^{ab}	0.44^{c}	0.32	<0.001	<0.001	<0.001	MS,KI
benzeneacethaldehyde	1.75^{a}	0.72^{b}	0.69^{b}	1.51^{a}	0.85^{b}	0.31^{b}	0.87^{b}	0.42^{b}	0.08	<0.001	0.002	<0.001	MS,KI
nonanal	9.95^{a}	4.05^{b}	3.02^{b}	2.82^{b}	11.88^{a}	0.89^{b}	11.51^{a}	0.85^{b}	0.77	0.001	<0.001	<0.001	MS
hept-(*Z*)-4-enal	1.41^{c}	0.42^{c}	0.47^{c}	0.51^{c}	11.07^{a}	0.63^{c}	7.37^{b}	0.40^{c}	0.64	<0.001	<0.001	<0.001	MS,KI
hepta-(*E,E*)-2,4-dienal	1.09^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.97^{a}	0.00^{b}	0.94^{a}	0.00^{b}	0.09	<0.001	<0.001	<0.001	MS,KI
pent-4-enal	0.27^{c}	0.32^{c}	0.68^{bc}	0.39^{c}	5.03^{a}	0.30^{c}	2.04^{b}	0.26^{c}	0.27	<0.001	<0.001	<0.001	MS
oct-(*E*)-2-enal	3.32^{b}	0.65^{c}	0.40^{c}	0.32^{c}	7.23^{a}	0.00^{c}	1.06^{c}	0.19^{c}	0.40	<0.001	<0.001	<0.001	MS,KI
nona-2,4-dienal	2.19^{b}	0.51^{c}	0.47^{c}	0.00^{c}	3.99^{a}	0.00^{c}	3.66^{a}	0.00^{c}	0.26	<0.001	<0.001	<0.001	MS,KI
non-(*Z*)-2-enal	4.74^{b}	0.79^{cd}	0.67^{cd}	0.11^{cd}	7.56^{a}	0.00^{d}	1.69^{c}	0.00^{d}	0.43	<0.001	<0.001	<0.001	
hexa-2,4-dienal	0.61^{c}	0.00^{c}	0.00^{c}	0.00^{c}	4.90^{a}	0.00^{c}	1.98^{b}	0.00^{c}	0.27	<0.001	<0.001	<0.001	MS,KI
dec-(*E*)-2-enal	4.65^{b}	0.29^{c}	0.38^{c}	0.34^{c}	8.99^{a}	1.09^{c}	2.07^{bc}	0.19^{c}	0.51	<0.001	<0.001	<0.001	MS,KI
deca-(*E,E*)-2,4-dienal	1.43^{ab}	0.00^{c}	0.35^{c}	0.00^{c}	2.26^{a}	0.00^{c}	0.67^{bc}	0.00^{c}	0.14	<0.001	<0.001	<0.001	MS,KI
deca-(*E,Z*)-2,4-dienal	3.67^{b}	0.00^{c}	0.40^{c}	0.00^{c}	8.64^{a}	0.00^{c}	3.48^{b}	0.00^{c}	0.50	<0.001	<0.001	<0.001	MS,KI
hydrocarbons													
decane	2.30^{b}	1.73^{bc}	3.30^{a}	1.35^{cd}	1.73^{bc}	0.59^{de}	1.38^{c}	0.45^{e}	0.15	<0.001	<0.001	0.003	MS
non-1-en-3-yne	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	3.19^{a}	0.00^{c}	1.78^{b}	0.99^{bc}	0.19	<0.001	<0.001	<0.001	MS
furans													
dihydro-furan-2-one	0.62^{b}	0.00^{c}	0.00^{c}	0.00^{c}	1.45^{a}	0.00^{c}	1.28^{a}	0.00^{c}	0.09	<0.001	<0.001	<0.001	MS,KI

	CONTROL		BHT		SAGE		ROSEMARY			p-value[2]			
Compds	white	Iberian	white	Iberian	white	Iberian	white	Iberian	SEM	A	O	AxO	MI[3]
2-pentyl-furan	0.66^{bc}	0.33^{c}	0.29^{c}	0.16^{c}	3.93^{a}	0.45^{bc}	1.62^{b}	0.10^{c}	0.21	<0.001	<0.001	<0.001	MS,KI
2,5-dihydro-furan	0.94^{bc}	0.37^{c}	0.58^{c}	0.00^{c}	6.41^{a}	0.00^{c}	2.47^{b}	0.00^{c}	0.35	<0.001	<0.001	<0.001	MS,KI
nitrogen compounds													
2-methyl-piridine	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.83^{a}	0.64^{ab}	0.70^{ab}	0.50^{b}	0.06	<0.001	<0.001	0.043	MS
others													
1-ethyl-2,3-dimethyl-benzene	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	1.49^{a}	0.00^{c}	0.42^{b}	0.00^{c}	0.08	<0.001	<0.001	<0.001	MS,KI
1-methyl-4-methyl-ethyl benzene	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	80.22^{ab}	96.69^{a}	75.30^{b}	73.66^{b}	6.76	<0.001	0.266	0.186	MS,KI
BHT	0.00^{b}	0.00^{b}	1584.65^{a}	1679.90^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	190.93	<0.001	0.356	0.292	MS

[1] Values are means (area units x 10^6) of five analyses.
[2] P-values for the studied factors; A: antioxidant, O: pâté origin, AxO: interaction antioxidant x pâté origin.
[3] Method of Identification; MS: Mass spectrum comparison using wiley and NIST libraries, KI: Kovats index in agreement with literature values.
[a,b,c,d,e] In the same line, means with different superscript resulted statistically different.

Table V.3. Volatile terpenes detected in headspace of liver pâtés from white and Iberian pigs with added sage and rosemary essential oils[1].

	SAGE		ROSEMARY				
	white	Iberian	white	Iberian	sem	p-value[2]	MI[3]
monoterpene hydrocarbons							
α-thujene	5.27^{b}	7.56^{a}	2.45^{c}	2.41^{c}	0.54	<0.001	MS
α-pinene	83.22^{c}	107.82^{bc}	211.17^{ab}	251.65^{a}	20.84	0.002	MS,KI
β-1-pinene	69.16	64.62	72.63	44.46	5.92	0.354	MS
β-2-pinene	1.13^{b}	0.78^{b}	1.66^{a}	1.72^{a}	0.11	<0.001	MS
δ-3-carene	1.46^{a}	0.84^{b}	0.56^{b}	0.58^{b}	0.11	0.001	MS,KI
α-fenchene	8.64	8.91	8.36	9.66	0.56	0.885	MS,KI
camphene	84.58	102.23	58.56	61.67	6.86	0.066	MS,KI
γ-terpinene	1.46^{a}	1.16^{a}	0.00^{b}	0.00^{b}	0.16	<0.001	MS
β-terpinene	14.80^{a}	14.59^{a}	2.27^{b}	1.53^{b}	1.89	0.002	MS
α-terpinene	1.48^{b}	1.76^{ab}	2.18^{ab}	2.32^{a}	0.12	0.041	MS
β-myrcene	62.85^{ab}	89.01^{a}	48.00^{b}	51.33^{b}	5.53	0.021	MS
tricyclene	3.46^{a}	2.49^{ab}	2.08^{b}	1.81^{b}	0.20	0.005	MS,KI
1-limonene	301.23^{a}	391.69^{a}	119.20^{b}	122.88^{b}	30.91	<0.001	MS,KI
(*E*)-ocimene	3.55^{b}	4.74^{a}	0.00^{c}	0.00^{c}	0.47	<0.001	MS
β-ocimene	5.29	6.75	6.10	6.82	0.25	0.099	MS
α-terpinolene	2.85^{c}	5.39^{a}	3.35^{bc}	4.13^{b}	0.25	<0.001	MS,KI
isoterpinolene	6.54^{b}	9.04^{a}	7.96^{ab}	6.74^{b}	0.32	0.008	MS
alloo-cimene	20.07^{a}	21.17^{a}	0.00^{b}	0.00^{b}	2.45	<0.001	MS,KI
sesquiterpene hydrocarbons							
α-cubenene	20.07^{a}	21.17^{a}	0.00^{b}	0.00^{b}	0.13	<0.001	MS,KI
farnesol	1.44^{b}	2.11^{a}	0.00^{c}	0.00^{c}	0.25	<0.001	MS,KI
α-ylangene	2.40^{a}	1.90^{b}	0.00^{c}	0.00^{c}	0.09	<0.001	MS
α-copaene	0.00^{b}	0.00^{b}	0.75^{a}	0.76^{a}	0.24	<0.001	MS,KI
α-gurjunene	0.89^{a}	0.85^{a}	0.00^{b}	0.00^{b}	0.11	<0.001	MS,KI
junipene	2.09^{a}	2.32^{a}	0.00^{b}	0.00^{b}	0.26	<0.001	MS
(*E*)-caryophyllene	24.62^{b}	27.72^{b}	56.40^{a}	60.50^{a}	3.89	<0.001	MS,KI
β-selinene	6.15	7.20	5.82	6.15	0.22	0.134	MS
α-elemene	0.00^{b}	0.00^{b}	2.13^{a}	2.11^{a}	0.25	<0.001	MS
δ-cadinene	1.46^{ab}	1.12^{b}	1.86^{a}	1.91^{a}	0.11	0.014	MS,KI
terpenoid alcohols							
linalool	73.70^{a}	81.88^{a}	37.63^{b}	37.17^{b}	5.07	<0.001	MS,KI
p-menth-3-en-1-ol	5.34^{a}	5.03^{a}	1.36^{b}	1.57^{b}	0.45	<0.001	MS,KI
α-terpineol	3.14	2.79	3.30	2.87	0.15	0.630	MS
endo-borneol	28.87	30.40	32.63	31.87	0.84	0.432	MS,KI
terpinene-4-ol	13.20^{a}	14.24^{a}	7.95^{b}	7.84^{b}	0.77	<0.001	MS
p-cymen-8-ol	2.87^{a}	2.76^{a}	0.59^{b}	0.59^{b}	0.27	<0.001	MS,KI
terpenoid esters							
linalyl acetate	161.76^{a}	152.26^{a}	6.48^{b}	7.25^{b}	17.64	<0.001	MS
linalyl propionate	64.24^{ab}	73.73^{a}	52.69^{b}	55.09^{b}	2.46	0.002	MS
endo-bornyl acetate	16.13	16.53	19.80	21.10	1.16	0.365	MS,KI

	SAGE		ROSEMARY				
	white	Iberian	white	Iberian	sem	p-value[2]	MI[3]
geranyl propionate	0.00[c]	0.00[c]	1.02[b]	1.17[a]	0.22	<0.001	MS,KI
			terpenoid carbonyls				
camphor	1007.11[a]	1026.35[a]	496.87[b]	499.16[b]	64.74	<0.001	MS,KI
			other terpenoids				
α-phellandrene epoxide	26.88[a]	34.90[a]	0.00[b]	0.00[b]	3.68	<0.001	MS,KI
1,8-cineole	766.18[b]	665.81[b]	1012.46[a]	1009.49[a]	58.50	0.046	MS,KI

[1] Values are means (area units x 10^6) of five analyses.
[2] Statistical significance.
[3] Method of Identification; MS: Mass spectrum comparison using wiley and NIST libraries, KI: Kovats index in agreement with literature values.
[a,b,c] In the same line, means with different superscript resulted statistically different in a Tukey test.

CHAPTER VI

Effectiveness of natural and synthetic antioxidants as inhibitors of lipid and protein oxidation: contradictory effects in different types of porcine liver pâtés [♣]

[♣] Submitted to *Food Chemistry* (10 July, 2005)

VI. 1. Abstract

The effect of natural (sage and rosemary essential oils) and synthetic (BHT) antioxidants on lipid and protein oxidation and the increase of non-heme iron (NHI) during refrigeration (+4ºC/90 days) of liver pâtés from intensively reared white pigs, was studied. These results were subsequently compared to those obtain from a parallel evaluation of the same antioxidants on liver pâtés from free-range reared Iberian pigs. Liver pâtés with no added essential oil were used as controls. The addition of BHT enhanced the oxidative stability of liver pâtés from Iberian and white pigs significantly reducing the generation of lipid and protein oxidation products. Conversely, the effect of the sage and rosemary essential oils was different depending on the type of liver pâté in which they were added. In liver pâtés from white pigs, they acted as prooxidants, significantly increasing the generation of TBA-RS whereas no effect was detected on hexanal counts, protein oxidation and NHI content. In liver pâtés from Iberian pigs, sage and rosemary essential oils successfully inhibited the development of lipid and protein oxidation and inhibited, in addition, the release of iron from the heme molecule. The large differences in liver pâtés from Iberian and white pigs in terms of fatty acid composition, tocopherol contents and susceptibility to undergo oxidative reactions could have influenced on the activity of the essential oils.

VI.2. Keywords: Rosemary, sage, BHT, tocopherols, lipid oxidation, protein oxidation, non-heme iron, liver pâté.

VI. 3. Introduction

The understanding of the mechanisms of the lipid oxidation and the factors influencing its occurrence and intensity in meat products has allowed food technologists design strategies to control the development of oxidative reactions during meat handling, processing and storage. These strategies include the modification of the muscle lipid characteristics through dietary means in order to enhance its oxidative stability (Kerry *et al.*, 2000). For instance, pigs are commonly fed using high MUFA/PUFA diets in order to

decrease PUFA levels together with the supplementation with tocopherol (Ruiz & López-Bote, 2002). In certain traditional feeding systems pigs are fed outdoors on natural resources such as grass, acorns and chestnuts which have been demonstrated to provide substances with proven antioxidant activity such as tocopherols and phenolic compounds (Nilzén *et al*., 2001; Estévez *et al*., 2004a; Cantos *et al*., 2005). On the other hand, some additives are directly added to the meat product to inhibit its oxidative deterioration. Synthetic phenolic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl, octyl and dodecyl gallates (PG, OG, DG), have been largely used in different food products though their possible mutagenicity (Clayson *et al*., 1986) have caused the rejection by consumers. Consequently, great scientific efforts have been exerted to find natural antioxidants in plant kingdom in order to use them as an alternative to synthetic antioxidants (Kanner *et al*., 1994). Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officianalis*) are popular *Labiatae* herbs commonly used in meat and fat products to reduce the adverse effects of lipid and protein oxidation (McCarthy *et al*., 2001; Yu *et al*., 2002; Viljanen *et al*., 2004). However, recent studies have reported the complexity associated to the use of herbs or plant extracts as inhibitors of oxidative reactions (Kähkönen *et al*., 1999; Zheng & Wang, 2001; Masuda *et al*., 2002). The antioxidant activity of these substances are affected by many factors including the total number and location of hydroxyl groups on aromatic rings, the nature of the extracts, their concentration and the characteristics of the system in which they are added (Huang & Frankel, 1997; Kähkönen *et al*., 1999; Zheng & Wang, 2001; SKerget *et al*., 2005). In addition, phenolic compounds from plants can interact with other substances such as tocopherols leading to synergist effects (Wong *et al*., 1995; Skerget *et al*., 2005). Kähkönen *et al*. (1999) suggested that the antioxidant activity of plant phenolics could be also affected by the oxidation conditions and lipid characteristics of the system. Furthermore, plant phenolics have shown unexpected prooxidant properties in biological materials and food systems (Laughton *et al*., 1989; Yen *et al*., 1997). Considering the large variety of

food systems and the complexity of their compositions, to approach the study of the effect of plant phenolics on some particular foods involves additional difficulties.

The liver pâté is an emulsion-type cooked product made with meat, liver, adipose tissue and several additives (Estévez *et al*., 2004a). Pâté contains high levels of fat and iron which considerably increases its oxidative instability during processing and subsequent refrigerated storage. The development of lipid and protein oxidation in liver pâtés leads to heme pigment degradation and colour and texture deterioration (Russell *et al*., 2003; Estévez & Cava, 2004). The addition of synthetic or natural antioxidants in liver pâtés could be an interesting option to inhibit the unpleasant effects of lipid and protein oxidation. Considering, however, that the compositional characteristics of the pâté lipids and the concentration of endogenous antioxidants, mainly tocopherols, vary considerably depending on the source of the pig tissues (Estévez *et al*., 2004a), the effect of added antioxidants in different types of liver pâtés seems to be unexpected. The present work was carried out to evaluate the effect of the addition of natural (rosemary and sage essential oils) and synthetic (BHT) antioxidants in liver pâtés produced with tissues from intensively reared white pigs fed on a tocopherol non-supplemented mixed diet and compare the obtained effects with those found in liver pâtés manufactured with tissues from free-range reared Iberian pigs fed on natural resources (acorns and grass) (Estévez *et al*. submitted a).

VI.4. Material and Methods

Raw material

The animals were slaughtered at ~150 Kg and an age of 12 months. Seven white pigs (Large-white x Landrace) were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at ~85 kg live weight and at the age of 7 months. Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed were free-range reared and fed on natural

resources (grass and acorns) following traditional livestock farming procedures for Iberian pigs (Estévez *et al*. submitted a). Iberian pigs and white pigs were slaughtered at the same slaughterhouse one week apart. After slaughter, back fat, muscle *quadriceps femoris*, and liver were removed from carcasses, vacuum packaged and stored at -80ºC until the manufacture of the pâté.

Manufacture of the liver pâté

The experimental pâtés were manufactured in a pilot plant. Depending on the origin of the raw material two types of liver pâtés were produced: liver pâtés from free-range reared Iberian pigs and liver pâtés from intensively reared white pigs. Muscles *quadriceps femoris*, livers and adipose tissues from seven animals from each pig breed were used. The same formulation was used for all liver pâtés except for the addition of antioxidants. In the basic formulation the ingredients were as follows per 100g of manufactured product: 28 g liver, 40 g subcutaneous fat, 5 g muscle, 23 g distilled water, 2 g sodium caseinate, and 2 g sodium chloride. The concentrations of all added additives and antioxidants were calculated in the basis of total ingredients. Sodium di- and tri-phosphates (0.3%), sodium ascorbate (0.05%), and sodium nitrite (0.03%) (all from ANVISA additives, Madrid, Spain) were included. Depending on the experimental batch, different antioxidants were added to liver pâtés from both Iberian and white pigs: rosemary extract (ROSE) (0.1%), sage extract (SAGE) (0.1%), and BHT (BHT) (0.02%). The essential oils (Soria Natural S.L., Soria, Spain) are considered as GRAS (Generally Recognised as Safe) and were added at a level (0.1%) at which the highest antioxidant activity would be exhibited (McCarthy *et al*., 2001). BHT (Sigma-Aldrich, Steinheim, Germany) was added up to the highest level allowed by the Spanish law for this kind of product (0.02%) (BOE, 2002). Control pâtés (CON) containing no added antioxidants were also prepared. The protocol followed for the manufacture of liver pâtés has been explained elsewhere (Estévez *et al*., 2004a). Following the aforementioned recipe, 1.5 kg of raw material was used to produce each of the 8 sets of experimental pâtés which

were manufactured in eight independent production processes. The day before to the manufacture of the liver pâté, the subcutaneous fat was chopped into small cubes (1.5 cm^3) and scalded in distilled water to an internal temperature of +65ºC. Liver and muscle were also sliced into small cubes (1.5 cm^3) and mixed with the sodium chloride, sodium nitrite and the sodium ascorbate in order to allow the nitrification of the samples. The scalded fat and the nitrification mixture were separately kept under refrigeration (+4ºC) in the darkness, before the manufacture of the liver pâtés (24 hours). The day of the production, the sodium caseinate was totally dissolved in hot water (+75ºC) and then added to the scalded fat and mixed during mincing in a Foss Tecator Homogeniser (mod. 2094) during 3 minutes. After that, the nitrification mixture was added to the cutter bowl, together with the water, the sodium di- and tri-phosphates and the antioxidants previously dissolved in 10 mL ethanol. For the production of the control batch, 10 mL of ethanol with no antioxidant was added. The whole mixture was completely minced during 3 minutes until a homogenous raw batter was obtained. Finally, the mixture was packed in glass containers (~50g of pâté per container; 5 containers per group) and heated in a hot water bath (+80ºC/30 min.). After cooling at room temperature, pâtés were stored at +4ºC for 90 days in the dark. Liver pâtés were analysed at days 0, 30, 60, and 90 for TBA-RS numbers, protein carbonyls, concentrations of NHI and hexanal counts. At sampling times, samples were stored at -80ºC until the other analytical experiments were conducted.

Analytical methods

Compositional analysis of liver pâté

Moisture, total protein, and ash were determined using AOAC methods (AOAC, 2000a, b, c). The method of Bligh & Dyer (1959) was used for the extraction and quantification of the fat from liver pâtés.

Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a FFAP-TPA fused-silica column (Hewlett Packard 30m long, 0.53 mm internal diameter and 1.0 μm film thickness). The injector and the detector temperature were held at +230ºC. Oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma). The quantification of fatty acids was carried out by using C13 as an internal standard. Results are expressed as g fatty acid $100g^{-1}$ total fatty acid analysed.

Tocopherols content

α- and γ-tocopherols were extracted from porcine tissues according to the method described by Rey *et al*. (1997). The analysis was carried by reverse phase HPLC (HP 1050, with a UV detector, HPIB 10) (Hewlett-Packard, Waldbronn, Germany). Results are expressed as μg tocopherols/ g tissue.

TBA-RS measurement

Malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al*. (1996). Results were expressed as mg MDA/Kg liver pâté.

Hexanal analysis

The SPME fibre, coated with a divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30μm, was preconditioned prior analysis at +220ºC during 45 min. The HS sampling was performed following a method previously described (Estévez *et al*., 2004b). 1 g of frankfurter was placed in 2.5 mL vials and the SPME fibre was exposed to the headspace of the pâté while the sample equilibrated during 30 minutes immersed in water

at +50ºC. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm id., 1.0mm film thickness). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40 ^{o}C. The SPME fibre was desorbed and maintained in the injection port at 220 ^{o}C during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at +40ºC and then raised at the rate of +7ºC min^{-1} to 250 ^{o}C, and held for 5 min. The GC/MS transfer line temperature was +270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Hexanal was identified by comparing its retention time with that from the standard compound. Results from the volatiles analysis are provided in area units (AU).

Protein oxidation measurement

Protein oxidation as measured by the total carbonyl content was assessed following the 2,4-dinitrophenylhydrazine (DNPH) coupling method described by Oliver *et al* (1987). DNP hydrazones were quantified by measuring absorbance values at 370 nm. Protein concentration was determined by spectrophotometry at 280 nm using bovine serum albumin (BSA) as standard. The amount of carbonyls was expressed as nM carbonyls/ mg protein.

Iron analysis

Nonheme iron (NHI) content was determined by spectrophotometry following the method described by Rhee *et al*. (1987). The amounts of iron were expressed as µg iron/g pâté.

Data analysis

Means and deviations from 5 measurements within each batch were obtained from all analytical experiments. Results from the experiments were used as

variables and analysed by using an Analysis of Variance (ANOVA) from SPSS software in order to assess the effect of the addition of antioxidants and the effect of refrigerated storage on liver pâtés. When statistically significant differences were found, Tukey test's were performed. Statistical significance was set at $p<0.05$.

VI.5. Results and discussion

Effect of antioxidants on liver pâtés from intensively reared white pigs

TBA-RS numbers significantly ($p<0.05$) increased throughout 90 days of refrigerated storage (Figure VI.1.). The oxidative deterioration suffered by liver pâtés as assessed by TBA-RS numbers clearly surpass the values at which the sensory perception of rancidity would be perceived. Gray & Pearson (1987) reported that rancid flavour is initially detected in meat products with TBA-RS values between 0.5 and 2.0. On the other hand, a peculiar warmed-over flavour (WOF) is developed during refrigerated storage of cooked meats as a result of the oxidation of PUFA (Tim & Watts, 1958). In accordance to Boles & Parrish (1990) WOF could be perceived in meat products at TBA-RS values above 1.0.

The addition of BHT significantly inhibited the development of lipid oxidation in liver pâtés since at all days of analysis TBA-RS numbers were significantly lower in BHT pâtés than in the CON ones. Conversely, the addition of the essential oils significantly increased the generation of TBA-RS at days 60 and 90 of storage. The sage essential oil exhibited a more intense prooxidant effect than rosemary since SAGE pâtés had significantly higher TBA-RS numbers than ROSE ones at all days of storage. Particularly remarkable is the effect of BHT of the generation of hexanal which has been considered as a lipid oxidation indicator (Shahidi & Pegg, 1993) (Figure VI.2.). BHT pâtés showed significantly smaller hexanal counts than CON ones at days 0 and 90 of storage which is in agreement with results obtained for TBA-RS numbers. In opposition, the plant essential oils showed no effect since SAGE and ROSE had similar hexanal counts than control ones. Hexanal is mainly generated as

a consequence of the oxidative decomposition of PUFA and has been related to rancid odours and used as an indicator of lipid oxidation (Shahidi & Pegg, 1993).

Protein oxidation is considered to be linked to lipid oxidation. In the presence of free-radicals generated in the primary steps of PUFA oxidation, protein oxidation is manifested by free radical chain reactions similar to those for lipid oxidation (Gardner, 1979). Accordingly, significant correlations have been found between lipid oxidation as measured by TBA-RS numbers and protein carbonyls (Mercier *et al*., 1995; Batifoulier *et al*., 2002; Estévez & Cava, 2004). In agreement with results from lipid oxidation, the addition of BHT significantly reduced the generation of carbonyls from protein oxidation at days 30, 60 and 90 of refrigerated storage (Figure VI.3.). On the contrary, sage essential oil showed a prooxidant effect significantly increasing the amount of protein carbonyls at days 30 and 60 whereas no significantly effect was detected at days 0 and 90. Rosemary essential oil showed no effect on protein oxidation except at day 60 in which a prooxidant effect was observed. The effect of added antioxidants on protein oxidation has been scarcely studied. Recently, Viljanen *et al*. (2004) have described the protective role of plant phenolics against lipid and protein oxidation in liposomes. The similarity between the effects of the added BHT and essential oils on lipid and protein oxidation supports the idea that both oxidative processes are developed upon common chemical mechanisms. Therefore, the protective role of BHT against lipid oxidation would enhance the stability of proteins through the reduction of the generation of reactive molecules such as radicals. Consistently, the prooxidant effect of the plant essential oils on lipid oxidation had the reflection on the oxidation of proteins, significantly increasing the generation of protein carbonyls.

The occurrence of protein oxidation in liver pâtés has been recently associated to heme pigments degradation and release of iron from the porphyrin ring (Estévez & Cava, 2004). The present results support the idea that some disruption of the heme molecule happened during refrigerated storage of liver pâtés since the amount of NHI significantly increased over

time (Figure VI.4.). From day 0 to day 60, all groups of liver pâté contained similar NHI amounts. During the last period of refrigerated storage (days 60-90), the addition of BHT significantly inhibited the release of iron from the heme molecule as a likely consequence of its protective role against protein oxidation. Plant essential oils had no effect on the release of iron since the evolution of the NHI content was similar among CON, SAGE and ROSE pâtés and contained at all days of storage similar NHI contents. The present results suggest that the role played by BHT on protein oxidation influenced on the stability of the heme pigments, significantly reducing the release of iron from the heme molecule. In addition, the lack of effect of the sage and rosemary essential oils as far as the amount of NHI is concerned is in agreement with the overall lack of effect of those on protein oxidation. The prooxidant effect of plant essential oils at day 60 had no reflection on the release of iron.

The effectiveness of BHT as inhibitor of lipid oxidation in meat products is supported by numerous studies (McCarthy *et al*., 2001; Formanek *et al*., 2001; Ansorena & Astiasarán, 2004; Sebranek *et al*., 2005). According to the present results, BHT is also an effective antioxidant regarding protein oxidation and the release of iron from heme molecule. Despite of their efficiency as antioxidants, the use of synthetic additives has been questioned due to their potential risk (Clayson *et al*., 1986). Plant essential oils have been successfully used as alternatives to inhibit the oxidative deterioration of meat and fat products (McCarthy *et al*., 2001; Yu *et al*., 2002*;* Ahn *et al*., 2002). Chen *et al*. (1999), Formanek *et al*. (2001) and McCarthy *et al*. (2001) described the high effectiveness of natural antioxidants in meat products and reported that natural antioxidants showed similar antioxidant effects than synthetic antioxidants such as BHT. Sebranek *et al*. (2004) reported similar antioxidant activities of rosemary extracts and synthetic ones (BHT/BHA) regarding MDA generation in refrigerated sausages. Results from the present study disagree with those obtained by the aforementioned authors, suggesting even the possibility that the addition of plant phenolics could promote the development of oxidative reactions in the liver pâtés. Phenolic compounds have been found to autoxidise and allow the production of

reactive oxygen species by reducing iron from the ferric form (Fe^{3+}) to the more active ferrous form (Fe^{2+}) which is able to generate hydroxyl radicals (Laughton *et al*., 1989). This prooxidative mechanism has been also described for two recognised antioxidant substances such as α-tocopherol (Pokorny, 1991) and ascorbic acid (Fukuzawa *et al*., 1993). Though the information concerning the role of plant phenolics as oxidation promoters is scarce, several studies have reported prooxidant effects of plant phenolics in food systems (Huang & Frankel, 1997; Yen *et al*., 1997; Chen *et al*., 1999; Škerget *et al*., 2005).

Effect of antioxidants in liver pâtés from free-range reared Iberian pigs

In previous works (Estévez *et al*., 2004b; Estévez *et al*., submitted a) the effect of the addition of BHT and sage and rosemary essential oils on the oxidative stability of liver pâtés produced with tissues from free-range reared Iberian pigs was reported. In those papers, the effect displayed by BHT was similar to that found in the present study. BHT significantly inhibited the oxidation of lipids and proteins during refrigerated storage of liver pâtés from Iberian pigs (Estévez *et al*., 2004b; Estévez *et al*., submitted a). Figures VI.5-8 show the comparison between the activities of the sage and rosemary essential oils in liver pâtés from Iberian and white pigs. In clear opposition to the effect of BHT, the effect of the essential oils seems to be influenced by the characteristics of the liver pâté. The addition of sage and rosemary essential oil enhanced the generation of TBA-RS in liver pâtés from white pigs at day 90 whereas significantly reduced TBA-RS numbers in liver pâtés from Iberian pigs (Figure VI.5a vs. VI.5b). In liver pâtés from white pigs, the essential oils had no effect on the hexanal counts while greatly influenced on liver pâtés from Iberian pigs significantly reducing the generation of hexanal (Figure VI.6a vs. VI.6b). Similar results were obtained on protein oxidation since no effect of essential oils was observed in liver pâtés from white pigs and the same essential oils significantly inhibited the generation of protein carbonyls in liver pâtés from Iberian pigs at day 90 (Figure VI.7a vs. VI.7b). This contradictory effect was also observed regarding the release of iron from the

heme molecule since control and treated pâtés from white pigs contained similar amounts of NHI and the addition of sage and rosemary oils in liver pâtés from Iberian pigs significantly reduced the release of iron from the heme molecule (Figure VI.8a vs. VI.8b).

Results from the present work suggest that the activity of the rosemary essential oil was dependent on the compositional characteristics of the food matrix. In fact, the effect of plant phenolics has been considered to be influenced by the compositional characteristics of the food system and the presence of other active substances (Yen *et al*., 1997; Huang & Frankel, 1997). Food systems, and particularly comminuted meat products such as liver pâtés, are very complex in the number and the type of chemicals in the mixture, and a particular combination of these compounds might behave differently from the individual components. In this sense, Wong *et al*. (1995) and Fang & Wada (1993) reported possible interactions between phenolic compounds from sage and rosemary essential oils and tocopherols, leading to different effects depending on the individual amounts of these substances in the food system. In the present work, muscles, adipose tissues and livers used for the production of liver pâtés from Iberian pigs contained significantly higher amounts of α-tocopherol than those from white pigs (Figure VI.9.). The high levels of tocopherols in tissues and elaborated products from free-range reared Iberian pigs is explained by the intake of natural resources (mainly grass and acorns) during the outdoors rearing of Iberian pigs (Estévez *et al*., 2004a). Therefore, the presence of a certain amount of endogenous antioxidants (tocopherols) in the raw material and manufactured product could influence on the activity of exogenous active extracts, leading to antioxidant or pro-oxidant effects. In this sense, similar effects have been reported in foods when mixtures of two antioxidants were joined at different proportions. For instance, β-carotene acted as a prooxidant in refrigerated stored chicken when the levels of tissue tocopherols were low, whereas it showed antioxidant effects at higher concentrations of tocopherol (Esteve-Garcia *et al*., 1998) which is in clear coincidence with the present results.

In addition, when testing natural antioxidants it is important to consider the system composition in terms of lipid substrate and degree of unsaturation (Frankel & Meyer, 2000). In accordance to Huang & Frankel (1997), whether phenolic compounds act as antioxidants or prooxidants appears to be dependant on the lipid characteristics of the model system. These authors reported antioxidant activities of tea chatechins in corn oil triglycerides whereas in oil in water emulsions, these compounds were all prooxidants. Liver pâtés from Iberian pigs had significantly higher percentages of oleic and total monounsaturated fatty acids (MUFA) than liver pâtés from white pigs which contained higher proportions of saturated (SFA) and polyunsaturated fatty acids (PUFA) (Table VI.1.). The analysis of the fatty acid composition of the tissues used for liver pâtés manufacture revealed similar results (Estévez *et al.*, 2004a). The high levels of oleic acid in tissues and meat products from Iberian pigs is due to the deposition of high amounts of such fatty acid derived from the intake of acorns (Estévez *et al.*, 2004a). The fatty acid profile of the tissues and liver pâté from white pigs reflected the fatty acid composition of the mixed diet on which white pigs were fed (Estévez *et al.*, 2004a). The different fatty acid composition between liver pâtés (Table VI.1.) affects the physical state of the lipids that could have affected the dispersion and antioxidant activity of the sage and rosemary essential oils leading to different effects. As long as liver pâtés from Iberian and white pigs showed similar proximate compositions (data not shown), the influence of other major component of liver pâtés supporting the contradictory effect of added antioxidants is unlikely.

Finally, the activity of the rosemary essential oil could have been affected by the initial oxidation state of the liver pâtés in which it was added (Frankel & Meyer, 2000). In systems with higher oxidative instability, the activity of plant phenolics could be reduced since phenolic compounds can be oxidised and the oxidation products could act as prooxidants promoting oxidative reactions (Huang & Frankel, 1997). The high oxidative instability in liver pâtés from white pigs would explain the prooxidant effect of the essential oils regarding TBA-RS numbers and the lack of effect on protein oxidation

whereas in Iberian liver pâtés, which showed considerably higher oxidative stability, the essential oil acted as a potent antioxidant. Furthermore, the oxidation of phenolics in Iberian pâtés could have been inhibited by the presence of high levels of tocopherols with which plant phenolics interact leading to regeneration and synergist effects (Wong *et al.*, 1995; Hupia *et al.*, 1996; Zhu *et al.*, 1999). The results obtained in the present work are in agreement with those obtained in a previous study in which a rosemary essential oil (600 ppm) showed an antioxidant effect when added on frankfurters from Iberian pigs and exhibited the opposite (prooxidant) effect in liver pâtés from white pigs (Estévez *et al.*, submitted b). Differences between liver pâtés from Iberian and white pigs reported in that study in terms of fatty acid composition and tocopherol contents are consistent with those reported in the present study which support the hypothesis and mechanisms suggested.

VI.6. Conclusion

In accordance to the present results, the effect of added sage and rosemary essential oils on liver pâtés is unpredictable depending on the characteristics of the liver pâté. Therefore, the use of plant materials with antioxidant properties in commercial liver pâtés should be preceded by systematic studies to assure the desirable antioxidant effects. The synthetic antioxidant (BHT) was not affected by the characteristics of the liver pâtés which could be considered as an advantage in comparison to essential oils. In the absence of the knowledge of precise mechanisms of interaction between the essential oil components and the liver product, further experiments would be required to shed light on the specific interactions between plant extracts and food components and to evaluate the influence of the chemical composition of tissues in terms of fatty acids and tocopherols on the activity of these substances.

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VI.8. References

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Table VI.1. Fatty acid composition (means ± standard deviation) of liver pâtes from extensively reared Iberian and intensively reared white pigs.

	Iberian	White	p[1]
C16:0[2]	20.69±0.08	22.65±0.05	<0.001
C18:0	10.58±0.00	13.40±0.03	<0.001
ΣSFA	32.87±0.09	37.98±0.12	<0.001
C16:1 (n-7)	2.00±0.01	2.44±0.00	<0.001
C18:1 (n-9)	53.43±0.07	43.57±0.07	<0.001
C20:1 (n-9)	1.83±0.02	1.08±0.04	<0.001
ΣMUFA	57.52±0.06	47.58±.010	<0.001
C18:2 (n-6)	7.71±0.11	12.23±0.04	<0.001
C18:3 (n-3)	0.49±0.01	0.61±0.01	<0.001
C20:2 (n-6)	0.57±0.02	0.45±0.23	0.296
C20:4 (n-6)	0.56±0.00	0.70±0.03	<0.001
ΣPUFA	9.63±0.29	14.40±0.37	<0.001

[1] Statistical significance in a student 't' test for independent variables.
[2] Fatty acids expressed as percentages of total fatty acids analysed.
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Figure VI.1. Evolution of TBA-RS numbers of during refrigerated storage of liver pâtés from white pigs with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

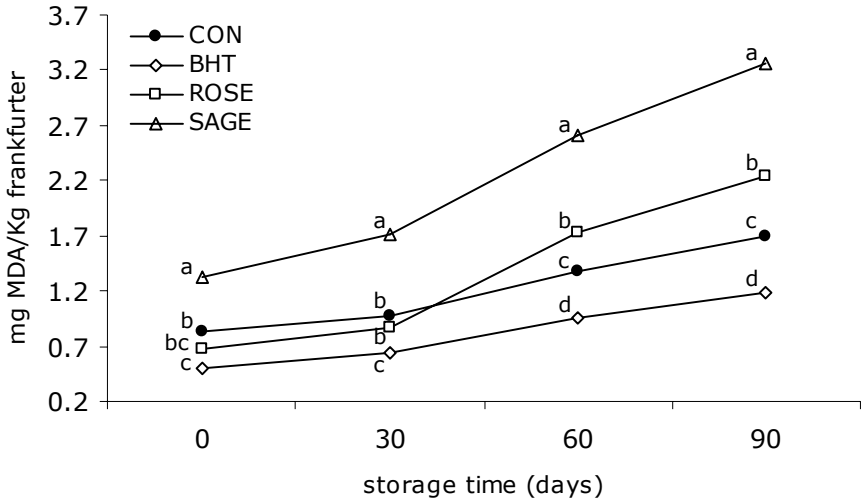


Figure VI.2. Hexanal counts at days 0 and 90 of refrigerated storage of liver pâtés from white pigs with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

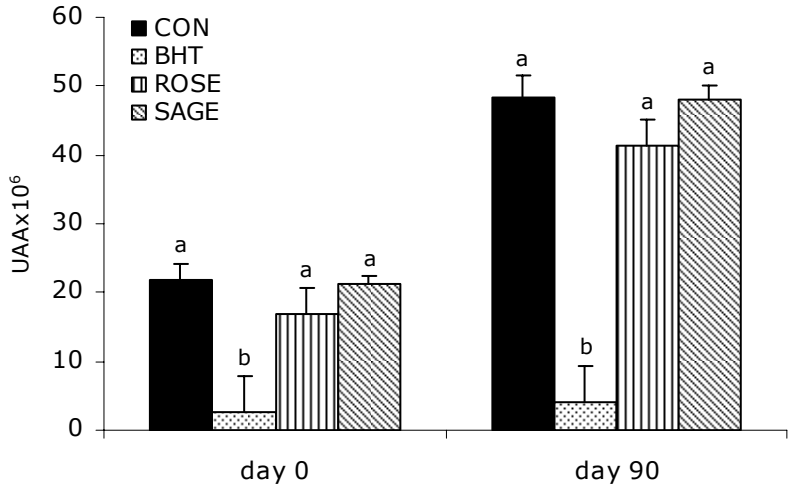


Figure VI.3. Evolution of protein oxidation during refrigerated storage of liver pâtés from white pigs with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

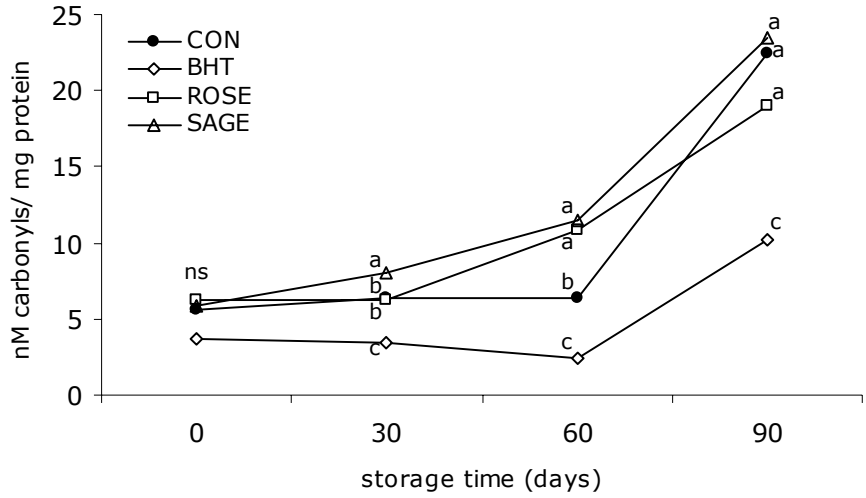


Figure VI.4. Evolution of NHI content during refrigerated storage of liver pâtés from white pigs with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

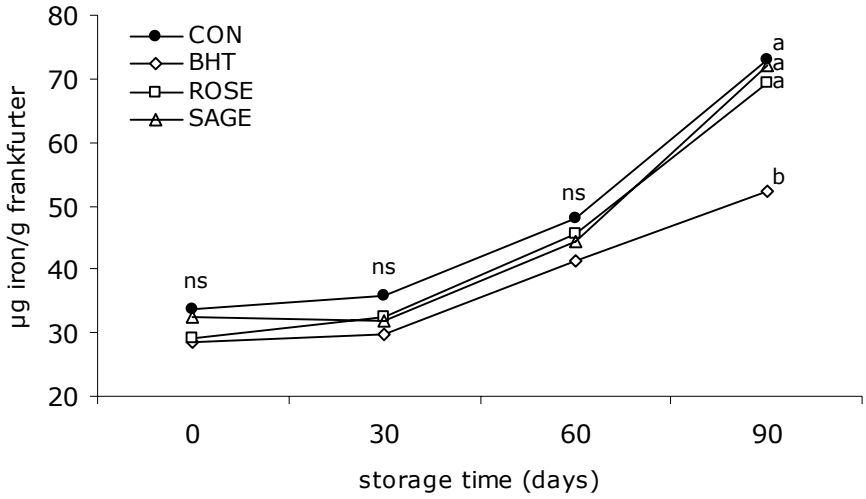


Figure VI.5. TBA-RS numbers in liver pâtés from white (A) and Iberian pigs (B) treated with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

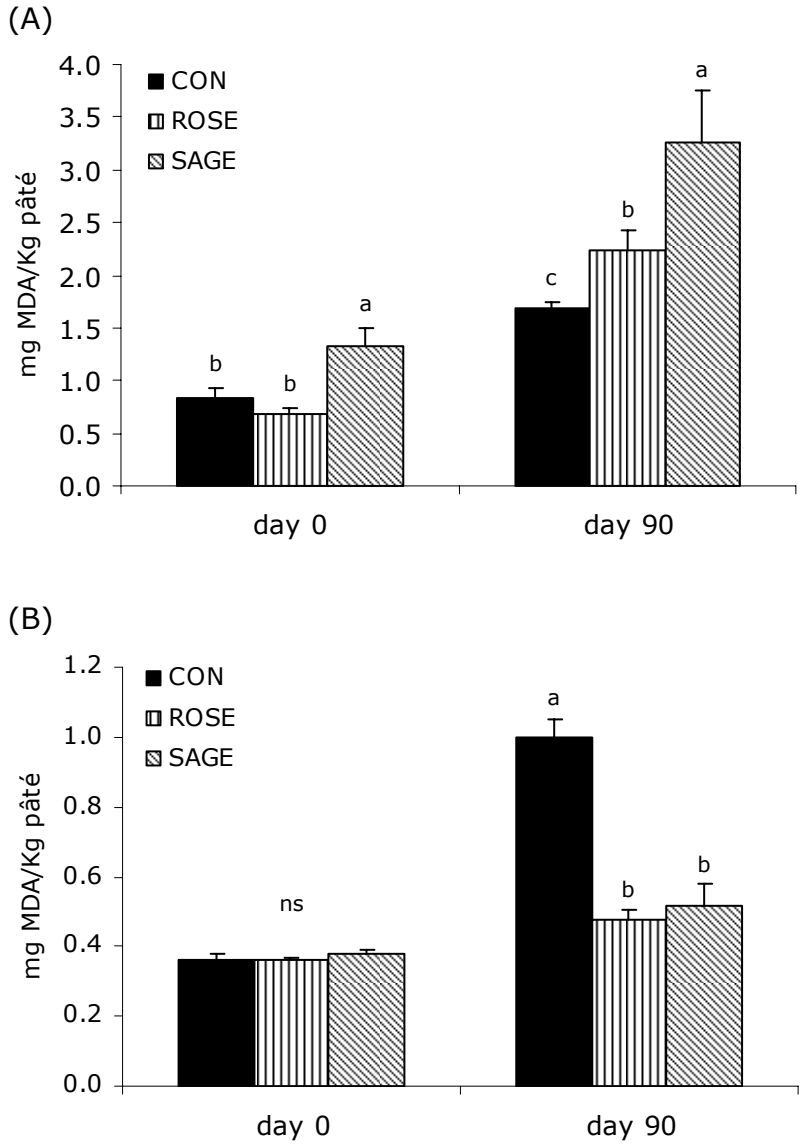


Figure VI.6. Hexanal counts in liver pâtés from white (A) and Iberian pigs (B) treated with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

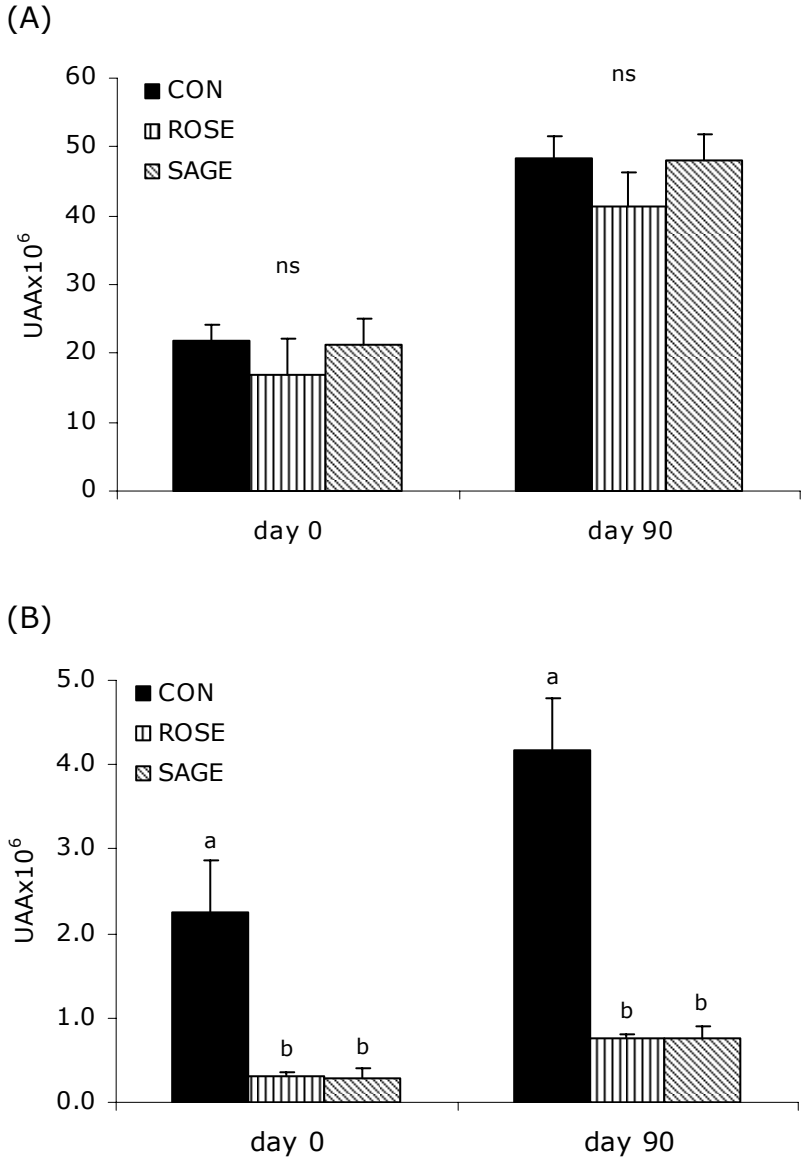


Figure VI.7. Protein carbonyls in liver pâtés from white (A) and Iberian pigs (B) (Results taken from Estévez *et al*. (submitted a)) treated with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

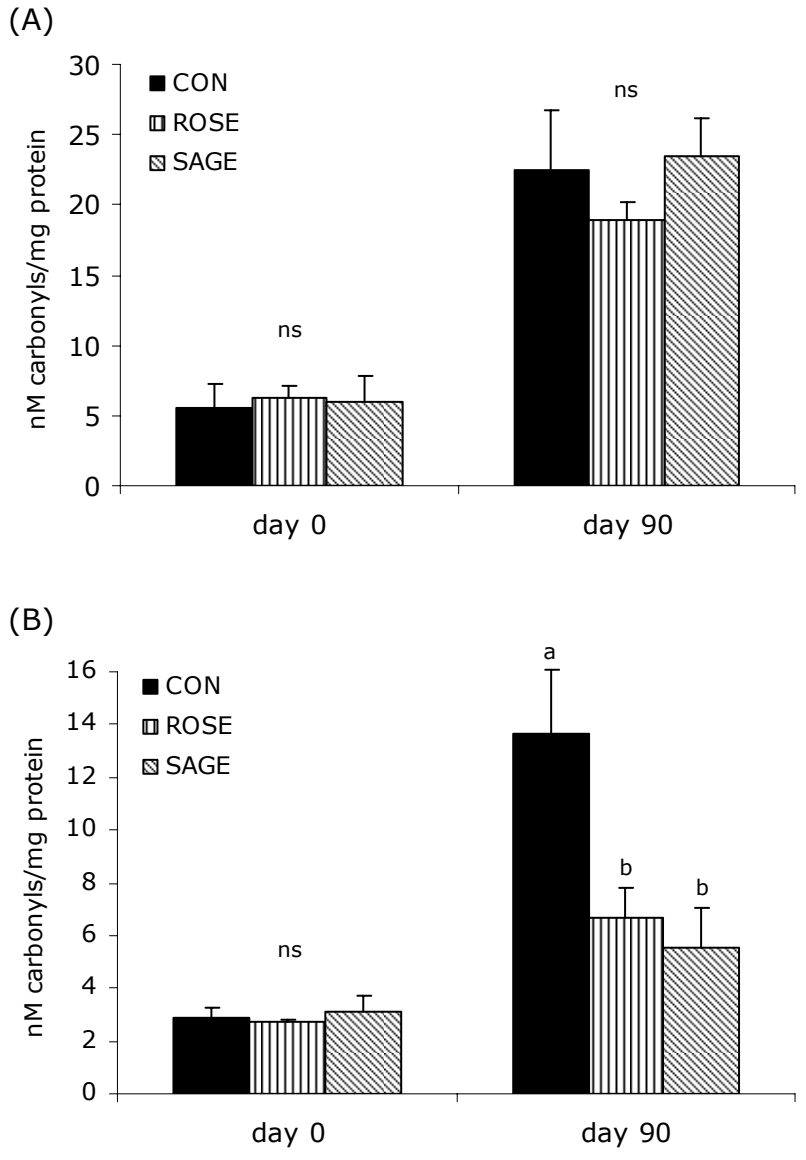


Figure VI.8. NHI content in liver pâtés from white (A) and Iberian pigs (B) (Results taken from Estévez *et al*. (submitted a)) treated with added BHT and sage and rosemary essential oils. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

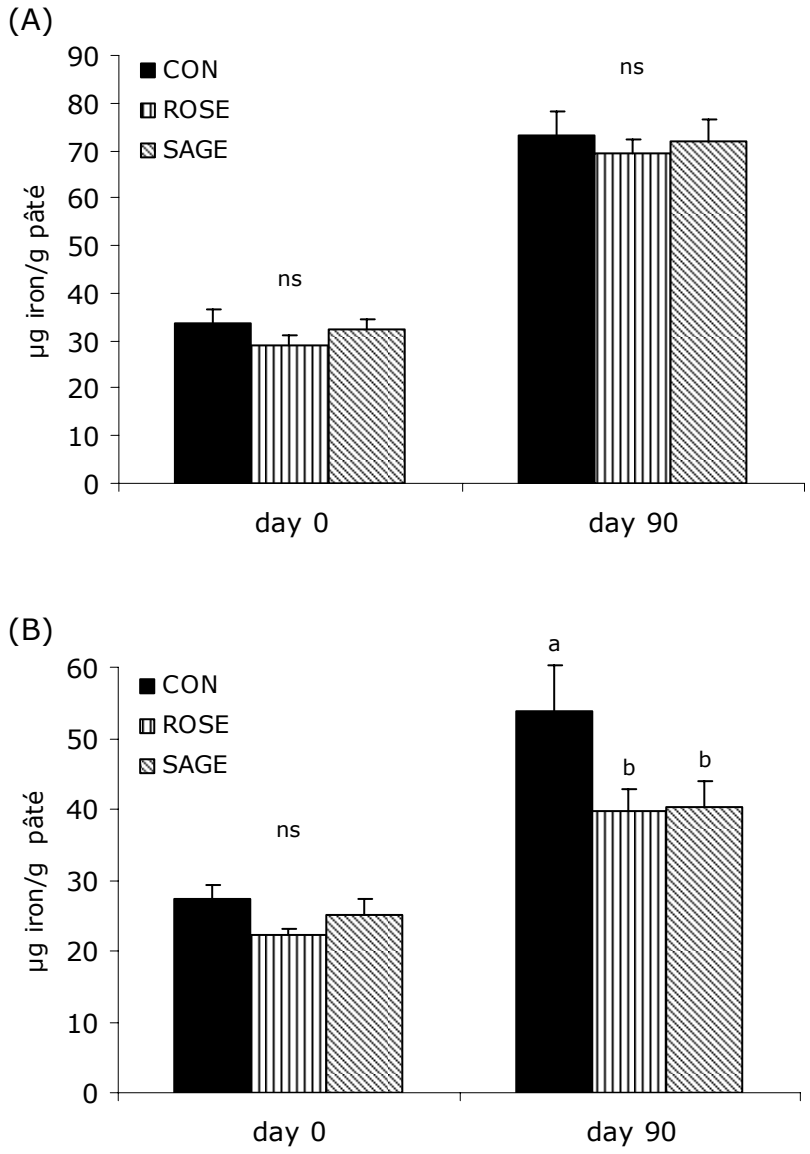
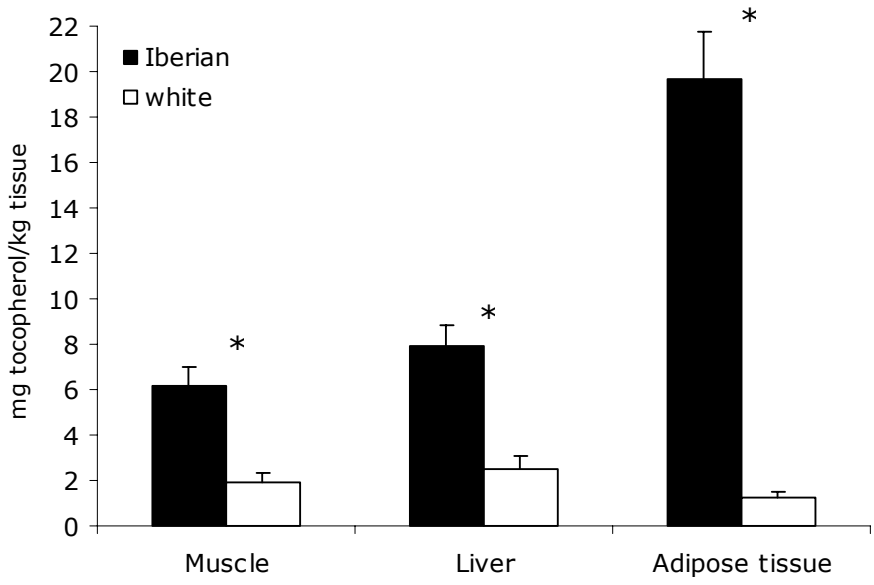


Figure VI.9. Tocopherol content in tissues from extensively reared Iberian pigs and intensively reared white pigs (Significant differences, $p<0.001$, between groups within a tissue are denoted with an asterisk).



CHAPTER VII

Extensively reared Iberian pigs versus intensively reared white pigs for the manufacture of frankfurters[♣]

[♣] Accepted for publication in *Meat Science* (18 July, 2005)

VII. 1. Abstract

Physico-chemical characteristics and different quality traits of the raw material (meat and adipose tissue) and the frankfurters elaborated from extensively reared Iberian pigs (IF) and intensively reared white pigs (WF) were evaluated. Hybrid frankfurters (HF) made with meat from white pigs and adipose tissue from Iberian pigs were also studied. The differences found between muscles and adipose tissues from Iberian and white pigs largely influenced the characteristics displayed by the frankfurters. Particularly remarkable are the higher amounts of substances with proven antioxidant activity such as tocopherols and phenolic compounds in tissues from Iberian pigs than in those from white pigs. No significant differences were found amongst frankfurters for their proximate composition though IF presented a higher iron content than WF and HF. IF exhibited a redder and darker colour than WF and HF. The latter were paler and showed higher hue values than IF. Though no significant differences were found amongst frankfurters for their texture profile, a clear trend was detected, with the HF showing intermediate texture characteristics between IF and WF. Concerning their fatty acid composition, IF had higher proportions of oleic acid and MUFA and smaller of SFA and PUFA than WF. From a nutritional point of view, IF had a lower n-6/n-3 value than WF. The addition of adipose tissue from Iberian pigs to the HF modified its fatty acid composition compared to that of WF, significantly increasing the percentages of MUFA and reducing the proportions of PUFA, SFA and the n-6/n-3 value.

VII.2. Keywords: Frankfurters, Iberian pigs, white pigs, iron, tocopherol, colour, texture profile, fatty acids.

VII.3. Introduction

As far as the Spanish livestock farming is concerned, the Iberian pigs are considered authentic gems. The products traditionally obtained from these animals such as dry-cured hams and dry-cured loins are highly appreciated by Spanish consumers. The reasons why these products from Iberian pigs are so

highly prized compared to those from other pigs are thought to be related to their extraordinary sensory characteristics. Particular genetic traits, the extensive rearing in oak forests and the use of natural resources such as acorns and grass for the feeding of Iberian pigs are carefully taken into consideration in order to achieve high-quality products and, therefore, fulfil consumer's expectations (Ventanas *et al*., 2001).

As a result of the activity of slaughterhouses and meat factories, a large amount of by-products (back fat, boneless meat and some viscera) with high nutritional value are generated. In Spain, in the year 2000, 24,300 metric tons of fat was obtained as a result of the slaughter of 600,000 outdoor reared Iberian pigs (revised by Cava *et al*., 2004). This foodstuff is either used in the local industry for the manufacture of low-quality products such as restructured meats or cured lards or removed, when the capacity of production is exceeded, using high cost processes, assuming sometimes environmental pollution. Estévez *et al*. (2004) suggested using the back fat, meat and livers from Iberian pigs for the manufacture of liver pâtés, which is something that is being done in Spain. The consumers could be influenced by the image of quality of the Iberian dry-cured products and will pay more money for the Iberian liver pâté as a high quality product manufactured using low-cost materials. In fact, according to Estévez *et al*., (2004) and Estévez & Cava (2004), liver pâtés from Iberian pigs have higher quality characteristics than those from white pigs, based on compositional and nutritional points of view.

Frankfurters are non-fermented, emulsion type cooked sausages produced with meat and fat from beef or pork. The production of frankfurters in Spain is around 10% of the total cooked products (AICE, 2004). The higher levels of oleic acid and tocopherol in the tissues from Iberian pigs, compared to those from white pigs, are some of their most remarkable quality traits (López-Bote & Rey, 2001; Estévez *et al*., 2004). It is reasonable to consider that frankfurters from Iberian pigs must be rather different from commercial frankfurters, generally produced using material from intensively reared white pigs. Nevertheless, there is no information available concerning the quality

characteristics of the Iberian frankfurters no a study comparing these characteristics with those displayed by frankfurters from white pigs.

The aim of this piece of work was to study the physico-chemical characteristics of the raw material (meat and adipose tissue) and the manufactured product (frankfurters) from extensively reared Iberian pigs and intensively reared white pigs. The effect of the addition of fat from Iberian pigs in frankfurters produced with meat from white pigs was also investigated.

VII.4. Material and Methods

Animals, feeds and sampling

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered at ~150 Kg live weight and an age of 12 months.

The analysis of the acorns (Table VII.1.) revealed a high content of ether extract (5.0%) and oleic acid (67.3%) agreeing with previous reports (Cava *et al.*, 1997; Ruiz *et al.*, 1998). The grass had high levels of moisture (89.3%) and low of ether extract (0.33%) being the linolenic acid (n-3) the most abundant (57.8%), in agreement with other authors (López-Bote & Rey, 2001).

Seven white pigs were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at 85 kg live weight and at the age of 7 months. The analysis of the mixed diet revealed a higher content of protein (mixed diet: 15.2%; acorn: 4.3%; grass: 4.3%) and lower of ether extract (mixed diet: 2.32%; acorn: 5.05%; grass: 6.26%), compared to the feed given to Iberian pigs (Table VII.1.). The palmitic acid (16.7%), oleic acid (31.3%) and linoleic acid (39.7%) were the major fatty acids.

Iberian pigs and white pigs were slaughtered at the same slaughterhouse one week apart. After slaughter, adipose tissues and meat were removed from the

carcasses, vacuum packaged and stored at -80ºC until the day of their analysis and the manufacture of the frankfurters.

Manufacture of the frankfurters

The experimental frankfurters were manufactured in a pilot plant. The same formulation was used for all frankfurters. Three different types of frankfurters were considered depending on the source of the raw material: frankfurters from Iberian pigs (IF), frankfurters from white pigs (WF) and hybrid frankfurters (HF) elaborated with meat from white pigs and adipose tissue from Iberian pigs. For the manufacture of the sausages, meat and adipose tissues from seven different animals were used for each of the groups (Iberian and white pigs). The ingredients were as follows per 100g of elaborated product: 50 g meat, 10 g adipose tissue, 37 g distilled water, 2 g sodium caseinate, 1 g potato starch. Sodium chloride (2%), Sodium di- and tri-phosphates (0.5%) sodium ascorbate (0.05%) and sodium nitrite (0.03%) (all from ANVISA, Madrid, Spain) were also added. Following the aforementioned recipe, 1.3 kg of raw material was used for each group, to produce the experimental frankfurters. Firstly, the meat was chopped into small cubes (1 cm^3) and mixed with the sodium chloride, sodium nitrite and the sodium ascorbate in order to allow the nitrification of the samples 2 hours before the manufacture. Then, the meat was minced in a cutter (Foss Tecator Homogeniser, mod. 2094) for 2 minutes together with the starch and the 50% of the sodium caseinate which was previously dissolved in water (+75ºC). After that, the adipose tissue was added together with the remaining dissolved sodium caseinate and minced for 4 more minutes until a homogenous raw batter was obtained. Finally, the mixture was stuffed into 18 mm diameter cellulose casings, handlinked at 10 cm intervals and given the thermal treatment in a hot water bath (+80ºC/30'). The frankfurters were kept frozen (-80ºC) until required for analytical experiments.

Analytical methods

Compositional analysis of raw material and frankfurters

Moisture, total protein and ash were determined using official methods (AOAC, 2000). The method of Bligh & Dyer (1959) was used for determining fat content of raw material and frankfurters. Total iron was determined following the procedure described by Miller *et al*. (1994).

Tocopherol content

The levels of tocopherol in meat and adipose tissues were determined according to the method described by Rey *et al*. (1997).

Phenolics compounds content

The Folin Ciocalteau reagent was used for the quantification of total phenolics as described by Turkmen *et al*. (in press) with minor modifications as follows: 0.5 g of meat (1 g of adipose tissue) was homogenised with 10 mL of water (meat) or 80% methanolic water (adipose tissue) and centrifuged for 5 minutes at 3000 rpm and +4ºC. Phenolics were extracted from the pellets following the same procedure. The supernatants were combined and 1 mL aliquot was mixed with 5 mL of Folin Ciocalteau reagent (10% in distilled water) in test tubes. After 5 minutes, 4 mL of sodium carbonate (7.5% in distilled water) was added, the test tubes were screw-capped and the samples allowed to stand for 2 hours at room temperature in the darkness. A standard curve with ethanolic gallic acid (ranged from 0.625×10^{-3} mg/mL to 0.02 mg/mL) was used for quantification. Results were expressed as mg of gallic acid equivalents (GAE) per gram of sample.

Instrumental colour measurement

Instrumental colour (CIE L* a* b*; CIE, 1976) was measured in triplicate on the cross section of the frankfurters using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ) with illuminant D_{65} and 0º standard observer. CIELAB L*, a*, b* values were determined as indicators of lightness, redness and yellowness, respectively. Chroma (C) and

Hue angle (Hº) values were obtained by using the following equations: $C= (a^{*2} + b^{*2})^{0.5}$; Hº= arctg b*/a* x (360/6.28).

Texture profile analysis (TPA)

The textural characteristics of the frankfurters were determined using a texturometer TA-XT2 TEXTURE ANALYSER (Stable Micro Systems Ltd., Surrey, England, UK). Uniform portions of 2 cm in length from the middle of the frankfurters were used as the test samples. The samples were compressed to 50% their original height at a crosshead speed of 5 mm/s through a two-cycle sequence. A 5 cm diameter probe was used in TPA measurements. Textural variables from force and area measurements were (Bourne, 1978): Hardness (N/cm^2)= maximum force required to compress the sample (peak force during the first compression cycle); Fracturability (N/cm^2)= the force during the first compression at which the material fractures; Adhesiveness (N s)= work necessary to pull the compressing plunger away from the sample; Springiness (cm)= height that the sample recovers during the time that elapses between the end of the first compression and the start of the second; Cohesiveness (dimensionless)= extent to which the sample could be deformed prior to rupture (A_1/A_2, A_1 being the total energy required to for the first compression and A_2 the total energy required for the second compression); Gumminess (N/cm^2)= the force needed to disintegrate a semisolid sample to a steady state of swallowing (hardness x cohesiveness); Chewiness (N s)= the work needed to chew a solid sample to a steady state of swallowing (gumminess x springiness); Resilience (dimensionless) = how well the product regains its original height , measured on the first withdrawal of the cylinder (area under the curve during the withdrawal of the first compression divided by the area of the first compression).

Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification with methanol in sulphuric acid (5%) and sodium methylate, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard,

mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a semi-capillary column (Hewlett Packard FFAP-TPA fused-silica column, 30m long, 0.53 mm internal diameter and 1.0 μm film thickness). The injector and the detector temperature were held at +230ºC. Column oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma). Fatty acid composition was expressed as percent of total fatty acid methyl esters.

Data analysis

All experiments were carried out in quindublicate. The results from the experiments were used as variables and analysed using an Analysis of Variance (ANOVA) (SPSS, 1997) in order to compare physico-chemical characteristics of meat and adipose tissue and frankfurters from Iberian pigs and white pigs. Statistical significance was predetermined at 0.05.

VII.5. Results and Discussion

General composition of meat and adipose tissue

The chemical composition of meat and adipose tissue from Iberian pigs and white pigs is shown in Table VII.2. No significant differences were found between groups which had similar contents of moisture (60.1%-58.9%), fat (18.3%-19.6%) and protein (17.0%-16.9%). According to previous studies (Estévez *et al.*, 2003; Estévez *et al.*, 2004), the higher content of iron in meat from Iberian pigs compared to that from white pigs (29.8 μg/g vs. 22.9 μg/g; $p<0.05$) was expected because of genetic traits, the higher age and weight at slaughter of Iberian pigs and the physical exercise performed by animals in extensive systems (Lawrie, 1998). Meat from Iberian pigs has been described as an excellent source of high bioavailable iron, even though this fact could imply the promotion of oxidative processes in this meat after cooking and refrigeration (Estévez *et al.*, 2003). Compared to meat from white pigs, meat

from Iberian pigs had a significantly higher amount of α- (3.8 μg/g vs. 1.8 μg/g) and γ-tocopherol (0.22 mg/kg vs. 0.08 mg/kg) as a likely result of the intake of grass with high levels of tocopherol by Iberian pigs (Rey *et al.*, 1998). Samples were also analysed for the total amount of phenolic compounds. In accordance to results of tocopherol content, meat from Iberian pigs contained a higher amount of total phenolics than that from white pigs. The information concerning the occurrence of phenolic compounds in animal tissues is extremely scarce since such compounds are widespread in the plant kingdom and therefore, their presence in animal tissues is principally relegated to the intake of natural resources by animals and their subsequent accumulation. The intake of grass and acorns by Iberian pigs could explain the higher amount of phenolic compounds in their tissues than in those from white pigs. In fact, Cantos *et al.* (2003) have recently reported elevated polyphenol levels in acorns.

As expected, fat was the principal component of adipose tissue from Iberian and white pigs (83.2% and 74.9% respectively; $p<0.05$) while moisture (Iberian: 9.7%; white 12.9%; $p<0.05$) and protein (Iberian: 2.6%; white 4.0%; $p<0.05$) presented smaller extents. Large differences were found between groups as far as the tocopherol content is concerned. Similar to the results presented above for meat, adipose tissue from Iberian pigs had significantly higher amounts of α- (17.2 μg/g vs. 3.9 μg/g) and γ-tocopherol (1.1 mg/kg vs. 0.0 mg/kg) than the adipose tissue from white pigs. The intake of pasture and acorns by free-range reared pigs is thought to increase the tocopherol levels in the animal tissues (Rey *et al.*, 1998; Nilzén *et al.*, 2001) which is consistent with the present results. Tocopherols are the most important natural antioxidants in meat and meat products and their protective activity against oxidation have been largely described in meat and several meat products (López-Bote & Rey, 2001; Nilzén *et al.*, 2001). Results from the present study suggest that phenolic compounds are, contrary to tocopherols, mainly accumulated in muscles rather than in the adipose tissue. The adipose tissue from Iberian pigs had also higher amount of phenolic compounds than that from white pigs. González *et al.* (2004) have recently

reported data in relation to the amount of phenolic compounds in adipose tissue from Iberian pigs. The adipose tissue from Iberian pigs fed exclusively on natural resources (grass and acorns) had significant higher amounts of phenolic compounds than those fed with mixed diets, which is in accordance with results from the present study. Amongst phenolic compounds, some particular polyphenols derived from plants, are substances with proven antioxidant activity and the presence of such compounds in the animal tissues could protect them and the products elaborated with them from oxidative deterioration.

Colour characteristics of meat and adipose tissue

Table VII.2. shows colour characteristics of the raw material (meat and adipose tissues) from Iberian and white pigs. Compared to the meat from white pigs, the meat from Iberian pigs exhibited a redder colour (higher a* value) which was more intense (higher chroma value) and closer to the true red axis (lower hue value). Meat from white pigs had, additionally, significantly higher L* values than those from Iberian pigs. In previous studies (Serra *et al.*, 1998; Estévez *et al.*, 2003; Estévez *et al.*, 2004), similar results comparing colour characteristics between muscles from Iberian and white pigs were obtained. The red colour in muscles is caused by the presence of a certain amount of heme pigments in meat, and therefore, the CIE a* values in meat are positively correlated with heme pigments and iron contents (Warris *et al.*, 1990). The present results are consistent with those previously reported by Serra *et al.*, (1998); Lindahl *et al.* (2001) and ourselves (Estévez *et al.*, 2003) who described a higher a* value, chroma and iron content in muscles from rustic pig breeds than in those from selected ones. In addition to the breed effect, the characteristics of the Iberian pigs' livestock farming could have influenced since the pigments and iron concentrations and the red colour of the muscles increase with the animal age and the physical exercise (Lawrie, 1998).

The colour characteristics of the adipose tissues were also different between groups since those from Iberian pigs exhibited significantly higher L* values

and those from white pigs showed a more intense (higher chroma) and redder colour (higher a* values). These results agree with those reported in a previous paper in which the colour traits of adipose tissues from Iberian and white pigs were investigated (Estévez *et al.*, 2004).

Fatty acid composition of meat and adipose tissue

The tissues from Iberian and white pigs showed clear differences in their fatty acid composition (Table VII.3.). Compared to meat from white pigs, meat from Iberian pigs had a larger proportion of monounsaturated fatty acids (MUFA), mainly oleic acid (52.4% vs. 42.8%; $p<0.05$) and smaller percentages of saturated fatty acids (SFA), such as palmitic (22.5% vs. 24.8%; $p<0.05$) and stearic (10.8% vs. 15.5%; $p<0.05$) acids. Meat from white pigs had a higher proportion of polyunsaturated fatty acids (PUFA), such as linoleic (9.1% vs. 6.7%; $p<0.05$) and arachidonic (0.47% vs. 0.37%; $p<0.05$) acids than that from Iberian pigs. Meat from Iberian pigs had smaller values in particular ratios closely associated to healthy characteristics of meat (Okuyama & Ikemoto, 1999)such as the n-6/n-3 ratio and that between hyper- (lauric, myristic, palmitic) and hypocholesterolemic (oleic, linoleic) fatty acids (Table VII.3.).

The fatty acid profiles of the adipose tissues are presented in Table VII.3. Four fatty acids (palmitic, stearic, oleic and linoleic acids) comprised more than the 90% of the total fatty acids analysed. As expected, oleic acid was the most abundant (57.4%-46.8%) followed by palmitic (18.3%-22.1%), stearic (8.0%-12.8%) and linoleic acid (8.9%-10.7%). In agreement with the results aforementioned for the meat, the fatty acid compositions of the adipose tissues were largely different between Iberian pigs and white pigs (Table VII.3.). Compared to adipose tissues from Iberian pigs, those from white pigs showed significantly higher percentages of saturated fatty acids (36.6% vs. 27.8%; $p<0.05$) and polyunsaturated fatty acids (12.7% vs. 10.8%; $p<0.05$). On the contrary, Iberian pigs presented higher percentages of monounsaturated fatty acids (61.4% vs. 50.7%; $p<0.05$).

The fatty acid composition of the animal tissues can be generally attributed to the compositional characteristics of the fat from the feeds given to the animals (Miller *et al.*, 1990; Enser *et al.*, 2000). Consistently, raw material from Iberian pigs reflected the fatty acid composition of acorns (with high levels of oleic acid). Contrarily, tissues from white pigs reflected the general composition of the mixed diet, with relative high proportion of linoleic acid and PUFA. According to previous studies, these results represent the general pattern of fatty acid composition of different tissues from Iberian pigs fed extensively with natural resources (Cava *et al.*, 1997; Ruiz *et al.*, 1998; Timón *et al.*, 2001) and white pigs fattened intensively with mixed diets (Flachowsky *et al.*, 1997; Serra *et al.*, 1998).

The livestock production system, the feed given to the animals and the genetic traits could have had an influence on the different quality characteristics of the tissues from Iberian and white pigs. Furthermore, the large differences in the age and weight of the animals at slaughter might have influenced, since these factors surely affect the physiology and biochemical maturation of porcine tissues.

General composition of frankfurters

The general composition of the frankfurters is shown in Table VII.4. No differences for moisture (61.7%-63.4%, $p>0.05$), fat (18.4%-18.9%, $p>0.05$) protein (10.7%-10.9%, $p>0.05$) or ash (1.1%-1.4%) contents were detected. The values obtained for the proximate composition in this work are within the range considered as acceptable in frankfurters (Matulis *et al.*, 1995). In fact, the chemical composition of the experimental frankfurters from the present study is similar to that reported by González-Viñas *et al.* (2004) for commercial frankfurters obtained from Spanish supermarkets. Agreeing with results from meat, IF had a significantly higher amount of iron compared with that from WP (16.6 µg/g vs. 11.5 µg/g; $p<0.05$). Since the source of iron for the frankfurter is the meat, the HF had a similar iron content to WF. Red meats are an essential source of heme iron for humans and, in addition, enhance the absorption of non-heme iron from vegetables and other foods

when included at the same time in the diet (Mulvihill & Morrissey, 1997). Meat from Iberian pigs has been previously described as a good source of high available iron and, based on the present results; the frankfurters produced with such raw material keep considerably high levels of iron, with those being significantly higher than those from white pigs and other cooked meats (Miller *et al.*, 1994; Kosse *et al.*, 2001).

Colour characteristics of frankfurters

Cie L*, a*, b*, chroma and hue angle measured in frankfurters are shown in Table VII.4. IF presented different colour characteristics to WF and HF, as suggested by the parameters measured. IF exhibited a more intense, redder and darker colour compared to that from WF. The red colour in the frankfurters is caused by the presence of heme pigments supplied by the meat, which is the main ingredient. This explains the differences found between types of frankfurters since meat from Iberian pigs presented a higher a* and chroma values than those from white pigs. Though WF and HF exhibited similar colour characteristics, the latter were paler as a likely result of the colour traits of the raw material used for their manufacture. The meat from white pigs was paler than that from Iberian pigs whereas adipose tissues from Iberian pigs had higher L* values than those from white pigs. In conclusion, the colour traits of the frankfurters are influenced by the colour characteristics of the main ingredients as has been previously described in liver pâté and other liver and meat products (Estévez *et al.*, 2004; Estévez *et al.*, submitted).

Fatty acid composition of frankfurters

The fatty acid profile of frankfurters is shown in Table VII.5. Large differences among types of frankfurters were detected for most of the fatty acids analysed. IF had significantly smaller proportions of palmitic (19.8% vs. 23.4%, $p<0.05$) stearic (9.3% vs. 14.3%) and SFA (30.7% vs. 39.6%, $p<0.05$) than WF. The differences for MUFA (59.5% vs. 48.6%, $p<0.05$) are particularly remarkable since IF had 10 percent more oleic acid than WF

(55.2% vs. 44.5%, $p<0.05$). The latter had larger percentages of PUFA (9.9% vs. 11.8%, $p<0.05$) such as linoleic (7.9% vs. 9.8%, $p<0.05$) acid. As expected, fatty acid composition of frankfurters reflected the fatty acid composition of the raw material used for their production. As aforementioned for the tissues, the differences are mainly explained by the different fatty acid composition of the feeds given to the animals during the fattening period. Large proportions of oleic acid have been considered as one of the main characteristics of Iberian pig products including meat, dry-cured products and liver pâtés (Timón *et al.*, 2001; Estévez *et al.*, 2003; Estévez *et al.*, 2004). The HF had an intermediate fatty acid profile between IF and WF. Replacing 10% fat from white pigs with fat from Iberian pigs in the HF significantly influenced the fatty acid profile, significantly reducing the proportion of SFA (from 39.6% to 35.1%) and PUFA (from 11.8% to 11.0%) and increasing the percentages of oleic acid (from 44.5% to 49.6%) and MUFA (from 48.6% to 53.7%) compared to results from WF.

Focusing on nutritional and technological aspects, using raw material from extensively reared Iberian pigs improved the lipid characteristics of the frankfurters. Contrary to MUFA, PUFA are very prone to oxidation, leading to the generation of unpleasant odours and reducing nutritional value of meat and fat products (Morrissey *et al.*, 1998). Thus, compared to SFA, MUFA are hypocholesterolemic, but, unlike PUFA, they do not decrease high-density lipoproteins (HDL) cholesterol which protects against coronary heart disease (Mattson & Grundy, 1985). The nutritional ratio between SFA hypercholesterolemic fatty acids (C12, C14, C16) and the unsaturated hypocholesterolemic ones (C18:1 n-9; C18:2 n-6) was also lower in IF (0.33 vs. 0.45; $p<0.05$). Great importance has been given to long chain PUFA in meat products because of the role played by the ratio n-6/n-3 in the development of coronary heart diseases (CHD) (Okuyama & Ikemoto, 1999). The ratio n-6/n-3 was lower in IF than in WF (9.7 vs. 11.8; $p<0.05$) as a result of the higher content of C18:2 (n-6) in those from white pigs. The content of long chain PUFA in tissues of pigs reared outdoors with access to pasture are thought to increase because of the intake of grass with high

content of n-3 PUFA (Nilzén *et al*., 2001) though it was not generally detected in the present work. Using adipose tissue from Iberian pigs for the manufacture of frankfurters significantly improved the fatty acid profile of frankfurters since HF presented better nutritional and n-6/n-3 ratios than those from WF.

Texture profile of frankfurters

The values of the texture parameters obtained from the TPA analysis of frankfurters are shown in Table VII.6. The range of values obtained for the texture profile of frankfurters in the present study are in agreement with those previously reported in similar cooked products (Fernández-López *et al*., 2003; González-Viñas *et al*., 2004). The results suggest that the three types of frankfurters had similar texture characteristics since similar values of hardness (15.1-16.0 N/cm^2), fracturability (0.08-0.09 N/cm^2), adhesiveness (-0.13- 0.17 N s), springiness (0.87-0.92), gumminess (8.46-10.0 N/cm^2), chewiness (7.8-9.4 N s) and resilience (0.33-0.34) were detected. Cavestany *et al*. (1994) reported that differences in texture properties among meat products are influenced by a variety of factors such as differences in formulations, functionality of proteins and amount and characteristics of fat. There have been many studies of the effect of the reduction of fat and the addition of some particular fat replacers and additives on the texture characteristics of frankfurters-type sausages (Grigelmo-Miguel *et al*., 1999; Crehan *et al*., 2000; Pappa *et al*., 2000). The fact that the experimental frankfurters in the present study were produced following the same recipe and had similar proximate composition could explain the lack of significant differences among them for the texture characteristics. Nevertheless, a clear trend was observed since IF tended to be harder and showed higher values of adhesiveness ($p>0.05$), springiness ($p>0.05$), cohesiveness ($p<0.05$), gumminess ($p>0.05$) and chewiness ($p>0.05$) than WF. HF tended to present intermediate texture characteristics between IF and WF. These trends could be attributed to the different characteristics of the meat and particularly those related to the fatty acid composition of their lipids. The modification of the

fatty acid composition of frankfurters, increasing monounsaturated fatty acids (MUFA) and reducing saturated (SFA) and polyunsaturated fatty acids (PUFA) levels through replacing pork back-fat with olive oil led to sausages with higher values of hardness, springiness, cohesiveness, gumminess and chewiness (Bloukas & Paneras, 1993). In addition, liver pâtés produced with tissues from Iberian pigs presented higher values of instrumental hardness than those produced with tissues from white pigs (Unpublished data). In the aforementioned work, pâtés from Iberian and white pigs had no different proximate composition but showed, however, significant different fatty acid composition (Estévez *et al.*, 2004) which agrees with results obtained in the present work.

VII.6. Conclusions

The large differences detected among tissues from Iberian and white pigs explain the differences found between types of frankfurters. Using meat and adipose tissue from Iberian pigs for the manufacture of frankfurters results in a high quality product, with higher iron content and different colour characteristics compared to frankfurters from white pigs. IF are characterised by large percentages of MUFA, a small proportion of hypercholesterolemic fatty acids and presented lower values of the ratio n-6/n-3 than WF. The quality characteristics and fatty acid composition of WF can be improved by replacing 10% fat with adipose tissue from Iberian pigs.

VII.7. Acknowledgements

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Table VII.1. Compositional analysis of the finishing diets of Iberian pigs (acorns and grass) and white pigs (mixed diet).

	Grass[2]	Acorn[2]	Mixed diet
% Moisture	89.24	46.10	10.56
% Fat	6.26	5.05	2.32
% Protein	4.34	4.31	15.19
% Ash	0.91	1.17	9.09
% FAMEs[1]			
c14	3.64	0.18	0.36
c16	13.95	11.82	16.72
c16:1 (n-7)	2.40	0.10	0.15
c18	1.99	0.56	7.57
c18:1 (n-9)	5.24	67.28	31.30
c18:2 (n-6)	11.42	18.70	39.70
c18:3 (n-3)	57.80	0.25	2.84
c20	2.40	0.25	0.35
c20:1 (n-9)	0.17	0.51	0.70
c20:2 (n-6)	0.03	0.09	0.18
c20:4 (n-6)	0.94	0.26	0.12

Fat, protein and ash expressed as percentage on fresh matter.
[1] FAMEs expressed as % of total fatty acids analysed.
[2] Previously published in Estévez *et al*. (2004).

Table VII.2. Chemical composition and instrumental colour of meat and adipose tissue from Iberian and white pigs.

	Muscle			Adipose tissue		
	Iberian	White	p[1]	Iberian	White	p
Moisture[2]	60.12±1.65	58.92±1.13	0.216	9.74±0.39	12.94±0.54	<0.001
Fat[2]	18.33±1.93	19.60±1.17	0.243	83.19±3.72	74.87±2.02	0.002
Protein[2]	17.02±0.37	16.89±0.74	0.745	2.56±0.49	4.06±0.52	0.002
Ash[2]	0.81±0.10	0.74±0.17	0.457	0.11±0.02	0.14±0.04	0.203
Iron[3]	29.79±1.09	22.99±1.52	<0.001	traces5	traces	-
α-tocopherol[3]	3.75±0.13	1.80±0.83	0.001	17.17±2.48	3.87±1.53	<0.001
γ-tocopherol[3]	0.22±0.02	0.08±0.04	0.024	1.07±0.35	0.00±0.00	<0.001
Total phenolics[4]	1.53±0.30	0.99±0.06	0.004	0.46±0.03	0.37±0.02	<0.001
L*	50.15±1.42	58.32±1.69	<0.001	82.08±1.29	79.14±1.71	0.015
a*	24.19±1.70	20.72±0.47	0.002	3.31±0.43	4.86±0.59	0.001
b*	13.16±1.17	13.66±0.64	0.422	5.93±0.46	5.96±0.37	0.930
Chroma	27.55±1.80	24.82±0.40	0.011	6.79±0.57	7.69±0.65	0.049
Hue	28.57±2.12	33.41±1.59	0.004	60.97±2.32	50.90±1.95	<0.001

[1] Statistical significance in a student's t-test for independent variables.
[2] g/100g of raw material.
[3] μg/g of raw material.
[4] mg GAE/g of raw material.
[5] traces.

Table VII.3. Fatty acid composition (means ± standard deviation) of muscle, liver and adipose tissue from extensively reared Iberian pigs and intensively reared white pigs[1].

	Muscle			Adipose tissue		
	Iberian	White	p[2]	Iberian	White	p
C12	0.01±0.00	0.02±0.02	0.278	0.01±0.00	0.01±0.00	0.005
C14	1.11±0.01	1.22±0.02	<0.001	1.04±0.03	1.03±0.03	0.851
C16	22.54±0.03	24.82±0.20	<0.001	18.32±0.25	22.05±0.18	<0.001
C17	0.21±0.01	0.42±0.01	<0.001	0.29±0.00	0.48±0.01	<0.001
C18	10.75±0.07	15.54±0.23	<0.001	8.01±0.31	12.83±0.47	<0.001
C20	0.12±0.06	0.25±0.06	0.009	0.16±0.02	0.21±0.02	0.006
Σ SFA	34.75±0.13	42.27±0.46	<0.001	27.82±0.58	36.61±0.53	<0.001
C16:1 (n-7)	3.02±0.03	2.48±0.01	<0.001	2.01±0.05	2.36±0.08	<0.001
C17:1 (n-7)	0.21±0.00	0.38±0.00	<0.001	0.29±0.00	0.47±0.02	<0.001
C18:1 (n-9)	52.38±0.20	42.78±0.49	<0.001	57.36±0.43	46.79±0.28	<0.001
C20:1 (n-9)	1.08±0.01	1.03±0.03	0.007	1.69±0.04	1.08±0.02	<0.001
C22:1 (n-9)	0.02±0.01	0.02±0.00	0.548	0.03±0.00	0.02±0.00	<0.001
Σ MUFA	56.71±0.18	46.69±0.52	<0.001	61.38±0.47	50.73±0.33	<0.001
C18:2 (n-6)	6.74±0.05	9.05±0.07	<0.001	8.85±0.11	10.66±0.13	<0.001
C18:3 (n-6)	0.10±0.00	0.16±0.01	<0.001	0.14±0.00	0.18±0.01	<0.001
C18:3 (n-3)	0.46±0.01	0.51±0.02	0.002	0.65±0.02	0.60±0.02	0.003
C20:2 (n-6)	0.20±0.06	0.44±0.02	0.010	0.58±0.01	0.48±0.05	0.003
C20:3 (n-3)	0.15±0.01	0.05±0.01	<0.001	0.06±0.00	0.15±0.00	<0.001
C20:3 (n-6)	0.04±0.01	0.04±0.01	0.350	0.02±0.00	0.03±0.00	<0.001
C20:4 (n-6)	0.47±0.03	0.37±0.01	<0.001	0.12±0.00	0.19±0.01	<0.001
C20:5 (n-3)	0.10±0.00	0.10±0.01	0.713	0.18±0.01	0.12±0.00	<0.001
C22:2 (n-6)	0.04±0.00	0.07±0.01	0.001	0.04±0.01	0.05±0.01	0.019
C22:4 (n-6)	0.02±0.01	0.03±0.01	0.739	0.03±0.00	0.04±0.00	0.003
C22:5 (n-3)	0.09±0.00	0.11±0.00	<0.001	0.05±0.02	0.08±0.00	0.036
C22:6 (n-3)	0.12±0.01	0.12±0.01	0.327	0.08±0.00	0.09±0.01	0.030
Σ PUFA	8.54±0.09	11.04±0.13	<0.001	10.80±0.15	12.69±0.17	<0.001
n-6/n-3	8.38±1.36	11.46±0.37	0.001	9.56±0.38	11.45±2.20	0.096
Nutritional ratio[3]	0.40±0.00	0.50±0.01	<0.001	0.29±0.01	0.40±0.01	<0.001

[1] Fatty acids expressed as percentages of total fatty acids analysed.
[2] Statistical significance in student t-test for independent variables.
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids;[3] Nutritional ratio: (C12:0 + C14:0 + C16:0) / (C18:1 + C18:2).

Table VII.4. Chemical composition and instrumental colour from experimental frankfurters.

	IF	WF	HF	p
Moisture	63.44±1.57	62.33±1.51	61.68±0.43	0.068
Fat	18.38±0.50	18.69±0.71	18.86±1.02	0.611
Protein	11.43±0.62	10.88±0.58	10.70±0.22	0.054
Ash	1.28±0.11	1.36±0.21	1.09±0.42	0.302
Iron	16.30^{a}±1.11	11.70^{b}±2.63	11.22^{b}±1.07	0.001
pH	8.08^{a}±0.02	7.91^{c}±0.03	7.98^{b}±0.01	<0.001
L*	71.59^{c}±0.63	73.05^{b}±0.42	77.09^{a}±0.62	<0.001
a*	13.57^{a}±0.17	11.66^{b}±0.29	11.64^{b}±0.97	<0.001
b*	9.21±0.03	9.02±0.15	9.21±0.23	0.131
Chroma	16.40^{a}±0.15	14.74^{b}±0.24	14.84^{b}±0.35	<0.001
Hue	34.18^{b}±0.30	37.75^{a}±0.88	38.38^{a}±0.29	<0.001

See footnotes of Table 2.

Table VII.5. Fatty acid composition (means ± standard deviation) from experimental frankfurters[1].

	IF	WF	HF	p[2]
C12	0.04±0.02	0.06±0.00	0.06±0.00	0.058
C14	1.04[b]±0.02	1.13[a]±0.01	1.12a±0.01	<0.001
C16	19.81[c]±0.09	23.35a±0.08	21.50[b]±0.17	<0.001
C17	0.26[c]±0.01	0.46[a]±0.00	0.38[b]±0.01	<0.001
C18	9.28[c]±0.02	14.33[a]±0.08	12.00[b]±0.38	<0.001
C20	0.23±0.09	0.29±0.02	0.26±0.02	0.365
Σ SFA	30.68[c]±0.10	39.62[a]±0.13	35.31[b]±0.56	<0.001
C16:1 (n-7)	2.39[b]±0.01	2.44[a]±0.01	2.27[c]±0.03	<0.001
C17:1 (n-7)	0.26[c]±0.00	0.43[a]±0.00	0.35[b]±0.00	<0.001
C18:1 (n-9)	55.23[a]±0.14	44.50[c]±0.06	49.57[b]±0.57	<0.001
C20:1 (n-9)	1.53[a]±0.09	1.16[c]±0.05	1.42[b]±0.02	<0.001
C22:1 (n-9)	0.05[a]±0.00	0.04[b]±0.00	0.04[a]±0.00	<0.001
Σ MUFA	59.46[a]±0.09	48.56[c]±0.07	53.65[b]±0.59	<0.001
C18:2 (n-6)	7.97[c]±0.02	9.79[a]±0.10	9.05[b]±0.04	<0.001
C18:3 (n-6)	0.11[c]±0.01	0.16[a]±0.01	0.14[b]±0.01	<0.001
C18:3 (n-3)	0.56±0.01	0.57±0.02	0.57±0.01	0.795
C20:2 (n-6)	0.47[b]±0.01	0.50[a]±0.01	0.51[a]±0.01	<0.001
C20:3 (n-3)	0.06[a]±0.00	0.05[b]±0.00	0.05[ab]±0.01	0.028
C20:3 (n-6)	0.08[b]±0.00	0.09[a]±0.00	0.08[ab]±0.01	0.007
C20:4 (n-6)	0.28[b]±0.01	0.30[a]±0.01	0.26[c]±0.01	<0.001
C20:5 (n-3)	0.14[a]±0.00	0.10[c]±0.00	0.13[b]±0.00	<0.001
C22:2 (n-6)	0.03±0.01	0.05±0.02	0.04±0.02	0.172
C22:4 (n-6)	0.02±0.01	0.02±0.01	0.01±0.01	0.398
C22:5 (n-3)	0.08[b]±0.00	0.10[a]±0.00	0.09[a]±0.00	<0.001
C22:6 (n-3)	0.09[b]±0.01	0.10[a]±0.01	0.10[a]±0.01	0.013
Σ PUFA	9.88[c]±0.04	11.83[a]±0.11	11.04[b]±0.08	<0.001
n-6/n-3	9.66[c]±0.12	11.83[a]±0.27	10.67[b]±0.12	<0.001
Nutritional ratio[3]	0.33[c]±0.00	0.45[a]±0.00	0.39[b]±0.01	<0.001

See footnotes of Table 3.

Table VII.6. Texture profile from experimental frankfurters.

	IF	WF	HF	p[1]
Hardness[1]	16.04±0.97	15.10±1.96	15.71±2.39	0.732
Fracturability[1]	0.08±0.01	0.09±0.01	0.09±0.01	0.435
Adhesiveness[2]	-0.17±0.03	-0.13±0.05	-0.14±0.04	0.352
Springiness[3]	0.92±0.02	0.87±0.04	0.88±0.02	0.105
Cohesiveness[4]	0.59[a]±0.00	0.59[ab]±0.01	0.58[b]±0.01	0.042
Gumminess[1]	10.04±0.56	8.46±1.44	9.09±1.31	0.139
Chewiness[2]	9.44±0.57	7.78±1.94	8.03±1.12	0.149
Resilience[4]	0.34±0.01	0.33±0.01	0.34±0.01	0.051

[1] Newtons/cm^2.
[2] Newtons x s.
[3] cm.
[4] Dimensionless.

CHAPTER VIII

Oxidation of lipids and proteins in frankfurters with different fatty acid composition and tocopherols and phenolics contents[♣]

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VIII.1. Abstract

In the present work, lipid and protein oxidation and the stability of heme iron (HI) in refrigerated frankfurters (60 days/+4ºC) were studied. Lipid oxidation was evaluated as TBA-RS and hexanal counts isolated with solid-phase microextraction (SPME) whereas protein carbonyls groups were quantified following the DNPH coupling method. Frankfurters were produced using meat and fat from either extensively reared Iberian pigs (IF) or intensively reared white pigs (WF). Hybrid frankfurters (HF) manufactured with meat from white pigs and fat from Iberian pigs were also considered. Frankfurters had significantly different tocopherol and total phenolics contents with the IF having the highest antioxidants content followed by HF and WF. Compared to WF, IF had significantly higher amounts of monounsaturated fatty acids (MUFA) and smaller of saturated (SFA) and PUFA. HF presented an intermediate fatty acid composition between IF and WF. Results suggest an intense oxidative deterioration of frankfurters during refrigeration since lipid oxidation products such as TBA-RS and hexanal were generated. Proteins were also damaged since carbonyls derived from their oxidation and non-heme iron content increased during refrigeration suggesting also oxidative instability of the heme molecule. Mainly explained by the different fatty acid composition and antioxidant status, IF showed a higher oxidative stability compared to WF. Using fat from Iberian pigs for the production of HF improved the oxidative stability of frankfurters, with that being significantly higher than those from WF and IF. IF had a significantly higher amount of iron compared to WF and HF which could have played an important role in the development of the oxidative reactions. Significant correlations were established between protein and lipid oxidation and protein oxidation and heme degradation, suggesting likely interactions between such processes.

VIII.2. Keywords: Lipid oxidation, protein oxidation, non-heme iron, fatty acid composition, tocopherols, phenolics.

VIII.3. Introduction

Lipid oxidation is the major factor reducing quality and acceptability of meat and fat products (Morrissey *et al.*, 1998). The oxidation process involves the degradation of polyunsaturated fatty acids (PUFA), vitamins and other tissue components and the generation of free radicals, which lead to the development of rancid odours and changes in colour and texture in foodstuffs (Kanner, 1994). Lipid oxidation is thought to promote the oxidative damage of proteins through the prooxidant activity of primary (hydroperoxides) and secondary (aldehydes, ketones) lipid oxidation products (Gardner, 1979, Kikugawa *et al.*, 1999). Protein oxidation mainly occurs via free radical reactions in which peroxyl radicals generated in the first stages of PUFA oxidation can abstract hydrogen atoms from protein molecules leading to the formation of protein radicals. The formation of non-covalent complexes between lipid oxidation products and reactive amino acids residues as well as the presence of some particular metal such as copper and iron can also lead to protein radicals' generation (revised by Viljanen *et al.*, 2004). Though relationships between lipid and protein oxidation have been established in microsomal, emulsion or liposome models (Howell *et al.*, 2001; Batifoulier *et al.*, 2002; Viljanen *et al.*, 2004a; Viljanen *et al.*, 2004b), the development of protein oxidative reactions in food systems is still largely unknown. In addition, the effect of the oxidation of proteins on meat quality needs to be elucidated. Protein oxidation has been linked to a decrease of protein solubility and functionality and colour and texture changes in model systems (Howell *et al.*, 2001; Karel *et al.*, 1975). More recently, Mercier *et al.* (2004) and ourselves (Estévez & Cava, 2004) have reported data on the oxidative stability of proteins in meat and liver products, establishing close relationships between lipid and protein oxidation phenomena. Nevertheless, the effect of antioxidant strategies (e.g. supplementation of antioxidants in animal diets, addition of antioxidants in muscle foods) on the quality and functionality of muscle proteins has been poorly studied.

Concerning oxidation promoters in animal foods, iron is thought to have a high catalytic activity (Kanner, 1994). However, knowledge of the

approximate proportion between the chemical forms of iron is of great importance because of the strong differences displayed by heme iron (HI) and non heme iron (NHI) in terms of promotion of lipid oxidation and bioavailability. NHI is considered one of the most important oxidation promoters in meat systems (Kanner, 1994) whereas HI is thought to be more bioavailable (Carpenter & Mahoney, 1992). An increase in the amount of NHI in meat systems has been considered to be a reflection of the decrease of heme iron (HI) as a consequence of the breakdown of heme molecule during cooking or storage (Miller *et al*., 1994; Lombardi-Boccia *et al*., 2002; Estévez & Cava, 2004) and has been eventually linked to a disruption of the porphyrin ring of myoglobin (Schricker *et al*., 1982; Estévez & Cava, 2004). However, the particular causes of the heme molecule disruption and the effects of the increase of non-heme iron in cooked products have not been considered.

Frankfurters are widely marketed and consumed meat products. The physico-chemical characteristics of frankfurters are influenced by the quality of the ingredients, particularly, the meat and adipose tissue. The tissues from extensively reared Iberian pigs are different to those from intensively reared white pigs as far as the proximate and fatty acid composition is concerned (Estévez *et al*., 2004). The large proportion of oleic acid (50- 60%) in tissues from Iberian pigs is considered as one of the most peculiar quality traits and is caused by the consumption of acorns by animals while traditional extensive rearing (Ruiz *et al*., 1998; Estévez *et al*., 2004). The consumption of fresh natural feeds by pigs is also associated to increasing levels in their tissues of minority substances with proven antioxidant activity such as tocopherols (Cava *et al*., 2000; Estévez *et al*., 2004; Daza *et al*., 2005). In addition, the intake of grass and acorns by free-range reared pigs has been recently associated to the incorporation of plant phenolics to the animal tissues which could contribute to enhance their oxidative stability (González *et al*., 2004). The differences between frankfurters from Iberian and white pigs in terms of their fatty acid composition and antioxidative status are expected to influence on their oxidative deterioration during refrigerated storage. The amount of protein in some commercial frankfurters is even higher than the fat content

(González-Viñas *et al.*, 2004) suggesting that the development of oxidative reactions will affect to proteins as well as to lipids, leading to a loss of quality. The occurrence of protein oxidation and the relationships between oxidative processes and the release of iron from myoglobin in frankfurters has not been previously described. The aim of the present work was to study the oxidative reactions in frankfurters elaborated with tissues from extensively reared Iberian and intensively reared white pigs which presented different fatty acid composition and antioxidant status. Relationships between the different measured parameters were also established.

VIII.4. Material and Methods

Animals and sampling

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered at ~150 Kg and an age of 12 months. Seven white pigs (Large-white x Landrace) were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed feed and slaughtered at ~85 kg live weight and at the age of 7 months. Iberian pigs and white pigs were slaughtered at the same slaughterhouse one week apart. After slaughter, meat and adipose tissues were removed from carcasses, vacuum packaged and stored at -80ºC until the manufacture of the frankfurters.

Frankfurter manufacture

The experimental frankfurters were manufactured in a pilot plant. The same formulation was used for all frankfurters. Three different types of frankfurters were considered depending on the source of the raw material: frankfurters from Iberian pigs (IF), frankfurters from white pigs (WF) and hybrid frankfurters (HF) elaborated with meat from white pigs and adipose tissue from Iberian pigs. For the manufacture of the sausages, meat and adipose

tissues from seven different animals were used for each of the groups (Iberian and white pigs). The ingredients were as follows per 100g of elaborated product: 50 g meat, 10 g adipose tissue, 37 g distilled water, 2 g sodium caseinate, 1 g potato starch. Sodium chloride (2%), Sodium di- and tri-phosphates (0.5%) sodium ascorbate (0.05%) and sodium nitrite (0.03%) (all from ANVISA, Madrid, Spain) were also added. Following the aforementioned recipe, 1.3 kg of raw material was used for each group, to produce the experimental frankfurters. Firstly, the meat was chopped into small cubes (1 cm^3) and mixed with the sodium chloride, sodium nitrite and the sodium ascorbate in order to allow the nitrification of the samples 2 hours before the manufacture. Then, the meat was minced in a cutter (Foss Tecator Homogeniser, mod. 2094) for 2 minutes together with the starch and the 50% of the sodium caseinate which was previously dissolved in water (+75ºC). After that, the adipose tissue was added together with the remaining dissolved sodium caseinate and minced for 4 more minutes until a homogenous raw batter was obtained. Finally, the mixture was stuffed into 18 mm diameter cellulose casings, handlinked at 10 cm intervals and given the thermal treatment in a hot water bath (+80ºC/30').

Refrigeration

In order to allow the development of oxidative reactions, the frankfurters were refrigerated stored (+4ºC/60 days) in the darkness. Sampling was carried out at days 0, 20, 40 and 60 for analytical experiments, with the day 0 being the day of the manufacture. After each refrigeration stage, frankfurters were frozen (-80ºC) until experiments were carried out.

Analytical methods

Compositional analysis of frankfurters

Moisture, total protein and ash were determined using AOAC methods (AOAC, 2000a, b, c). The method of Bligh and Dyer (1959) was used for isolating fat from frankfurters.

Iron analysis

Total iron was determined following the procedure described by Miller *et al*. (1994). Nonheme iron (NHI) content was determined following the method described by Rhee *et al*. (1987). The amount of heme iron (HI) was calculated by difference between total and NHI. The amounts of iron were expressed as µg iron/g frankfurter.

Tocopherols content

α- and γ-tocopherols were extracted from frankfurters according to the method described by Rey *et al*. (1997). The analysis was carried by reverse phase HPLC (HP 1050, with a UV detector, HPIB 10) (Hewlett-Packard, Waldbronn, Germany).

Phenolics compounds content

The Folin Ciocalteau reagent was used for the quantification of total phenolics as described by Turkmen *et al*. (*in press*) with minor modifications as follows: 0.5 g of frankfurter was homogenised with 10 mL of water, methanol or 80% methanolic water and centrifuged for 5 minutes at 3000 rpm and +4ºC. Phenolics were extracted from the pellets following the same procedure. The supernatants were combined and 1 mL aliquot was mixed with 5 mL of Folin Ciocalteau reagent (10% in distilled water) in test tubes. After 5 minutes, 4 mL of sodium carbonate (7.5% in distilled water) was added, the test tubes were screw-capped and the samples allowed to stand for 2 hours at room temperature in the darkness. A standard curve with ethanolic gallic acid (ranged from 0.625 x 10-3 mg/mL to 0.02 mg/mL) was used for quantification. Results were expressed as mg of gallic acid equivalents (GAE) per gram of sample.

Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas

chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a FFAP-TPA fused-silica column (Hewlett Packard 30m long, 0.53 mm internal diameter and 1.0 µm film thickness). The injector and the detector temperature were held at +230ºC. Oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma). The quantification of fatty acids was carried out by using C13 as an internal standard. Results are expressed as g fatty acid $100g^{-1}$ total fatty acid analysed.

Protein oxidation measurement

Protein oxidation as measured by the total carbonyl content was assessed following the method described by Oliver *et al*. (1987). Protein concentration was determined by spectrophotometry using bovine serum albumin (BSA) as standard. The amount of carbonyls was expressed as nM carbonyls/ mg protein.

TBA-RS numbers

Thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al*. (1996). Results are expressed as mg malondialdehyde (MDA)/ kg frankfurter.

Hexanal analysis

The SPME fibre, coated with a divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30µm, was preconditioned prior analysis at +220ºC during 45 min. The HS sampling was performed following a method previously described (Estévez *et al*., 2004). 1 g of frankfurter was placed in 2.5 mL vials and the SPME fibre was exposed to the headspace of the frankfurters while the sample equilibrated during 30 minutes immersed in water at +50ºC. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95%

dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm id., 1.0mm film thickness). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40 ^{o}C. The SPME fibre was desorbed and maintained in the injection port at 220 ^{o}C during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at 40 ºC and then raised at the rate of 7 ºC min^{-1} to 250 ^{o}C, and held for 5 min. The GC/MS transfer line temperature was 270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Hexanal was identified by comparing its retention time with that from the standard compound. Results from the volatiles analysis are provided in area units (AU).

Data analysis

The results of the experiments were used as variables and analysed by using the Analysis of Variance (ANOVA) (SPSS, 1997) in order to compare amongst types of frankfurters. The same statistical analysis was used to evaluate the effect of refrigerated storage on frankfurters. Statistical significance was set at 0.05.

VIII.5. Results and Discussion

Proximate and Fatty Acid Composition of Frankfurters

The analysis of the proximate composition revealed no statistically significant differences among types of frankfurters since they had similar moisture (61.7%-63.4%, $p>0.05$), fat (18.4%-18.9%, $p>0.05$) protein (10.7%-10.9%, $p>0.05$) and ash (1.1%-1.4%) contents. IF had, however, a significantly higher amount of iron (16.3 µg/g frankfurter) than WF (11.7 µg/g frankfurter) and WF (11.2 µg/g frankfurter). This result was expected since the meat from Iberian pigs contained a higher amount of iron than that from white pigs. Large differences among types of frankfurters were detected for most of the fatty acids analysed (Table VIII.1.). IF had significantly smaller amounts of

palmitic (20.1 vs. 23.5 g/100g) stearic (9.0 vs. 13.9 g/100g) and total SFA (31.0 vs. 39.8 g/100g) than WF. Compared to WF, IF contained significantly higher amounts of oleic (53.6 vs. 42.9 g/100g) and total MUFA (57.9 vs. 47.1 g/100g). WF had, on the contrary, larger amounts of PUFA (11.10 vs. 13.21 g/100g) such as linoleic (8.80 vs. 10.75 g/100g) acid. In addition, WF contained significantly higher amounts of minority n-3 and n-6 long chain PUFA than IF. As expected, fatty acid composition of frankfurters reflected the fatty acid composition of the tissues used for their elaboration. As described in a previous paper (Estévez *et al*., 2004), the differences are mainly explained by the different fatty acid composition of the feeds given to the animals during the fattening period, and therefore, meat and adipose tissues from Iberian pigs reflected the fatty acid composition of the acorns which had high levels of oleic acid. The HF had an intermediate fatty acid composition between IF and WF. Replacing 10% fat from white pigs with fat from Iberian pigs in the HF significantly influenced on their fatty acid composition, significantly reducing the proportion of SFA (from 39.8 to 35.6 g/100g) and PUFA (from 13.2 to 12.4 g/100g) and increasing the percentages of oleic acid (from 42.9 to 47.7 g/100g) and MUFA (from 47.1 to 51.8 g/100g) compared to results from WF.

Antioxidant Content of Frankfurters

Results from the quantification of tocopherols and total phenolics in frankfurters are shown in Table VIII.2. IF presented higher levels of α- and γ-tocopherol compared to those from WF (3.7 vs. 1.3 µg/g and 0.23 vs. 0.05 µg/g, respectively), which is consistent with previously reported data regarding tocopherol contents in the tissues (meat and adipose tissue) from outdoor reared Iberian pigs and white pigs reared indoors (Cava *et al*., 2000; Estévez *et al*., 2004a; Daza *et al*., 2005). The HF had an intermediate amount of tocopherols suggesting that the addition of 10% fat from Iberian pigs in those frankfurters increased α- (from 1.31 to 2.74 µg/g) and γ-tocopherol (from 0.05 to 0.15 µg/g) levels in comparison with the WF. The high content of tocopherols in tissues and meat products from outdoor reared Iberian pigs

has been profusely described in previous works, and considered as one of the most appreciated quality traits (Cava *et al*., 2000; Estévez *et al*., 2004; Daza *et al*., 2005) as long as tocopherols enhance the oxidative stability of meats and meat products, improving their nutritional and technological properties (Morrissey *et al*., 1998; Rey *et al*., 1998). The α- and γ-tocopherol contents in animal tissues reflect the tocopherol concentration of the diets (Daza *et al*., 2005), and therefore, the high levels of tocopherols in the grass and acorns with which Iberian pigs were fed explain the high levels of such substances in their tissues. According to Cava *et al*., (2000) and Daza *et al*., (2005) meat from Iberian pigs fed on natural resources (grass and acorns) contained similar or even higher tocopherol levels than those fed with diets supplemented with α-tocopherol up to 200 mg/Kg. Furthermore, acorns have been shown to be important sources of γ-tocopherol for extensively reared pigs and the presence of such tocopherol isomer in pig muscles is almost restricted in tissues from pigs fed with that fruit (Rey *et al*., 1998; Daza *et al*., 2005) which is in agreement with results obtained in the present work. The relatively small amounts of tocopherols in WF were expected since white pigs were fed on a tocopherol non-supplemented mixed diet.

The amount of total phenolics varied considerably depending on the solvents used for their extraction ranging from 0.52 to 1.09 g/100g in IF, from 0.46 to 0.85 g/100g in WF and from 0.46 to 0.89 g/100g in HF. Regardless of the type of extraction, the amount of total phenolics in IF was significantly higher than in either WF or HF. The information concerning the occurrence of phenolic compounds in animal tissues is extremely scarce since such compounds are widespread in plant kingdom and therefore, their presence in animal tissues is principally relegated to the intake of natural resources and the subsequent accumulation in animal tissues. The intake of grass and acorns by Iberian pigs could explain the higher amount of phenolic compounds in their tissues and elaborated frankfurters than in those from white pigs. In fact, Cantos *et al*. (2003) have recently reported elevated polyphenol levels in acorns. In agreement with the present results, González *et al*. (2004) have recently reported data significantly higher amounts of

phenolic compounds in adipose tissue from Iberian pigs fed exclusively on natural resources (grass and acorns) than those fed with mixed diets. Amongst phenolic compounds, some particular polyphenols derived from plants, are substances with proven antioxidant activity and the presence of such compounds in the animal tissues could protect them and their transformed products from oxidative deterioration.

Lipid oxidation during refrigerated storage of frankfurters

TBA-RS numbers gradually increased in experimental frankfurters during 60 days of refrigerated storage at 4ºC (Figure VIII.1.). Significant changes ($p<0.05$) were detected for MDA content between day 0 and day 60 for IF (from 0.37 to 0.94 mg MDA/Kg frankfurter), WF (from 0.49 to 1.12 mg MDA/Kg frankfurter) and HF (from 0.25 to 0.78 mg MDA/Kg frankfurter). The hexanal counts also increased after 90 days of refrigerated storage (Figure VIII.2.). As expected, a statistically significant ($p<0.01$) correlation ($R^2=0.55$) was detected between TBA-RS and hexanal contents. These data revealed that, regardless of the addition of substances with proven antioxidant activity such as nitrite and phosphates, considerably intense lipid oxidation processes affected frankfurters during refrigeration. Based in findings by Gray & Pearson (1987), rancid flavour is initially detected in meat products with TBA-RS values between 0.5 and 2.0. Furthermore, Boles & Parrish (1990) reported that a warmed-over flavour (WOF) could be perceived in meat products at TBA-RS values above 1.0. On the other hand, the hexanal is mainly generated as a consequence of the oxidative decomposition of PUFA and has been related to rancid odours and used as an indicator of lipid oxidation (Shahidi & Pegg, 1993). Throughout the entire storage period, TBA-RS numbers in WF were significantly higher than in the IF. The chromatographic areas for hexanal were also significantly higher in WF than in the IF at days 0 and 60 of storage. These results agree with those obtained in previous works devoted to the comparison of the lipid oxidative stability between cooked meats and liver products from Iberian and white pigs (Estévez & Cava, 2004; Estévez *et al.*, 2004). The different fatty acid composition, with higher amount of PUFA in

WF, could explain these findings. High levels PUFA in frankfurters have been previously associated with high oxidative instability during storage (Jeun-Horng *et al.*, 2002). On the other hand, Bloukas & Paneras (1993) improved oxidative stability of frankfurters by increasing the amount of MUFA by replacing pork back fat with olive oil, which is in agreement with results from the present work. In addition, the higher amounts of antioxidants such as tocopherols and phenolic compounds in IF could also have influenced. The relationship between the nutritional background (pasture- and mixed diet finishing) and the fatty acid profile and oxidation stability of liver, pork and their based products is profusely documented (Rey *et al.*, 1998; Ruiz *et al.*, 1998; Cava *et al.*, 2000; Daza *et al.*, 2005). Including 10% adipose tissue from Iberian pigs in HF led to a remarkable improvement of their oxidative stability since HF had significantly smaller TBA-RS values and hexanal counts than WF. This improvement was achieved by the modification of the fatty acid composition of the frankfurters, significantly increasing MUFA contents and reducing PUFA content. In addition, the adipose tissue from Iberian pigs was a source of tocopherols for HF, which likely enhanced their oxidative stability. Surprisingly, HF had even smaller TBA-RS values than IF. This could be due to the different iron content between types of frankfurters. The meat and meat products from Iberian pigs contain higher amounts of iron than those from white pigs (Estévez *et al.*, 2004) which imply enhanced nutritional properties. On the other hand, iron is considered a potent oxidation promoter and, from that point of view, high levels of that metal in meat from Iberian pigs increase its oxidative instability. The manufacture of frankfurters with meat from white pigs and adipose tissue from Iberian pigs lead to a product with improved fatty acid composition and high levels of antioxidants without increasing the iron levels which explains its high lipid oxidative stability.

Protein oxidation during refrigerated storage of frankfurters

The accumulation of protein oxidation products was detected in refrigerated stored frankfurters (Figure VIII.3.). The amount of carbonyls significantly increased ($p<0.05$) from 3.7 to 5.4 nM carbonyls/mg protein, from 5.5 to 6.5

nM carbonyls/mg protein and from 3.2 to 4.8 nM carbonyls/mg protein in IF, WF and HF, respectively. Compared to WF, IF and HF had smaller amount of carbonyls at all days of analysis. These results agree with those obtained for lipid oxidation suggesting the possible relationship between lipid and protein oxidation. In fact, a statistically significant correlations were found between protein oxidation and TBA-RS (R^2= 0.75; $p<0.01$) and between protein oxidation and hexanal (R^2= 0.68; $p<0.01$) with those correlation coefficients being higher that that between TBA-RS and hexanal contents (Table VIII.3.). Mercier *et al*. (1995) originally reported the relationship between lipid and protein oxidation in beef muscles. Accordingly, Viljanen *et al*. (2004a, b) discussed in detail the likely relationships between the oxidation of some particular proteins (BSA, lactalbumin and casein) and the development of lipid oxidation in liposomes. We have recently reported statistically significant correlations between TBA-RS values and carbonyls contents in refrigerated stored liver pâtés (Estévez & Cava, 2004). The casualty relationship between lipid and protein oxidation is probable since primary and secondary oxidation products can interact with proteins leading to protein radicals (Gardner, 1979). The differences between frankfurters could be also explained by the likely protective effect of tocopherols and phenolics from IF and HF and the large differences in the fatty acid composition between types of frankfurters. In agreement with the present results, Mercier *et al*. (2004) reported smaller amounts of carbonyls in beef from cows finished with pasture than in those finished with mixed diets, suggesting the protective role of vitamin E from the grass against the oxidation of proteins. The antioxidant activity of plant phenolics against protein oxidation has been also described in model systems. Viljanen *et al*. (2004b) described the antioxidant activity of berry phenolics in proteins from liposomes whereas ourselves (Estévez *et al*., 2004) described the protective effect of added sage and rosemary essential oils against protein oxidation in liver pâtés. Phenolic compounds can inhibit the oxidation of proteins by retarding the lipid oxidative reactions and by binding to the proteins and by forming complexes with them (Siebert *et al*., 1996). Though a loss of protein functionality associated to protein oxidation has been described

(Karel *et al*., 1975; Howell *et al*., 2001), scarce information is available on the impact of protein oxidation on meat products quality.

Release of iron from heme during refrigerated storage of frankfurters

In the present work, the amount of HI gradually decreased in frankfurters during storage (Figure VIII.4a) and, as a likely reflection of this fact, the amount of NHI steadily increased (Figure VIII.4b). The breakdown of the heme molecule and the release of iron from the porphyrin ring have been reported to occur as a consequence of the high temperatures during cooking (Miller *et al*., 1994; Lombardi-Boccia *et al*., 2002). The evolution of non-heme iron contents suggest that the heme degradation mainly happened during refrigerated storage. In accordance with our results, Gómez-Basauri & Regenstein (1992) reported the rapid breakdown of the heme molecule during refrigerated storage of cod and mackerel flesh. Studying the heme stability in ground cooked pork; Miller *et al*. (1994) suggested a relationship between the release of iron from the heme molecule during refrigerated storage to the disruption of the porphyrin ring. Purchas *et al*. (2004) reported similar conclusions studying the variations in the form of iron during refrigerated storage of beef and lamb meat. Though the precise causes of the heme degradation were not elucidated, the release of iron from heme molecule has been related to oxidative reactions. In fact, Miller *et al*. (1994) described the use of the NHI quantification during refrigerated storage of meats as a sensitive, reliable and consistent analysis for the evaluation of lipid oxidative changes. Accordingly, the aforementioned authors and ourselves (Estévez & Cava, 2004) reported significant correlations between NHI content and TBA-RS which is in agreement with the correlations found in the present study between TBA-RS and NHI (R^2= 0.67; $p<0.01$) and between hexanal counts and NHI (R^2=0.51; $p<0.01$). In addition, a significant correlation was found between the carbonyl content derived from protein oxidation and the NHI (R^2=0.51; $p<0.01$). Though relatively small, this correlation reasonably suggests that the oxidative deterioration of some particular proteins such as the myoglobin could promote the degradation of the heme group and the

subsequent release of iron. In fact, results from the release of iron are consistent with those previously reported concerning the oxidative stability of frankfurters since WF had from day 20 to day 60 significantly higher amounts of NHI than IF. The accurate knowledge of the levels of the chemical forms of iron in meat products is of a great important from nutritional and technological points of view. From a nutritional point of view, HI has a higher bioavailability than NHI and represents the primary source of iron in human's diet (Carpenter & Mahoney, 1992). Consequently, the degradation of heme iron would reduce the nutritional value of the frankfurters in terms of iron bioavailability. IF contained, during the entire storage period, a significantly higher amount of HI than WF and HF which represents an important nutritional benefit. This difference is mainly explained by the higher amount of total iron in IF since the amount of NHI was similar among types of frankfurters. On the other hand, iron is considered as one of the most important oxidation promoters in meat systems (Kanner, 1994). The forms of NHI including ferritin, lactoferrin, cytosolic iron-dependant enzymes and low molecular weight (LMW) chelatable iron ions enhance lipid peroxidation in meat to a higher extent than HI (Kanner, 1994). Consequently, the increase of NHI content as a result of the release of the iron from the heme group would increase the oxidative instability of the frankfurters promoting the formation of further TBA-RS, hexanal and carbonyls from proteins.

VIII.6. Acknowledgements

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Table VIII.1. Fatty acid composition of frankfurters[1].

	IF	WF	HF	sem[2]	p[3]
C12	0.11[b]	0.13[a]	0.13[a]	0.00	0.004
C14	1.25[b]	1.36[a]	1.34[a]	0.01	<0.001
C16	20.07[c]	23.52[a]	21.71[b]	0.38	<0.001
C17	0.36[c]	0.63[a]	0.51[b]	0.03	<0.001
C18	9.02[c]	13.86[a]	11.63[b]	0.53	<0.001
C20	0.23	0.28	0.25	0.01	0.385
SFA	31.04[c]	39.77[a]	35.57[b]	0.96	<0.001
C16:1 (n-7)	2.59[a]	2.62[b]	2.45[c]	0.02	<0.001
C17:1 (n-7)	0.26[c]	0.42[a]	0.35[b]	0.02	<0.001
C18:1 (n-9)	53.58[a]	42.94[c]	47.69[b]	1.16	<0.001
C20:1 (n-9)	1.41[a]	1.06[c]	1.31[b]	0.04	<0.001
C22:1 (n-9)	0.04[a]	0.03[b]	0.04[a]	0.00	<0.001
MUFA	57.88[a]	47.08[c]	51.83[b]	1.18	<0.001
C18:2 (n-6)	8.80[c]	10.75[a]	9.95[b]	0.21	<0.001
C18:3 (n-6)	0.14[c]	0.20[a]	0.17[b]	0.01	<0.001
C18:3 (n-3)	0.71	0.71	0.71	0.00	0.945
C20:2 (n-6)	0.51[b]	0.54[a]	0.55[a]	0.01	<0.001
C20:3 (n-3)	0.06[a]	0.04[b]	0.05[ab]	0.00	0.025
C20:3 (n-6)	0.09[b]	0.11[a]	0.10[ab]	0.00	0.009
C20:4 (n-6)	0.41[b]	0.44[a]	0.38[c]	0.01	<0.001
C20:5 (n-3)	0.15[a]	0.11[c]	0.14[b]	0.00	<0.001
C22:2 (n-6)	0.03	0.05	0.05	0.01	0.176
C22:4 (n-6)	0.03	0.03	0.03	0.00	0.469
C22:5 (n-3)	0.09[c]	0.11[a]	0.10[b]	0.00	<0.001
C22:6 (n-3)	0.09[b]	0.12[a]	0.13[a]	0.01	0.001
PUFA	11.10[c]	13.21[a]	12.35[b]	0.23	<0.001

[1] g/100g fatty acids.
[2] Standard error of the means.
[3] Statistical significance.
Different letters in the same row denote statistical differences among types of frankfurters.
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Table VIII.2. Tocopherols and total phenolics contents in frankfurters.

	IF	WF	HF	sem[4]	p[5]
Tocopherol[1]					
α-tocopherol	3.72[a]	1.31[c]	2.74[b]	0.28	<0.001
γ-tocopherol	0.23[a]	0.05[b]	0.15[a]	0.02	0.001
Total Phenolics[2]					
Water[3]	1.04[a]	0.66[b]	0.79[b]	0.05	<0.001
Methanol	1.09[a]	0.85[b]	0.89[b]	0.04	0.012
80% Methanol water	0.52[a]	0.46[b]	0.46[b]	0.01	0.045

[1] mg/kg frankfurter.
[2] g/100g frankfurter.
[3] Solvents used for phenolic compounds extraction.
[4] Standard error of the mean.
[5] Statistical significance.
Different letters in the same row denote statistical differences among types of frankfurters.

Table VIII.3. Pearson's correlation coefficients (R^2)[1]

	R^2
TBA-RS vs. hexanal	0.55*
TBA-RS vs. Pox[2]	0.75*
TBA-RS vs. NHI	0.67*
Pox vs. NHI	0.51*
Pox vs. hexanal	0.68*
NHI vs. hexanal	0.51*

[1] n= 10 frankfurters for correlation coefficients taken from measurements at days 0 and 90 of storage.
[2] Protein oxidation as assessed by total carbonyl content.
*Significant correlations at $p<0.01$.

Figure VIII.1. Evolution of TBA-RS numbers during refrigerated storage of frankfurters. (Significant differences, $p<0.05$, among types of frankfurters within a day of storage are denoted by different letters).

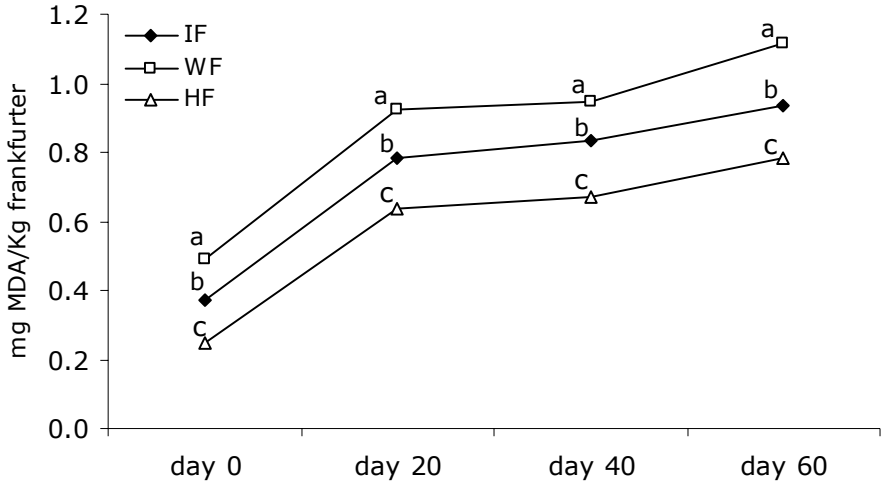


Figure VIII.2. Increase of hexanal counts during refrigerated storage of frankfurters. (Significant differences, $p<0.05$, among types of frankfurters within a day of storage are denoted by different letters).

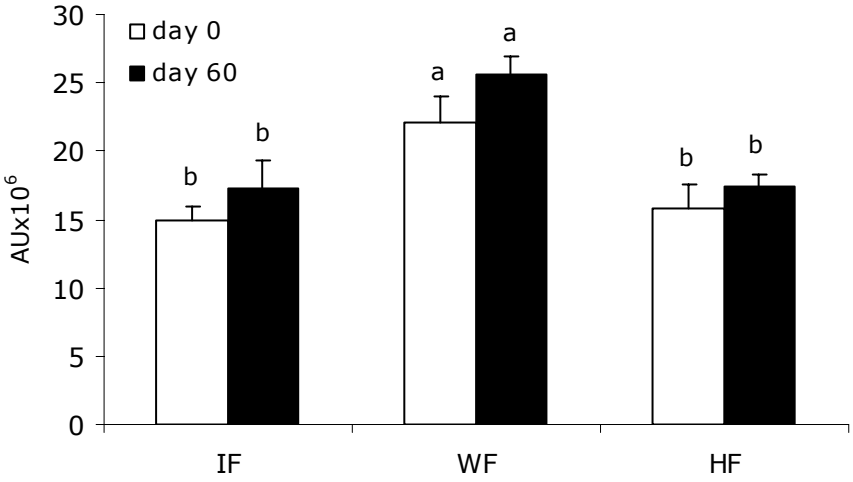


Figure VIII.3. Evolution of carbonyls content during refrigerated storage of frankfurters. (Significant differences, $p<0.05$, among types of frankfurters within a day of storage are denoted by different letters).

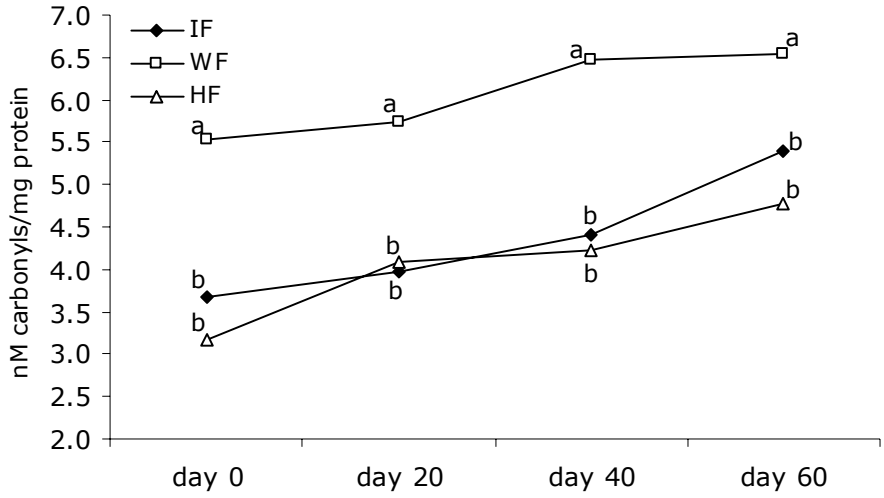
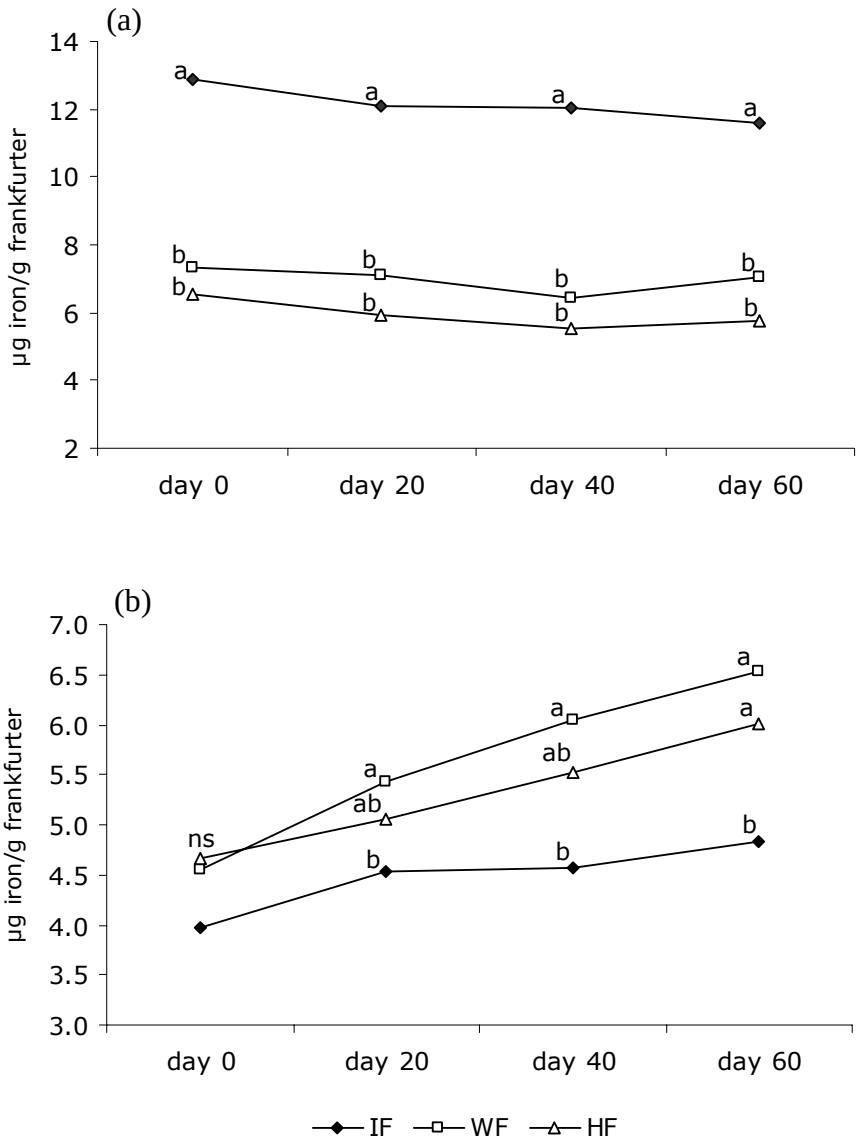


Figure VIII.4. Evolution of heme (a) and non-heme iron (b) contents during refrigerated storage of frankfurters. (Significant differences, $p<0.05$, among types of frankfurters within a day of storage are denoted by different letters).



CHAPTER IX

Protein oxidation in frankfurters with different levels of added rosemary essential oil: effect on colour and texture deterioration[♣]

[♣] *Journal of Food Science* (In press)

IX.1. Abstract

The development of protein oxidation as assessed by the total carbonyl content and its influence on colour and texture deterioration during the refrigerated storage (+4ºC/60 days) of frankfurters, were studied. The effect of the addition of a rosemary essential oil at different levels (150, 300 and 600 ppm) on the protein oxidative stability of the frankfurters was also evaluated. Frankfurters with no added essential oil were used as controls. The amount of carbonyls from protein oxidation significantly increased during refrigerated storage, and this increase was significantly higher in control frankfurters than in those with added 300 and 600 ppm rosemary essential oil. Rosemary essential oil at levels of 300 and 600 ppm successfully protected heme molecule from degradation as significantly inhibited the increase of NHI in refrigerated stored frankfurters. Colour changes were directly related to oxidation processes since frankfurters with added antioxidants (300 and 600 ppm) suffered less colour modifications than the controls ones. The addition of rosemary essential oil significantly enhanced texture characteristics of frankfurters, significantly reducing hardness, adhesiveness, gumminess chewiness and controlling the lost of elasticity during refrigeration. Statistically significant correlations were calculated between protein oxidation and instrumentally measured parameters suggesting that the alteration of protein functionality caused by oxidation likely affected colour and texture characteristics of frankfurters.

IX.2. Keywords: Protein oxidation, frankfurters, non-heme iron, colour, texture, rosemary.

IX.3. Introduction

The development of oxidative reactions during manufacture, handling or storage of meat products is a major concern for food technologists. During several decades, the lipid oxidation has been considered a hot topic of study due to its influence on muscle foods quality. Lipid oxidation decreases nutritional properties of foods since involves the loss of essential fatty acids

and vitamins and the generation of toxic compounds such as the malondialdehyde (MDA) and cholesterol oxidation products (COPs) (Morrissey *et al*., 1998). In addition, lipid oxidation affects essential sensory traits of meat products, causing flavour, texture and colour deterioration (Gray, 1978). Some other components of meat such as proteins can also be affected by oxidative reactions. The attack of reactive oxygen species (ROS) on muscle proteins leads to the loss of sulphydryl groups and the generation of carbonyl compounds (Xiong, 2000). Protein carbonyls can be generated via four possible pathways: (i) direct oxidation of amino acid side chains, (ii) fragmentation of the peptide backbone, (iii) reactions with reducing sugars and (iiii) binding non-protein carbonyl compounds (Xiong, 2000). Some studies have demonstrated that ROS can cause meat protein polymerisation and degradation leading to a decrease of protein solubility and functionality in model systems (Pokorny *et al*., 1990; Howell *et al*., 2001). However, quality changes in muscle foods as a result of the oxidative modification of proteins are largely unknown. Recently, Rowe *et al*. (2004) have suggested the influence of early post-mortem protein oxidation on beef tenderness. Ourselves (Estévez & Cava, 2004) have lately described the relationship between the development of protein oxidative reactions in a cooked liver product and the release of iron from the heme molecule as a likely consequence of the degradation of the heme protein and the breakdown of the porphyrin ring. The modification of the heme molecule structure could have an impact on the colour of the cooked product though this point has not been certainly elucidated.

In order to diminish the intensity of oxidative reactions in meat products, additives with antioxidant activity have been commonly used in the meat industry. However, the image of some particular synthetic substances used as antioxidants in foods such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl, octyl, and dodecyl gallates (PG, OG, DG) has been worsened by the findings linking the use of those compounds to health risks (Clayson *et al*., 1986). Consequently, substances derived from the plant kingdom such as dried herbs and essential oils have been

successfully used to reduce lipid oxidation in meat products (Chen *et al.*, 1999; McCarthy *et al.*, 2001; Formanek *et al.*, 2001). Concerning the strategies to inhibit protein oxidation in meats, Mercier *et al.* (1998) reported decreased protein carbonyls formation in muscle from turkey fed vitamin E. Nevertheless, the effect of the addition of antioxidants in muscle foods on the quality and functionality of muscle proteins has been poorly studied.

Frankfurters are non-fermented, emulsion type sausages marketed and consumed worldwide. During the production of frankfurters, ingredients such as meat and adipose tissue are finely minced and cooked at moderate temperatures which greatly increase the development of oxidative reactions during following refrigerated storage. The study of the protein oxidation in frankfurters and the impact of that on essential quality traits are of high interest due to the lack of knowledge on this particular topic. Colour and texture of frankfurters are important quality attributes that influences consumer's acceptance (Grigelmo-Miguel *et al.*, 1999; Jo *et al.*, 2000). This work was designed to investigate the development of protein oxidation of frankfurters with different levels of added rosemary essential oil during refrigerated storage and to evaluate the relationship between extent of protein oxidation and colour and texture traits.

IX.4. Material and Methods

Animals, feeds and sampling

Seven Iberian pigs were free-range reared and fed on natural resources (grass and acorns) following traditional livestock farming procedures. The animals were slaughtered at ~150 Kg live weight and an age of 12 months. After slaughter, adipose tissues and meat were removed from the carcasses, vacuum packaged and stored at -80ºC until the day the manufacture of the frankfurters (less than 2 weeks).

Manufacture of the frankfurters

The experimental frankfurters were manufactured in a pilot plant. For the manufacture of the sausages, meat and adipose tissues from seven different animals were used. The same formulation was used for all frankfurters except for the addition of a rosemary essential oil. The ingredients were as follows per 100g of elaborated product: 50 g meat, 10 g adipose tissue, 37 g distilled water, 2 g sodium caseinate, 1 g potato starch. Sodium chloride (2%), Sodium di- and tri-phosphates (0.5%) sodium ascorbate (0.05%) and sodium nitrite (0.03%) (all from ANVISA, Madrid, Spain) were also added. Depending on the addition of rosemary essential oil (Soria Natural S.L., Soria, Spain) at 150 ppm (T#150), 300 ppm (T#300) and 600 ppm (T#600), four experimental groups were studied since a control (CON) group with no added essential oil was also considered. For the manufacture, the meat was firstly chopped into small cubes (1 cm^3) and mixed with the sodium chloride and the nitrification mixture (sodium nitrite and ascorbate) 2 hours before frankfurter's manufacture. Then, the meat was minced in a Foss Tecator Homogeniser (mod. 2094) for 2 minutes together with the starch and the 50% of the total amount of sodium caseinate which was previously dissolved in water (+75ºC). After that, the adipose tissue was added together with the remaining dissolved sodium caseinate and minced for 4 more minutes until a homogenous raw batter was obtained. Finally, the mixture was stuffed into 18 mm diameter cellulose casings, handlinked at 10 cm intervals and given the thermal treatment by immersion in a hot water bath (+80ºC/30'). After that, frankfurters were allowed to cool at +4ºC for 24 hours.

Refrigeration

In order to allow the development of oxidative reactions, frankfurters were over-wrapped in PVC films and refrigerated stored (+4ºC) in the darkness for 60 days. Sampling was carried out at days 0, 20, 40 and 60 for analytical experiments. After each refrigeration stage frankfurters were analysed for instrumental colour and texture and kept frozen (-80ºC) until the remaining experiments were carried out.

Analytical methods

Protein oxidation measurement

Protein oxidation as measured by the total carbonyl content was assessed following the 2,4-dinitrophenylhydrazine (DNPH) coupling method described by Oliver *et al*. (1987). DNP hydrazones were quantified by measuring absorbance values at 370 nm. Protein concentration was determined by measuring absorbance at 280 nm using bovine serum albumin (BSA) as standard. The amount of carbonyls was expressed as nM carbonyls/ mg protein. The percent inhibition against protein oxidation was calculated at day 60 as $[(C_{60} - T_{60})/C_{60})] \times 100$, where T_{60} is the amount of carbonyls in the treated frankfurter at day 60 and C_{60} is the amount of carbonyls in control frankfurters at day 60.

Iron analysis

Nonheme iron (NHI) content was determined by spectrophotometry following the method described by Rhee *et al*. (1987). The amounts of iron were expressed as µg iron/g frankfurter.

Instrumental colour measurement

Instrumental colour (CIE L* a* b*; CIE, 1976) was measured on the cross section of the frankfurters using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ) with illuminant D_{65} and 0º standard observer. CIELAB L*, a*, b* values were determined as indicators of lightness, redness and yellowness, respectively. Chroma (C) and Hue angle (Hº) values were obtained by using the following equations: $C= (a^{*2} + b^{*2})^{0.5}$; Hº= arctg b*/a* x (360/6.25). A numerical total colour difference (ΔE) between frankfurters at day 0 and day 60 of storage was calculated by: $\Delta E_{0-60}= [(L_{60}-L_0)^2+ (a_{60}-a_0)^2+ (b_{60}-b_0)^2)]^{1/2}$.

Texture profile analysis (TPA)

The textural characteristics of the frankfurters were determined using a texturometer TA-XT2 TEXTURE ANALYSER (Stable Micro Systems Ltd., Surrey,

England, UK). Uniform portions of 2 cm in length from the middle of the frankfurters were used as the test samples. The samples were compressed to 50% their original height at a crosshead speed of 5 mm/s through a two-cycle sequence. A 5 cm diameter probe was used in TPA measurements. Textural variables from force and area measurements were (Bourne, 1978): Hardness (N/cm^2)= maximum force required to compress the sample (peak force during the first compression cycle); Adhesiveness (J)= work necessary to pull the compressing plunger away from the sample; Springiness (cm)= height that the sample recovers during the time that elapses between the end of the first compression and the start of the second; Cohesiveness (dimensionless)= extent to which the sample could be deformed prior to rupture (A_1/A_2, A_1 being the total energy required to for the first compression and A_2 the total energy required for the second compression); Gumminess (N/cm^2)= the force needed to disintegrate a semisolid sample to a steady state of swallowing (hardness x cohesiveness); Chewiness (J)= the work needed to chew a solid sample to a steady state of swallowing (gumminess x springiness).

Data analysis

All experiments were carried out in quindublicate. In order to assess the addition of the essential oil, the results from the experiments were used as variables and analysed using an Analysis of Variance (ANOVA) (SPSS, 1997). When statistically significant differences were found, Tukey test's were performed. Pearson correlation coefficients were calculated to determine the linear relationship between carbonyl and iron contents and the instrumentally measured parameters. Statistical significance was predetermined at 0.05.

IX.5. Results and Discussion

Protein oxidation during refrigerated storage of frankfurters

Results from the analysis of the oxidative deterioration of proteins from frankfurters during refrigerated storage are shown in Figure IX.1. The amount of protein carbonyls significantly increased during refrigeration in CON (from

3.7 to 5.4 nM carbonyls/mg protein), T#150 (from 3.8 to 5.3 nM carbonyls/mg protein), T#300 (from 3.0 to 4.7 nM carbonyls/mg protein) and T#600 (from 3.0 to 4.2 nM carbonyls/mg protein) frankfurters. In general, the addition of the rosemary essential oil significantly inhibited the oxidation of proteins since T#300 and T#600 frankfurters had significantly smaller amount of carbonyls than the controls at days 0, 20 and 60 of storage. As expected, the antioxidant effect was more intense when higher levels of essential oil were added, with the percent inhibition against protein oxidation at day 60 being 1.6%, 13.2% and 22.8% in T#150, T#300 and T#600 frankfurters, respectively. Results suggest that the rosemary essential oil should be used at 300 ppm or at higher concentrations to significantly inhibit the development of protein oxidation in refrigerated frankfurters. The use of rosemary essential oil as inhibitor of lipid oxidation in meat products has been profusely documented (McCarthy *et al*., 2001; Djenane *et al*., 2003; Estévez *et al*., 2004). It is plausible that the mechanisms and reaction pathways for the oxidation of lipids and proteins are directly linked as both processes are affected by the same prooxidant and antioxidant factors. In fact, in the presence of oxidising lipids, protein oxidation is manifested by free radical chain reactions similar to those for lipid oxidation, which involve initiation, propagation, and termination (Gardner, 1979). In this sense, Mercier *et al*. (1995), Batifoulier *et al*. (2002) and ourselves (Estévez & Cava, 2004) reported a possible linkage between lipid and protein oxidation based on the significant correlation coefficients found between both processes. In agreement with results from the present study, Viljanen *et al*. (2004) reported the antioxidant activity of berry phenolics on proteins from liposomes whereas ourselves (Estévez *et al*., 2004) described the protective effect of added sage and rosemary essential oils against protein oxidation in liver pâtés. Phenolic compounds can inhibit the oxidation of proteins by retarding the lipid oxidative reactions and by binding to the proteins and by forming complexes with them (Siebert *et al*., 1996).

Closely associated to the development of oxidative reactions in meats and cooked products, the breakdown of the heme molecule and the subsequent

release of iron from the porphyrin ring has been reported to occur as a consequence of the high temperatures reached during cooking (Schricker *et al*., 1982; Lombardi-Boccia *et al*., 2002). Moreover, Miller *et al*., (1994) and ourselves (Estévez & Cava, 2004) established relationships between a gradual increase of NHI and the development of oxidative deterioration during refrigerated storage of meat and meat products. In the present study, the amount of NHI progressively increased in the four groups of frankfurters during refrigerated storage (Figure IX.2.). In accordance to results from the protein oxidation, the addition of rosemary essential oil significantly reduced the release of iron from the heme molecule. At all days of storage, T#300 and T#600 frankfurters contained significantly smaller amounts of NHI than the CON ones whereas the addition of 150 ppm did not affect the amount of NHI. Miller *et al*. (1994) suggested a relationship between the iron release from heme and the disruption of the porphyrin ring during refrigerated storage. Recently, Purchas *et al*., (2004) reported similar conclusions studying the variations in the form of iron during refrigerated storage of beef and lamb meat. Though the precise causes of the heme degradation were not elucidated, the release of iron from the heme molecule has been related to oxidative reactions. Accordingly, the aforementioned authors and ourselves (Estévez & Cava, 2004) reported significant correlations between NHI content and TBA-RS agreeing with the correlations found in the present study between protein carbonyls and NHI (R^2= 0.72; $p<0.01$). This correlation reasonably suggests that the oxidative deterioration of some particular proteins such as the myoglobin could promote the degradation of the heme group and the subsequent release of iron. In the present study, the effect of the rosemary essential oil on the amounts of NHI could be explained by a likely protective effect of plant phenolics on the heme molecule through the inhibition of protein oxidation, reducing the release of iron.

Colour changes during refrigerated storage of frankfurters

Frankfurters exhibited different colour depending on the addition of antioxidants (Table IX.1.). At day 0, treated frankfurters showed smaller L*-values and higher b*-values than CON frankfurters.

Colour characteristics of frankfurters significantly changed during refrigerated storage (Table IX.1.). Lightness (L*-values) gradually increased over time in all groups. At day 60, higher L*-values were measured in control frankfurters than in the treated ones. The redness (a*-value) decreased during refrigerated storage with this decrease being more intense in the control frankfurters than in the treated counterparts. At day 60, T#600 frankfurters showed higher a*-values than those from the other groups of frankfurters. The yellowness (b*-value) tended to increase over time and no differences were found among frankfurters at day 60. The discolouration of frankfurters during refrigerated storage was affected by the addition of rosemary essential oil since the total colour difference (ΔE_{0-60}) values in T#300 and T#600 frankfurters was significantly smaller than in the T#150 and control ones. According to Francis & Clydesdale (1975), the colour modifications instrumentally measured can be considered as noticeable visual changes when the total colour difference (ΔE_{0-60}) values are higher than 2. In this sense, the addition of rosemary essential oil at 600 ppm successfully inhibited the discoloration of frankfurters after 60 days since the changes instrumentally measured were not even visually noticeable. The colour changes described in this study agree with those previously reported in relation to the discoloration of cooked sausages and other cooked products during refrigerated storage (Carballo *et al.*, 1991; Perlo *et al.*, 1995; Jo *et al.*, 2000; Fernández-Ginés *et al.*, 2003). These authors suggested that colour deterioration during refrigerated storage of cooked meats is explained by the degradation of certain nitrosopigments caused by oxidative processes, though no precise mechanisms were reported. Some other authors linked the discolouration of cooked products with lipid oxidation (Akamittath *et al.*, 1990; Jo *et al.*, 1999). It is reasonable that the colour changes in cooked products are caused by oxidative reactions since the addition of substances with proven antioxidant

activity inhibit to some extents the discolouration of frankfurters and other meat products (Cava *et al.*, 2004; Sebranek *et al.*, 2005). The modification of the pigment structure, suggested in the present work by the degradation of the heme molecule and the release of iron, might affect the colour displayed by frankfurters. In fact, the significant correlations between the NHI content and the colour parameters L* (R^2= 0.73; $p<0.01$) and a* (R^2= -0.84; $p<0.01$) (Table IX.3.) suggest the possible relationship between the degradation of the heme molecule and the discolouration of the frankfurters causing the loss of colour intensity and increasing hue values. In addition, significant correlations were also found between protein oxidation and the same colour parameters (Table IX.3.) suggesting that the development of protein oxidation in frankfurters affected their colour characteristics through the degradation of the heme molecule and the release of iron. Therefore, the protective role of the rosemary essential oil on frankfurter proteins and particularly on the heme protein would explain why treated frankfurters (T#300 and T#600) showed a more stable colour during refrigerated storage than the CON ones.

Texture profile changes during refrigerated storage of frankfurters

Results from the texture profile analysis of frankfurters during refrigerated storage are shown in Table IX.2. All texture parameters were affected by the addition of the rosemary essential oil at all days of study except the cohesiveness. After 60 days of refrigeration, hardness of frankfurters significantly increased in all groups with this increase being significantly higher in the CON frankfurters than in the treated ones. At all days of storage, CON frankfurters were harder than those with added essential oil. Though loss of moisture during storage could explain the increase of hardness in frankfurters, it is not applicable in the present study since the proximate composition of frankfurters at day 0 was not different to that at day 60. In addition, no differences were found between control and treated frankfurters concerning moisture content neither at day 0, nor at day 60 (data not shown). Hardness increase during refrigerated storage of frankfurters and other food

emulsions has been previously described and related to the process of emulsion destabilisation due to water and fat separation from the protein matrix (Fernández-Ginés *et al*., 2003; Fernández-López *et al*., 2004). To form a stable emulsion, proteins must surround the finely chopped fat particles before cooking and, therefore, protein functionality is essential to yield stable products (Smith, 1988). Protein oxidation is believed to affect protein functionality and their emulsification ability (Xiong, 2000). In addition, the oxidative damage of proteins has an impact in protein solubility, leading to the aggregation and complex formation due to cross links (Karel *et al*., 1975). It is plausible that the protein oxidation caused an increase of hardness in frankfurters through the loss of protein functionality and the formation of cross links between proteins since the addition of the antioxidant significantly reduced the hardness in frankfurters. The secondary parameters of gumminess and chewiness behaved similarly to the hardness, on which they are dependant. Consistently, significant correlations were found between the protein carbonyls and hardness (R^2= 0.56; $p<0.01$) and between protein carbonyls and gumminess (R^2= 0.42; $p<0.01$).

Adhesiveness changed variably during storage and the differences among groups were the same during the whole storage period. CON frankfurters showed at all days of storage significantly higher adhesiveness values than the treated ones. The adhesiveness is related to the presence of liquid fat on the surface of the frankfurters and for this reason, the reduction of fat in frankfurters leads to decreased adhesiveness values (Crehan *et al*., 2000). In the present study, the lower values of adhesiveness in frankfurters with added essential oil could be a reflection of the antioxidant effect on proteins that allowed the formation of more stable emulsions reducing the presence of such fat in their surface. Though it was relatively small, a significant correlation was found between protein oxidation and frankfurter adhesiveness (R^2= -0.35; $p<0.05$). The addition of the rosemary essential oil significantly reduced the springiness in frankfurters at day 0. The decrease of springiness during refrigerated storage was particularly intense in the CON frankfurters so that at day 60, the springiness of the treated frankfurters was significantly higher

than that of the CON ones which suggest the effect of the rosemary essential oil on keeping the elastic characteristics of the frankfurters. The cohesiveness of T#600 frankfurters was lower than in the CON ones at days 0 and 20 which is in accordance with the significant correlation found between protein carbonyls and this texture parameter (R^2= -0.70; $p<0.01$). Nevertheless the decrease of cohesiveness measured during refrigeration affected the same extent to the four groups.

IX.6. Conclusions

The addition of rosemary essential oil at levels of 300 and 600 ppm successfully enhanced oxidation stability of proteins in frankfurters, significantly reducing, in addition, the release of iron from the heme molecule. According to the present results, reasonable mechanisms have been reported by which the development of protein oxidation would affect the colour and texture characteristics of frankfurters. The addition of antioxidants improved some quality traits of frankfurters, inhibiting their discolouration and texture deterioration during refrigerated storage. Future studies concerning the effect of protein oxidation on meat quality would be of interest.

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IX.8. References

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Figure IX.1. Evolution of protein carbonyls content during refrigerated storage of frankfurters. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

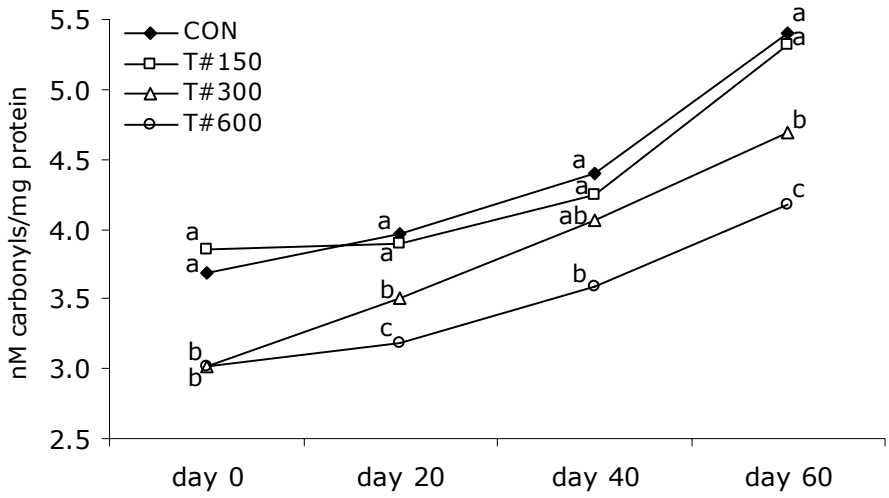

Figure IX.2. Evolution of non-heme iron content during refrigerated storage of frankfurters. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

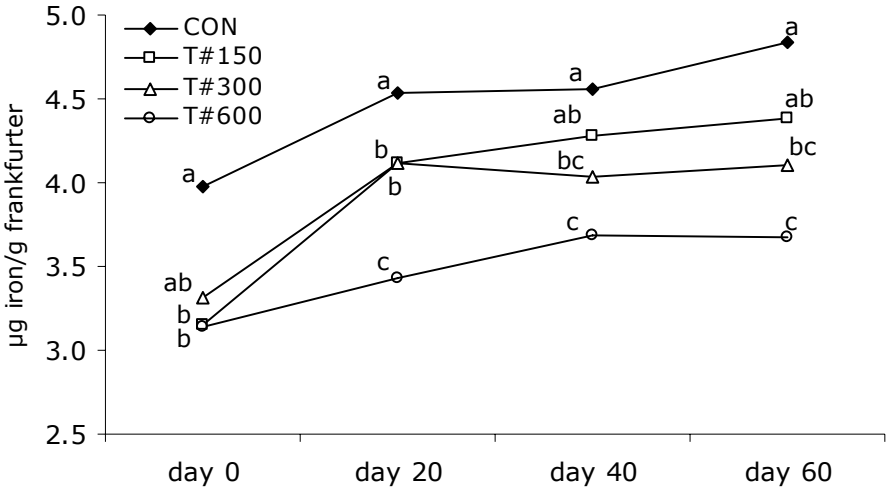

Table IX.1. Instrumental colour measured during refrigerated storage of frankfurters.

		CON	T#150	T#300	T#600	SEM[1]
L*	day 0	$71.59^{a,xy}$	$69.42^{b,z}$	$69.47^{b,y}$	$69.27^{b,y}$	0.24
	20	$70.98^{a,y}$	$68.40^{b,z}$	$68.87^{b,y}$	$68.54^{b,y}$	0.26
	40	$71.15^{a,y}$	$69.60^{b,y}$	$69.25^{b,y}$	$69.16^{b,y}$	0.21
	60	$72.17^{a,x}$	$71.48^{b,x}$	$71.39^{b,x}$	$70.05^{c,x}$	0.19
	SEM[2]	0.13	0.27	0.25	0.15	
a*	day 0	13.57^{x}	13.57^{x}	13.51^{x}	13.48^{x}	0.05
	20	$13.26^{b,x}$	$13.48^{b,x}$	$13.51^{ab,x}$	$13.77^{a,x}$	0.05
	40	12.36^{y}	12.41^{y}	12.36^{y}	12.36^{y}	0.08
	60	$11.17^{b,z}$	$11.39^{b,z}$	$11.52^{b,z}$	$12.63^{a,y}$	0.15
	SEM[2]	0.22	0.22	0.20	0.14	
b*	day 0	$9.21^{c,z}$	$9.18^{c,z}$	$9.46^{b,y}$	$9.73^{a,z}$	0.05
	20	$9.50^{b,z}$	$9.46^{b,yz}$	$9.60^{ab,y}$	$9.96^{a,yz}$	0.07
	40	9.97^{y}	9.93^{xy}	10.21^{x}	10.31^{xy}	0.06
	60	10.44^{x}	10.25^{x}	10.14^{x}	10.63^{x}	0.08
	SEM[2]	0.12	0.11	0.09	0.09	
Chroma	day 0	16.40^{x}	16.38^{xy}	16.49^{x}	16.62^{xy}	0.04
	20	$16.31^{b,xy}$	$16.51^{b,x}$	$16.58^{b,x}$	$16.99^{a,x}$	0.07
	40	15.88^{y}	15.91^{yz}	16.04^{y}	15.92^{z}	0.07
	60	$15.37^{b,z}$	$15.48^{b,z}$	$15.26^{b,y}$	$16.19^{a,yz}$	0.11
	SEM[2]	0.11	0.11	0.13	0.11	
Hue	day 0	$34.18^{c,z}$	$34.10^{c,z}$	$35.02^{ab,z}$	$35.86^{a,y}$	0.20
	20	35.62^{yz}	35.45^{z}	35.42^{z}	35.89^{y}	0.16
	40	40.42^{y}	38.81^{y}	39.59^{y}	38.92^{x}	0.28
	60	$43.02^{a.x}$	$41.56^{b,x}$	$41.66^{b,x}$	$41.18^{b,x}$	0.56
	SEM[2]	0.90	0.72	0.68	0.63	
ΔE^*		3.03^{a}	2.94^{a}	2.21^{b}	1.55^{c}	0.15

[1] Standard error of the mean within the same storage day (n=20).
[2] Standard error of the mean within the same antioxidant group (n=20).
Values with a different superscript (a-b) within a row of the same storage day are significantly different ($p<0.05$).
Values with a different superscript (x-z) within a column of the same antioxidant are significantly different ($p<0.05$).

Table IX.2. Texture parameters measured during refrigerated storage of frankfurters.

		CON	T#150	T#300	T#600	SEM[1]
Hardness	day 0	$15.91^{a,z}$	$13.29^{b,z}$	13.45^{b}	13.61^{b}	0.29
	20	$16.85^{a,yz}$	$12.99^{b,yz}$	13.04^{b}	13.47^{b}	0.40
	40	$17.51^{a,y}$	$14.18^{b,xy}$	13.50^{b}	13.81^{b}	0.40
	60	$18.71^{a,x}$	$14.66^{b,x}$	14.04^{b}	15.03^{b}	0.43
	SEM[2]	0.26	0.20	0.15	0.23	
Adhesiveness	day 0	$-0.17^{a,xy}$	-0.10^{ab}	$-0.09^{b,y}$	$-0.08^{c,y}$	0.01
	20	$-0.16^{a,y}$	-0.10^{b}	$-0.11^{b,x}$	$-0.08^{b,xy}$	0.01
	40	$-0.19^{a,x}$	-0.10	-0.10^{xy}	-0.09^{xy}	0.01
	60	$-0.18^{a,x}$	-0.09^{b}	$-0.10^{b,xy}$	$-0.09^{b,x}$	0.01
	SEM[2]	0.00	0.00	0.00	0.00	
Springiness	day 0	$0.92^{a,x}$	$0.88^{b,xy}$	0.88^{b}	$0.88^{b,x}$	0.00
	20	$0.92^{a.x}$	$0.88^{b,xy}$	0.88^{b}	$0.88^{b,xy}$	0.00
	40	$0.90^{a,y}$	$0.89^{ab,x}$	0.87^{c}	$0.88^{bc,xy}$	0.00
	60	$0.84^{b,z}$	$0.87^{a,y}$	0.87^{a}	$0.86^{a,y}$	0.00
	SEM[2]	0.01	0.00	0.00	0.00	
Cohesiveness	day 0	$0.59^{a,x}$	$0.59^{a,x}$	$0.59^{ab,x}$	$0.58^{b,x}$	0.00
	20	$0.60^{a,x}$	$0.59^{ab,x}$	$0.59^{b,x}$	$0.58^{c,x}$	0.00
	40	0.59^{x}	0.59^{x}	0.59^{x}	0.59^{x}	0.00
	60	0.56^{y}	0.56^{y}	0.56^{y}	0.56^{y}	0.00
	SEM[2]	0.00	0.00	0.00	0.00	
Gumminess	day 0	10.04^{a}	$7.71^{b,y}$	7.47^{b}	$7.68^{b,y}$	0.26
	20	9.80^{a}	$7.60^{b,y}$	7.33^{b}	$7.34^{b,y}$	0.26
	40	10.06^{a}	$8.25^{b,x}$	8.04^{b}	$7.69^{b,y}$	0.22
	60	10.16^{a}	$8.27^{b,x}$	8.00^{b}	$8.33^{b,x}$	0.21
	SEM[2]	0.10	0.09	0.13	0.10	
Chewiness	day 0	9.44^{a}	7.50^{b}	6.71^{b}	6.86^{b}	0.27
	20	9.44^{a}	7.27^{b}	6.96^{b}	6.61^{b}	0.26
	40	9.27^{a}	7.50^{b}	6.94^{bc}	6.81^{c}	0.24
	60	9.21^{a}	7.26^{b}	6.85^{b}	6.94^{b}	0.23
	SEM[2]	0.09	0.06	0.08	0.10	

[1] Standard error of the mean within the same storage day (n=20).
[2] Standard error of the mean within the same antioxidant group (n=20).
Values with a different superscript (a-b) within a row of the same storage day are significantly different ($p<0.05$).
Values with a different superscript (x-z) within a column of the same antioxidant are significantly different ($p<0.05$).

Table IX.3. Pearson's correlation coefficients (R^2)[1] between protein carbonyls and NHI and the instrumentally measured parameters.

	Carbonyls	NHI
Colour		
L*	0.73**	0.74**
a*	-0.84**	-0.74**
b*	0.59**	0.47**
chroma	-0.76**	-0.66**
hue	0.71**	0.64**
Texture		
Hardness	0.56**	0.65**
Adhesiveness	-0.35*	-0.53**
Springiness	-0.55**	-0.32*
Cohesiveness	-0.70**	-0.49**
Gumminess	0.42**	0.56**
Chewiness	0.28	0.50**

[1] n= 40 frankfurters for correlation coefficients taken from measurements at days 0 and 60 of refrigerated storage.

CHAPTER X

Analysis of volatiles in porcine frankfurters with increasing levels of added rosemary essential oil by using SPME-GC-MS[♣]

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X.1. Abstract

The effect of the addition of increasing levels of rosemary essential oil (150, 300 and 600 ppm) on the generation of volatile compounds in frankfurters from Iberian and white pigs, was analysed using SPME-GC-MS. Lipid-derived volatiles such as aldehydes (hexanal, octanal, nonanal) and alcohols (pentan-1-ol, hexan-1-ol, oct-1-en-3-ol) were the most abundant compounds in the headspace (HS) of porcine frankfurters. Frankfurters from different pig breeds presented different volatiles profiles due to their different oxidation susceptibility as a likely result of their fatty acid composition and tocopherol content. Rosemary essential oil showed a different effect on the generation of volatiles depending on the type of frankfurter in which they were added. In frankfurters from Iberian pigs, the antioxidant effect of the essential oil improved with increasing levels showing the highest activity at 600 ppm. In contrast, 150 ppm of the essential oil improved the oxidative stability of frankfurters from white pigs whereas higher levels led to no effect or a prooxidant effect. The activity of the essential oils could have been affected by the different fatty acid composition and tocopherol content between types of frankfurters. SPME successfully allowed the isolation and analysis of volatile terpenes from frankfurters with added rosemary essential oil including α-pinene, β-myrcene, 1-limonene, (*E*)-caryophyllene, linalool, camphor and 1,8-cineole, that might contribute to the aroma characteristics of frankfurters.

X.2. Keywords: Frankfurters, lipid-derived volatiles, volatile terpenes, rosemary essential oil, tocopherol, fatty acid composition.

X.3. Introduction

The SPME device has been successfully used for the analysis of volatile compounds in a large assortment of foods (Grimm *et al.*, 2001; Marco *et al.*, 2004; Estévez *et al.*, 2004a; Bonino *et al.*, 2003; Ziino *et al.*, in press). Concerning meat and meat products, the results obtained from this type of analysis include a large variety of information. For instance, the study of the volatiles profile from a particular meat product allows the achievement of

objective and valuable information regarding its aroma characteristics (Mottram, 1998). In fact, close relationships have been established between volatiles profiles and the aroma characteristics of different meat products, shedding light on the mechanisms of generation of volatile compounds (Mottram, 1998; Vergnais *et al*., 1998; Elmore *et al*., 1999; Chevance & Farmer, 1999). In addition, the analysis of the volatile components of a muscle food provides information about its deterioration during storage or manipulation since certain compounds are reliable indicators of particular enzymatic, microbial or biochemical alteration processes (Shahidi & Pegg, 1993; Vergnais *et al*., 1998; Vinauskiene *et al*., 2002; Estévez *et al*., 2003a). Lipid oxidation is one of the main causes of deterioration of the quality of meat products during storage and processing (Morrissey *et al*., 1998). However, some lipid-derived volatiles have been demonstrated to be potent odorants and contribute to the overall aroma of cooked and dry-cured meats (Elmore *et al*., 1999; Carrapiso *et al*., 2001; Machiels *et al*., 2004).

The use of herbs and spices has been largely spread in recent years in order to inhibit the development of oxidative reactions in food systems. Among the natural antioxidants, rosemary has been widely accepted as one of the spices with highest antioxidant activity (Hopia *et al*., 1996; Kähkönen *et al*., 1999; Ibáñez *et al*. 2003). The antioxidant activity of rosemary essential oil is primarily related to two phenolic diterpenes: carnosic acid and carnosol (Cuvelier *et al*., 1996; Ibáñez *et al*., 2003). Essential oils and extracts from rosemary and other *Labietae* herbs have been successfully used to reduce oxidative deterioration in a large variety of foods including frozen pork patties (McCarthy *et al*., 2001), refrigerated beef (Djenane *et al*., 2003), seed oils (Abdalla & Roozen, 1999), bread (Frutos & Fernández-Herrero, 2005), liver pâtés (Estévez *et al*., 2004a), cooked sausages (Sebranek *et al*., 2005) and deep-fat-fried potatoes (Chen Man & Tan, 1999). However, recent studies have described the complexity associated to the use of herbs or plant extracts as inhibitors of oxidative reactions (Kähkönen *et al*., 1999; Zheng & Wang, 2001). The antioxidant activity of these substances are affected by many factors including the total number and location of hydroxyl groups on aromatic

rings, the nature of the extracts, their concentration and the characteristics of the system in which they are added (Huang & Frankel, 1997; Kähkönen *et al*., 1999; Zheng & Wang, 2001; Škerget *et al*., 2005). Kähkönen *et al*. (1999) suggested that the antioxidant activity of plant phenolics could be also affected by the oxidation conditions and lipid characteristics of the system whereas Wong *et al*. (1995) and Skerget *et al*. (2005) reported that phenolic compounds from plants can interact with other substances such as tocopherols leading to synergist effects. Furthermore, plant phenolics have shown unexpected prooxidant properties in biological materials and food systems (Laughton *et al*., 1989; Yen *et al*., 1997). Nevertheless, most of these results have been reported in relatively simple model systems. Most of the studies carried out in order to evaluate the activity of rosemary essential oil in individual foods did not consider the effect of the compositional characteristics of the food. Meat and meat products from free-range reared Iberian pigs and intensively reared white pigs are considerably different in terms of fatty acid composition and tocopherol contents which could affect the activity of added rosemary essential oil though this extent has never been elucidated.

The purpose of the present study was to evaluate the effect of increasing levels of added rosemary essential oil on the generation of volatile compounds in frankfurters from free-range reared Iberian pigs and intensively reared white pigs using SPME-GC-MS.

X.4. Material and Methods

Animals, feeds and sampling

Seven Iberian pigs were free-range reared and fed on natural resources (grass and acorns) following traditional livestock farming procedures. The animals were slaughtered at ~150 Kg live weight and an age of 12 months. After slaughter, adipose tissues and meat were removed from the carcasses, vacuum packaged and stored at -80ºC until the day the manufacture of the frankfurters (less than 2 weeks).

Manufacture of the frankfurters

The experimental frankfurters were manufactured in a pilot plant. Depending on the origin of the raw material two types of frankfurters were produced: frankfurters from free-range reared Iberian pigs (IF) and frankfurters from intensively reared white pigs (WF). Meat and adipose tissues from seven animals from each pig breed were used. The same formulation was used for all frankfurters except for the addition of a rosemary essential oil. The ingredients were as follows per 100g of elaborated product: 50 g meat, 10 g adipose tissue, 37 g distilled water, 2 g sodium caseinate, 1 g potato starch. Sodium chloride (2%), Sodium di- and tri-phosphates (0.5%) sodium ascorbate (0.05%) and sodium nitrite (0.03%) (all from ANVISA, Madrid, Spain) were also added. Depending on the addition of rosemary essential oil (Soria Natural S.L., Soria, Spain) at 150 ppm (T#150), 300 ppm (T#300) and 600 ppm (T#600), four experimental groups within each pig breed were studied since a control (CON) group with no added essential oil was also considered. The eight set of frankfurters were independently produced in repeated manufacture processes. For the manufacture, the meat was firstly chopped into small cubes (1 cm^3) and mixed with the sodium chloride and the nitrification mixture (sodium nitrite and ascorbate) 2 hours before frankfurter's manufacture. Then, the meat was minced in a Foss Tecator Homogeniser (mod. 2094) for 2 minutes together with the starch and the 50% of the total amount of sodium caseinate which was previously dissolved in water (+75ºC). After that, the adipose tissue was added together with the remaining dissolved sodium caseinate and minced for 4 more minutes until a homogenous raw batter was obtained. Finally, the mixture was stuffed into 18 mm diameter cellulose casings, handlinked at 10 cm intervals and given the thermal treatment by immersion in a hot water bath (+80ºC/30'). After that, frankfurters (n=5 within each batch) were allowed to cool at +4 ºC for 24 hours.

Proximate composition of frankfurters

Moisture, total protein and ash were determined using AOAC methods (AOAC, 2000a, b, c). The method of Bligh and Dyer (1959) was used for isolating fat from frankfurters.

Iron analysis

Total iron was determined following the procedure described by Miller *et al*. (1994). The amount of iron was expressed as µg iron/g frankfurter.

Tocopherols content

α- and γ-tocopherols were extracted from frankfurters according to the method described by Rey *et al*. (1997). The analysis was carried by reverse phase HPLC (HP 1050, with a UV detector, HPIB 10) (Hewlett-Packard, Waldbronn, Germany).

Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a FFAP-TPA fused-silica column (Hewlett Packard 30m long, 0.53 mm internal diameter and 1.0 µm film thickness). The injector and the detector temperature were held at +230ºC. Oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma). The quantification of fatty acids was carried out by using C13 as an internal standard. Results are expressed as g fatty acid $100g^{-1}$ total fatty acid analysed.

SPME of Volatiles

The SPME fibre, coated with a divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30µm, was preconditioned prior

analysis at +220ºC during 45 min. The HS sampling was performed following a method previously described (Estévez *et al*., 2004a). 1 g of frankfurter was placed in 2.5 mL vials and the SPME fibre was exposed to the headspace of the frankfurters while the sample equilibrated during 30 minutes immersed in water at +50ºC. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm id., 1.0mm film thickness). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40 ^{o}C. The SPME fibre was desorbed and maintained in the injection port at 220 ^{o}C during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at +40ºC and then raised at the rate of +7ºC min^{-1} to +250^{o}C, and held for 5 min. The GC/MS transfer line temperature was 270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Compounds were tentatively identified by comparing their mass spectra with those contained in the Willey library and by comparison of their Kovats index with those reported by Kondjoyan & Berdagué (1996). Some compounds were identified by comparing their retention times with those from standard compounds (Sigma-Aldrich, Steinheim, Germany). Results from the volatiles analysis are provided in area units (AU).

Data Analysis

The proximate compositions, tocopherol contents and fatty acid compositions of frankfurters from Iberian and white pigs were compared using a student's 't' test for independent variables. (GLM) procedure of SPSS software (11.0 version). Chromatographic areas of all tentatively identified peaks were used as variables. In order to determinate the effect of the frankfurter origin (Iberian and white pigs) and the addition of rosemary essential oil (control, 150, 300 and 600 ppm) on the generation of volatiles, an ANOVA for a four

(rosemary) x two (origins) together with the interaction was used. Tukey's tests were used when ANOVA found significance differences between treatments. Significance was defined at $p<0.05$.

X.5. Results and Discussion

Proximate and fatty acid composition of frankfurters

No significant differences were detected between frankfurters concerning their proximate composition since they had similar moisture, fat, protein, and ash contents (Table X.1.). IF had, however, a significantly higher amount of iron (16.3 µg/g frankfurter) than WF (11.7 µg/g frankfurter). This result was expected since it is generally known that meat from Iberian pigs contains higher amounts of iron than that from white pigs due to the higher concentration of myoglobin pigments (Estévez *et al.*, 2003b; Estévez *et al.*, 2004b). The addition of rosemary essential oil did not affect the proximate composition of frankfurters (data not shown).

Large differences were found between types of frankfurters for most of the fatty acids analysed. IF had significantly smaller amounts of myristic (1.3 vs. 1.4 g/100g), palmitic (20.0 vs. 24.1 g/100g), stearic (9.2 vs. 14.2 g/100g) and total SFA (31.6 vs. 40.7 g/100g) than WF. Compared to WF, IF contained significantly higher amounts of oleic (54.5 vs. 43.9 g/100g) and total MUFA (58.9 vs. 48.1 g/100g). WF had, conversely, larger amounts of PUFA (11.3 vs. 13.5 g/100g) such as linoleic (9.0 vs. 11.0 g/100g) acid. Fatty acid composition of frankfurters and other composite meat products reflects the fatty acid composition of the ingredients, mainly meat and adipose tissue used for their elaboration (Estévez *et al.*, 2004b). The differences reported between frankfurters from Iberian and white pigs are mainly explained by the different fatty acid composition of the feeds given to the animals during the fattening period, and therefore, meat and adipose tissues from Iberian pigs reflected the fatty acid composition of the acorns which had high levels of oleic acid (Cava *et al.*, 2000; Estévez *et al.*, 2004b). On the other hand, white pigs were fed on commercial mixed diets with relatively high amounts of PUFA

which would explain the high levels of such fatty acids in their tissues and consequently, in the elaborated frankfurters.

Tocopherol content of frankfurters

Results from the quantification of tocopherols in frankfurters are shown in Table X.1. IF presented higher levels of α- and γ-tocopherol compared to those from WF (3.7 vs. 1.3 μg/g and 0.23 vs. 0.05 μg/g, respectively), which is consistent with previously reported data regarding tocopherol contents in the tissues (meat and adipose tissue) from free-range reared Iberian pigs and white pigs reared indoors (Cava *et al.*, 2000; Estévez *et al.*, 2004b; Daza *et al.*, 2005). The α- and γ-tocopherol contents in animal tissues reflect the tocopherol concentration of the diets (Daza *et al.*, 2005), and therefore, the high levels of tocopherols in the grass and acorns with which Iberian pigs were fed explain the high levels of such substances in their tissues and frankfurters. The relatively small amounts of tocopherols in WF were expected since white pigs were fed on a tocopherol non-supplemented mixed diet. The high content of tocopherols in tissues and meat products from free-range reared Iberian pigs has been profusely described in previous works, and considered as one of the most appreciated quality traits (Cava *et al.*, 2000; Estévez *et al.*, 2004b; Daza *et al.*, 2005) as long as tocopherols enhance the oxidation stability of meats and meat products, improving their nutritional and technological properties (Morrissey *et al.*, 1998; Rey *et al.*, 1998).

Analysis of volatiles from frankfurters

From the total volatile compounds detected in the HS, 95 of them were categorised into 12 classes depending on their chemical structure and shown in Table X.2. According to Chevance & Farmer (1999) volatile components of the HS of frankfurters are derived from the main ingredients (meat and adipose tissue) and from the addition of spices and other minoritary additives. The generation of volatile compounds in frankfurters will be discussed according to their apparent origin.

Analysis of volatiles generated from ingredients

Considering volatile compounds generated from main ingredients (meat and adipose tissue), lipid-derived volatiles such as aldehydes (hexanal, heptanal, octanal, nonanal, decanal, dodecanal), ketones (heptan-2-one, 1-phenyl-propanone) and alcohols (oct-1-en-3-ol) were the most abundant compounds in the HS of frankfurters. A relatively high amount of esters and aliphatic hydrocarbons were also detected while acids and furans were minority. Most of the volatile compounds detected in the present study have been previously described as components of the HS of cooked pork and beef (Wettasinghe *et al*., 2001; Estévez *et al*., 2003a; Machiels *et al*., 2004). The similarity between the volatiles profiles from cooked meats and frankfurters was expected since pork and porcine back fat were the major ingredients. Ahn *et al*. (1999) and Jo & Ahn (2000) described lipid-derived volatiles such as hydrocarbons, ketones, alcohols and aldehydes as the most abundant volatile compounds in porcine cooked sausages. Accordingly, Chevance & Farmer (1999) reported that the HS of frankfurters without spices and smoke were dominated by volatiles generated from lipid oxidation. The production of frankfurters involves meat handling, mincing and cooking which greatly enhances the development of oxidative reactions (Kanner, 1994; Morrissey *et al*., 1998). In addition, the high levels of fat and iron could explain the high levels of lipid-derived volatiles in the HS of frankfurters. Some of these compounds such as hexanal are useful indicators of lipid decomposition and have been commonly used to assess oxidative changes in meat, meat products and several food systems (Shahidi & Pegg, 1993; Elmore *et al*., 1999; Estévez *et al*., 2004a; Viljanen *et al*., 2004). In addition, some of the lipid-derived volatiles described in the present work are recognised odorants commonly isolated from frankfurters and other cooked meats. Hexanal is responsible of 'green' odours in frankfurters (Chevance & Farmer, 1999) though other authors have associated that volatile compound with rancidity and warmed-over flavours (Shahidi & Pegg, 1993; Estévez *et al*., 2003a; Im *et al*., 2004). Oct-1-en-3-ol contributes with 'mushrooms' odour notes

whereas unsaturated aldehydes derived from PUFA degradation are thought to contribute with 'unpleasant, stale, oily' odours (Chevance & Farmer, 1999). Volatiles derived from other chemical reactions were also detected. Strecker aldehydes (2- and 3-methylbutanal, benzaldehyde) and alcohols (2-methyl-propan-1-ol, 2-methyl-butan-1-ol, 3-methyl-butan-1-ol) were isolated from the HS of frankfurters. These compounds are common components of cooked meats and meat products contributing with desirable 'almond-like', 'toasted' aroma notes (Elmore *et al.*, 1999; Carrapiso *et al.*, 2001). The presence of sulphur and nitrogen volatile compounds derived from Maillard reactions was highly restricted which is in disagreement with the results obtained by Chevance & Farmer (1999) who described a large variety of these compounds in porcine frankfurters. Maillard products such as pyrazines and tiophenes are potent odorants which have been linked to desirable 'roasted meat' flavours. Though those authors suggested that the Maillard compounds isolated from the commercial frankfurters were generated from the main ingredients (meat and back fat) it is more likely than those could be added as volatile components of flavourings to enhance consumer's acceptability. In fact, these compounds were not detected when the addition of spices and smoking flavours in frankfurters was avoided. The strategy of improving the aroma characteristics of a foodstuff through the addition of particular volatile compounds has been recently described in liver products (Estévez *et al.*, in press).

Regardless of the addition of the rosemary essential oil, frankfurters from white pigs ('control' group) had, compared to those from Iberian pigs, a higher number of lipid derived volatiles since hexanoic and heptanoic acids, hex-2-enal, dec-(*E*)-2-enal, 2-methylbut-(*E*)-2-enal, 2,5-dihydrofuran, hexane-2,4-dione and octan-2-one were not detected in the HS of IF. Furthermore, WF showed significantly ($p<0.05$) higher chromatographic areas of certain compounds closely related to lipid oxidation and off-flavours such as octanoic (5.2 vs. 2.1 AU) and nonanoic acids (7.7 vs. 2.0 AU), pentanal (0.93 vs. 0.56 AU) and heptan-2-one (0.53 vs. 0.37 AU). Differences between types of frankfurters were also significant on hexanal (white: 22.1 AU, Iberian: 14.9

AU; $p<0.05$) that has been widely used on meat products as indicator of lipid oxidation (Mottram, 1998; Elmore *et al*., 1999; Estévez *et al*., 2003a). These results are in agreement with those obtained in previous works in which the oxidative stabilities of meat and meat products from Iberian and white pigs were evaluated (Estévez *et al*., 2004a; Estévez *et al*., 2004c). The significantly higher amount of iron in IF compared to that in WF, could have played a prooxidant role since that metal is considered the most potent oxidation promoter in muscle foods (Kanner, 1994). The present results and those from previous studies suggest that other circumstances should be considered to fully comprehend the considerably high oxidative stability of meats from Iberian pigs. A higher proportion of MUFA and lower of PUFA (more prone to be oxidised) and the presence of significantly ($p<0.05$) higher amounts of tocopherols in IF, compared to those in white pigs, could partly explain these results. More recently, some authors (González *et al*., 2004) have suggested the possibility that other substances with antioxidant activity such as plant phenolics could be accumulated in Iberian pig's tissues as a result of the intake of natural resources and hence, contribute to inhibit the development of oxidative reaction in meat and muscle foods from free-range reared Iberian pigs.

On the other hand, the large differences between types of frankfurters in terms of fatty acid composition could affect the aromatic characteristics of frankfurters as long as the pathways for the generation of volatile compounds from lipid oxidation are fairly specific for each fatty acid. In fact, the significant differences reported between types of frankfurters concerning some particular volatiles generated from PUFA such as hexanal were not perceived for volatiles mainly derived from oleic acid such as octanal, nonanal and octan-1-ol. More than 10 percent points higher of oleic acid in IF than in WF likely led to an intense generation of volatiles derived from the oxidation of that fatty acid in IF which would explain that lack of differences between types of frankfurters. Oleic acid-derived volatiles are associated to pleasant notes, described as 'floral' and 'sweet' (Specht & Baltes, 1994), while the aromatic notes of linoleic and PUFA-derived volatiles have been described as

intense 'grass-like' and related to rancidity in cooked meat and other food systems (Shahidi & Pegg, 1933; Im *et al*., 2004). Consistently to results from fatty acid compositions, the ratio between oleic-derived volatiles (octanal, nonanal and octan-1-ol) and linoleic-derived volatiles (hexanal, hex-2-enal and dec-(*E*)-2-enal) was significantly higher in IF than in WF (Iberian: 3.28, white: 2.12; $p<0.05$) suggesting a more pleasant aromatic profile in the former. The high content of oleic acid and its oxidation-derived aldehydes in meat products from Iberian pigs has been related to essential quality traits (Cava *et al*., 2000; Estévez *et al*., 2004a; Estévez *et al*., 2004b). In addition, significantly higher amounts of Strecker aldehydes (2- and 3-methylbutanal, benzaldehyde) and alcohols (2-methyl-propan-1-ol, 2-methyl-butan-1-ol, 3-methyl-butan-1-ol) were detected in IF compared to those in WF which could contribute also to define different aromatic profiles between types of frankfurters. Strecker volatiles have been described as quality indicators in Iberian dry-cured products in which they contribute with desirable 'almond-like', 'toasted' aroma notes (Ruiz *et al*., 1999; Carrapiso *et al*., 2001). Finally, IF contained also significantly higher amounts of certain aliphatic and aromatic hydrocarbons (heptane, 2-methylnonane, undecane, methylbenzene, 1,3-dimethylbenzene and 1-methyl-3(1-methylethyl)benzene) and volatile terpenes (α-pinene, I-limonene and linalool). These compounds are likely to have been derived from the direct deposition in animal tissues from grass which would explain the significantly higher amounts in IF.

In general, the addition of rosemary essential oil had a significant effect on the generation of major volatile compounds but this effect was different depending on the amount of essential oil and the type of frankfurter in which it was added. In fact, the interaction between 'origin of frankfurter' and 'rosemary' resulted significant for most volatiles (Table X.2.) suggesting that the effect of the addition of rosemary was influenced by the type of frankfurter. In agreement with previous research on several meats and meat products (Wong *et al*., 1995, Chen *et al*., 1999) the addition of rosemary essential oil had an antioxidant effect on frankfurters from Iberian pigs since

the generation of lipid-derived volatiles was inhibited as the amount of the added essential increased. The addition of 150 ppm of essential oil significantly inhibited the generation of certain lipid-derived volatiles such as octanoic and nonanoic acids, pentan-2-ol, octan-1-ol and pent-4-enal. Higher antioxidant effects were achieved with higher rosemary levels with the highest antioxidant effect being detected at 600 ppm. Compared to the control ones, frankfurters with 600 ppm of rosemary essential oil had significantly smaller amounts of octanoic and nonanoic acids, pentan-2-ol, oct-1-en-3-ol, octan-1-ol, hexanal, pent-4-enal, but-(*E*)-2-enal, heptanal, octanal and decanal. The rosemary essential oil also inhibited the generation of Strecker volatiles and certain hydrocarbons in IF.

In contrast, the addition of rosemary essential oil in WF had a different effect, with that changing with the amount of essential oil added. 150 ppm showed, in general, an antioxidant effect, significantly decreasing the amount of certain lipid-derived volatiles such as pentanal, hex-2-enal, hexanal, dec-(*E*)-2-enal, 2,5-dihydrofuran, heptan-2-one, 1-phenylethanone, 1-phenylpropanone and octan-2-one in WF. Rosemary essential oil added at 300 ppm had no effect on the generation of the major lipid-derived volatiles whereas 600 ppm addition levels resulted in clear prooxidant effect significantly increasing the production of a large variety of volatile compounds from lipid decomposition such as octanoic acid, hexan-1-ol, pentan-2-ol, oct-1-en-3-ol, octan-1-ol, but-(*E*)-2-enal, heptanal, dec-(*E*)-2-enal, dodecanal, 2,5-dihydrofuran, 1-phenylpropanone and octan-2-one.

Although the antioxidant activity of plant phenolics are generally recognised (Huang *et al*., 1996), the pro-oxidant properties of these substances have also been described, being able to generate reactive oxygen species and damage lipids, proteins and other cellular components (Aruoma *et al*., 1992; Yen *et al*., 1997). Results from the present work suggest that the activity of the rosemary essential oil was dependent on the compositional characteristics of the food matrix. In fact, the effect of plant phenolics has been considered to be influenced by the compositional characteristics of the food system and the presence of other active substances (Yen *et al*., 1997; Huang & Frankel,

1997). Food systems, and particularly comminuted meat products such as frankfurters, are very complex in the number and the type of chemicals in the mixture, and a particular combination of these compounds might behave differently from the individual components. In this sense, Wong *et al*. (1995) and Fang & Wada (1993) reported possible interactions between phenolic compounds from sage and rosemary essential oils and tocopherols, resulting in different activities depending on the individual amounts of these substances in the food system. In the present work, significant differences ($p<0.05$) were found between frankfurters from Iberian and white pigs regarding tocopherol contents (4.0 vs. 1.4 μg/g frankfurter). Therefore, the presence of a certain amount of endogenous antioxidants (tocopherols) in the raw material and manufactured product might influence on the activity of exogenous active extracts, leading to antioxidant or pro-oxidant effects. In addition, the different fatty acid composition between frankfurters from Iberian and white pigs could have also influenced. In accordance to Huang & Frankel (1997), whether phenolic compounds act as antioxidants or prooxidants appears to be dependant on the lipid characteristics of the model system. These authors reported antioxidant activities of tea catechins in corn oil triglycerides whereas in oil in water emulsions, these compounds were all prooxidants. Moreover, the prooxidant activity was stronger with higher concentrations which is in agreement with the results from the present study. The different fatty acid composition between frankfurters affects the physical state of the lipids that could have affected the dispersion and antioxidant activity of the rosemary essential oils leading to different effects.

Finally, the activity of the rosemary essential oil could have been affected by the initial oxidation state of the frankfurter in which it was added. In systems with higher oxidative instability, the activity of plant phenolics could be reduced since phenolic compounds can be oxidised and the oxidation products could act as prooxidants promoting oxidative reactions (Huang & Frankel, 1997). These would explain the prooxidant activity of the rosemary essential oil in frankfurters from white pigs, with higher oxidative instability than those from Iberian pigs. Furthermore, the oxidation of phenolics in IF could have

been inhibited by the presence of high levels of tocopherols with which plant phenolics interact leading to regeneration and synergist effects (Wong *et al*., 1995; Hupia *et al*., 1996; Zhu *et al*., 1999). The results obtained in the present work are in agreement with those obtained in a previous study in which sage and rosemary essential oils (1000 ppm) showed an antioxidant effect when added on liver pâtés from Iberian pigs and exhibited the opposite (prooxidant) effect in liver pâtés from white pigs (Estévez *et al*., 2004a). The differences between liver pâtés from Iberian and white pigs reported in that study in terms of fatty acid composition and tocopherol contents are consistent with those reported in the present study which supports the hypothesis and mechanisms suggested.

Analysis of volatiles from added rosemary essential oil

The higher chromatographic areas detected owned to volatile terpenes derived from the addition of the rosemary essential oil. SPME allowed the isolation and analysis of 33 volatile terpenes including monoterpenes hydrocarbons such as α-pinene, camphene, β-myrcene and 1-limonene, sesquiterpenes hydrocarbons such as α-cubebene and (*E*)-caryophyllene and oxygen-derivative terpenes such as alcohols (linalool, endo-borneol, terpinene-4-ol), esters (linalyl acetate, linalyl propionate), carbonyls (camphor), and ethers (1,8-cineole). Most of these compounds have been previously reported as volatile components of sage and rosemary essential oils and isolated in the HS of several spiced foods (Chevance & Farmer, 1999; Ibáñez *et al*., 1999; Paleari *et al*., 2004). In fact, Chevance and Farmer (1999) reported that the most abundant headspace compounds from frankfurters were terpenes originated from spices, with smaller quantities of volatiles derived from meat, fat and other ingredients. The rosemary essential oil also contributed with large quantities of aromatic hydrocarbons and alcohols such as 1-methyl-4(1-methylethyl) benzene, 1-methyl-4(1methylethenyl) ciclohexanol and 1-methoxi-2-methylbenzene. The chromatographic areas of these compounds enlarged with increasing levels of the added rosemary essential oil.

As expected, no differences were detected between treated IF and WF within each level of added essential oil as long as the same formulation was used for all of them. Several of the volatile terpenes detected are recognised odorants and are commonly used in the food industry as flavour and fragrance ingredients (Ibáñez *et al.*, 1999). Volatile terpenes such as α-pinene, 1,8-cineole and linalool have been related to 'spices, pine needles', 'medicinal, cough syrup' and 'flowers, carnation' odours, respectively, and have been reported as contributors to the aroma of spiced cooked sausages (Chevance & Farmer, 1999). In absence of olfactometry or sensory assessment of frankfurters, the contribution of these compounds to the overall aroma of frankfurters remains unknown, and therefore, the attitude of consumers towards frankfurters with odour notes referred to such aromatic herbs would be a future work of interest. On the other hand, using deodorised extracts of these plants would be also an interesting option in order to achieve antioxidant effects in meat and fat products without including unexpected aroma components (Dorman *et al.*, 2003). The present results suggest that further research would be needed to establish the optimal level of added essential oil to achieve antioxidant effects and pleasant aromatic characteristics considering the individuality of the food system in terms of fatty acid composition and endogenous antioxidant content.

X.6. Acknowledgment

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X.7. References

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Table X.1. Proximate, tocopherol and fatty acid composition of frankfurters from white and Iberian pigs.

	Iberian	White	SEM	p-value[1]
moisture[2]	63.44	62.33	0.39	0.161
fat[2]	18.38	18.69	0.19	0.444
protein[2]	11.43	10.88	0.17	0.096
ash[2]	1.28	1.36	0.05	0.448
iron[3]	16.3	11.7	0.88	<0.001
α-tocopherol[3]	3.72	1.31	0.41	<0.001
γ-tocopherol[3]	0.23	0.05	0.03	<0.001
fatty acids[4]				
C14:0	1.27	1.39	0.02	0.038
C16:0	20.41	24.05	0.58	0.004
C18:0	9.17	14.17	0.81	<0.001
Σ SFA	31.56	40.66	1.46	<0.001
C16:1	2.63	2.68	0.01	0.358
C18:1	54.48	43.91	1.77	0.006
C20:1	1.43	1.08	0.06	0.017
Σ MUFA	58.85	48.14	1.80	0.010
C18:2	8.95	10.99	0.33	0.001
C18:3	0.72	0.73	0.00	0.559
C20:2	0.52	0.55	0.01	0.097
C20:4	0.42	0.45	0.01	0.108
Σ PUFA	11.29	13.51	0.35	0.002

[1] Statistical significance in a student's t-test for independent variables.
[2] g/100g of raw material.
[3] μg/g of raw material.
[4] mg fatty acid/100g total fatty acids analysed.

Table X.2. Volatile compounds (AU x 10^6) detected in headspace of frankfurters from Iberian and white pigs with 150, 300 and 600 ppm of added rosemary essential oil[1].

	Iberian				white					*p*-value[2]			
Compound	Control	T#150	T#300	T#600	Control	T#150	T#300	T#600	SEM[1]	O	R	OxR	rel[3]
Acids													
hexanoic acid	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.99^{a}	0.00^{b}	1.04^{a}	0.94^{a}	0.06	<0.001	<0.001	<0.001	b
heptanoic acid	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	1.73^{a}	0.00^{b}	1.59^{a}	0.94^{ab}	0.15	<0.001	0.029	0.029	b
octanoic acid	2.07^{b}	0.00^{c}	0.00^{c}	0.00^{c}	5.20^{a}	0.00^{c}	5.88^{a}	8.05^{a}	0.53	<0.001	<0.001	<0.001	b
nonanoic acid	1.97^{bc}	0.00^{c}	0.00^{c}	0.00^{c}	7.70^{a}	0.00^{c}	4.77^{ab}	6.49^{a}	0.57	<0.001	<0.001	0.008	b
Alcohols													
ethanol	20.58^{a}	17.31^{a}	19.73^{a}	17.50^{a}	15.90^{a}	16.31^{a}	10.51^{b}	10.42^{b}	1.00	<0.001	<0.001	<0.001	a
2-methylprop-2-en-1-ol	3.47^{a}	0.33^{c}	0.81^{bc}	1.49^{b}	0.33^{c}	0.33^{c}	1.22^{b}	0.90^{bc}	0.16	<0.001	<0.001	<0.001	b
2-methylpropan-1-ol	1.07^{b}	0.56^{c}	0.82^{bc}	0.77^{bc}	1.39^{a}	0.28^{d}	0.41^{d}	0.39^{d}	0.10	<0.001	<0.001	<0.001	b
3-methylbutan-1-ol	3.63^{a}	0.00^{c}	0.00^{c}	0.00^{c}	0.75^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.20	<0.001	<0.001	<0.001	b
2-methylbutan-1-ol	1.12^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.07	0.001	<0.001	<0.001	b
hexan-1-ol	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.46^{b}	0.35^{b}	0.32^{b}	0.87^{a}	0.05	<0.001	<0.001	<0.001	a
pentan-2-ol	0.53^{ab}	0.30^{cd}	0.29^{cd}	0.14^{d}	0.45^{bc}	0.26^{cd}	0.56^{ab}	0.67^{a}	0.03	<0.001	<0.001	<0.001	b
oct-1-en-3-ol	2.34^{bc}	1.32^{cd}	1.87^{c}	0.40^{d}	3.12^{b}	2.06^{bc}	3.01^{b}	5.60^{a}	0.24	<0.001	<0.001	<0.001	a
1-methyl-4(1methylethenyl) ciclohexanol	0.00^{e}	7.32^{d}	12.78^{c}	19.87^{a}	0.00^{e}	5.58^{d}	12.57^{c}	16.06^{b}	1.10	0.001	<0.001	0.004	c
octan-1-ol	1.04^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.95^{b}	0.00^{c}	1.59^{ab}	1.84^{a}	0.12	<0.001	<0.001	<0.001	a
Aldehydes													
3-methylbutanal	2.22^{a}	0.43^{b}	0.46^{b}	0.75^{b}	0.42^{b}	0.39^{b}	0.38^{b}	0.36^{b}	0.10^{b}	<0.001	<0.001	<0.001	a
2-methylbutanal	1.03^{a}	0.50^{b}	0.56^{b}	0.60^{b}	0.47^{b}	0.45^{b}	0.53^{b}	0.42^{b}	0.03^{b}	<0.001	<0.001	<0.001	a
pentanal	0.56^{de}	0.36^{e}	0.56^{de}	0.41^{e}	0.93^{a}	0.65^{cd}	0.70^{bc}	0.84^{ab}	0.05	0.003	0.383	0.754	a
hex-2-enal	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.34^{b}	0.00^{c}	0.69^{a}	0.47^{b}	0.04	<0.001	<0.001	<0.001	b
hexanal	14.88^{bc}	6.68^{cd}	7.51^{cd}	5.31^{d}	22.12^{a}	11.02^{cd}	18.12^{ab}	22.17^{a}	1.18	<0.001	<0.001	0.012	a
pent-4-enal	0.41^{a}	0.29^{b}	0.29^{b}	0.17^{c}	0.37^{ab}	0.32^{ab}	0.29^{b}	0.32^{ab}	0.01	0.017	<0.001	<0.001	b
but-(*E*)-2-enal	0.30^{bc}	0.20^{cd}	0.21^{bc}	0.08^{d}	0.33^{b}	0.25^{bc}	0.22^{bc}	0.61^{a}	0.02	<0.001	<0.001	<0.001	a
heptanal	7.04^{bcd}	4.89^{de}	4.86^{de}	3.64^{e}	8.04^{bc}	5.65^{cde}	9.49^{ab}	11.63^{a}	0.46	<0.001	0.004	<0.001	a
benzaldehyde	3.24^{a}	1.65^{bcd}	2.50^{ab}	2.04^{bc}	2.03^{bc}	1.41^{cd}	1.54^{cd}	1.10^{d}	0.12	<0.001	<0.001	0.102	b

	Iberian				white					*p*-value[2]			
Compound	Control	T#150	T#300	T#600	Control	T#150	T#300	T#600	SEM[1]	O	R	OxR	rel[3]
octanal	11.70^{ab}	8.79^{bc}	8.79^{bc}	6.17^{c}	11.83^{ab}	9.55^{abc}	8.19^{bc}	13.50^{a}	0.46	0.006	0.007	<0.001	a
nonanal	36.07^{ab}	34.84^{ab}	24.89^{b}	24.52^{b}	36.37^{ab}	38.09^{a}	40.32^{a}	38.79^{a}	1.41	0.074	0.005	0.044	a
decanal	2.75^{bc}	1.77^{cd}	1.19^{cd}	0.85^{d}	3.81^{ab}	2.75^{bc}	4.38^{ab}	4.87^{a}	0.25	<0.001	0.098	0.001	a
2-methylbut-(*E*)-2-enal	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.70^{b}	0.00^{c}	0.55^{b}	1.33^{a}	0.08	<0.001	<0.001	<0.001	b
dec-(*E*)-2-enal	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.63^{b}	0.00^{c}	1.43^{a}	1.37^{a}	0.10	<0.001	<0.001	<0.001	a
dodecanal	2.30^{d}	2.09^{d}	5.10^{ab}	3.02^{cd}	2.45^{d}	1.40^{d}	4.69^{ab}	5.86^{a}	0.29	0.181	<0.001	0.004	b
Aliphatic Hydrocarbons													
2-methylpentane	2.16^{abc}	0.92^{cd}	3.17^{a}	1.17^{bcd}	1.44^{bcd}	0.62^{d}	2.43^{ab}	2.27^{ab}	0.16	0.408	<0.001	0.006	b
heptane	0.66^{ab}	0.43^{bc}	0.58^{abc}	0.61^{abc}	0.37^{c}	0.51^{abc}	0.69^{a}	0.75^{a}	0.03	0.817	0.002	0.001	a
2-methylnonane	14.90^{a}	2.80^{b}	2.78^{bc}	2.83^{b}	0.96^{e}	1.87^{d}	1.41^{de}	1.90^{cd}	0.69	<0.001	<0.001	<0.001	a
undecane	5.34^{a}	0.00^{c}	0.00^{c}	0.00^{c}	3.09^{b}	0.00^{c}	0.00^{c}	0.00^{c}	0.32	0.019	<0.001	0.002	a
dodecane	2.32^{c}	5.71^{bc}	9.84^{b}	7.77^{bc}	1.94^{c}	6.43^{bc}	7.95^{bc}	16.25^{a}	0.81	0.081	<0.001	0.003	a
tridecane	0.98^{de}	1.28^{abcd}	1.63^{a}	1.38^{ab}	0.72^{e}	0.93^{cde}	1.34^{abc}	1.18^{bcd}	0.05	<0.001	<0.001	0.811	a
Aromatic hydrocarbons													
methylbenzene	1.54^{a}	1.37^{abc}	1.46^{ab}	1.40^{ab}	0.96^{bc}	0.85^{c}	1.07^{abc}	1.46^{ab}	0.05	<0.001	0.064	0.036	b
ethylbenzene	6.57^{a}	2.94^{bc}	2.71^{bc}	2.82^{bc}	4.71^{ab}	1.20^{c}	3.64^{b}	4.35^{ab}	0.29	0.448	<0.001	0.003	b
1,2-dimethylbenzene	6.90^{b}	4.26^{bc}	4.13^{bc}	3.39^{bc}	15.84^{a}	1.60^{c}	1.20^{c}	0.94^{c}	0.79	0.727	<0.001	<0.001	b
1,3-dimethylbenzene	6.38^{b}	4.02^{c}	3.23^{cd}	3.12^{cd}	3.70^{cd}	1.06^{d}	3.05^{cd}	21.01^{a}	1.00	<0.001	<0.001	<0.001	b
1-methyl-4(1-methylethyl) benzene	0.00^{d}	231.09^{c}	380.19^{b}	536.45^{a}	0.76^{d}	212.22^{c}	360.12^{b}	534.11^{a}	31.80	0.277	<0.001	0.788	c
1-methyl-3(1-methylethyl) benzene	1.24^{bc}	1.15^{c}	1.06^{c}	1.95^{a}	0.00^{e}	0.57^{d}	1.30^{bc}	1.59^{ab}	0.09	<0.001	<0.001	<0.001	c
Amines													
N,N-diethylethanamine	4.74^{a}	2.83^{ab}	2.70^{ab}	2.89^{ab}	1.36^{c}	4.03^{a}	1.41^{c}	3.77^{ab}	0.25	0.096	0.058	<0.001	b
N,N-dimethyl-1-dodecanamine	3.46^{d}	4.83^{cd}	14.92^{ab}	12.80^{abc}	7.19^{bcd}	2.58^{d}	17.65^{a}	5.83^{cd}	1.01	0.570	<0.001	0.013	b
Esters													
acetic acid ethyl ester	0.41^{b}	0.53^{b}	0.00^{c}	0.00^{c}	0.58^{b}	0.59^{b}	0.67^{b}	1.07^{a}	0.06	<0.001	0.007	<0.001	b
hexanoic acid ethyl ester	7.06^{a}	4.81^{ab}	7.05^{a}	5.60^{ab}	5.05^{ab}	3.27^{b}	5.25^{ab}	3.64^{b}	0.28	<0.001	0.001	0.977	b
nitric acid hexyl ester	0.00^{c}	0.30^{c}	0.55^{c}	0.30^{c}	1.21^{b}	1.79^{ab}	1.81^{a}	1.59^{ab}	0.12	<0.001	0.001	0.712	c

	Iberian				white					*p*-value[2]			
Compound	Control	T#150	T#300	T#600	Control	T#150	T#300	T#600	SEM[1]	O	R	OxR	rel[3]
heptanoic acid etil ester	1.64	1.60	2.43	2.38	2.64	1.21	2.23	1.91	0.14	0.970	0.082	0.179	b
octanoic acid ethyl ester	5.28^{c}	7.85^{bc}	9.16^{bc}	9.90^{bc}	5.45^{c}	7.42^{bc}	11.11^{b}	18.55^{a}	0.74	0.004	<0.001	0.002	b
nitric acid nonyl ester	0.89^{c}	2.15^{abc}	3.24^{ab}	3.57^{a}	0.81^{c}	1.76^{bc}	1.92^{abc}	2.35^{abc}	0.19	0.006	<0.001	0.250	c
nonanoic acid ethyl ester	1.76^{c}	4.22^{b}	6.31^{a}	6.42^{a}	1.89^{c}	2.21^{c}	4.84^{b}	4.08^{b}	0.30	<0.001	<0.001	0.001	b
decanoic acid ethyl ester	2.20^{d}	3.00^{cd}	4.15^{ab}	4.13^{ab}	2.55^{cd}	2.67^{cd}	4.50^{a}	3.38^{bc}	0.15	0.552	<0.001	0.056	b
Furans													
2,5-dihydrofuran	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.56^{b}	0.00^{c}	0.44^{b}	1.07^{a}	0.06	<0.001	<0.001	<0.001	b
Ketones													
heptan-2-one	0.37^{b}	0.30^{bc}	0.23^{c}	0.27^{bc}	0.53^{a}	0.22^{c}	0.38^{b}	0.52^{a}	0.02	0.204	0.001	<0.001	a
1-phenyl-ethanone	0.70^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.53^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.05	0.185	<0.001	0.160	b
hexane-2,5-dione	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.42^{a}	0.35^{a}	0.38^{a}	0.42^{a}	0.03	<0.001	0.119	0.119	a
1-phenyl-propanone	9.05^{a}	6.62^{ab}	4.00^{bc}	2.28^{c}	10.78^{a}	4.10^{bc}	2.76^{c}	4.88^{bc}	0.44	<0.001	<0.001	0.004	b
octan-2-one	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.31^{b}	0.00^{c}	0.41^{b}	0.71^{a}	0.04	<0.001	<0.001	<0.001	a
Nitrogen compounds													
pyridine	1.89^{a}	0.55^{b}	0.41^{b}	0.26^{b}	1.64^{a}	0.70^{b}	0.81^{b}	0.64^{b}	0.10	0.110	<0.001	0.115	a
Terpenes													
δ-3-carene	0.00^{d}	1.07^{c}	2.38^{b}	3.59^{a}	0.00^{d}	1.34^{c}	1.98^{b}	3.83^{a}	0.21	0.125	<0.001	0.002	b
α-thujene	0.00^{d}	7.86^{c}	13.58^{b}	19.33^{a}	0.00^{d}	7.48^{c}	10.44^{bc}	16.70^{a}	1.12	0.046	<0.001	0.349	b
α-pinene	0.54^{d}	505.21^{c}	827.89^{b}	1281.79^{a}	0.00^{d}	475.40^{c}	770.11^{b}	1365.90^{a}	77.99	0.978	<0.001	0.272	b
α-fenchene	0.00^{d}	28.25^{c}	48.04^{b}	74.58^{a}	0.00^{d}	27.31^{c}	44.69^{b}	77.80^{a}	4.50	0.868	<0.001	0.541	b
camphene	0.00^{d}	173.36^{c}	285.08^{b}	447.79^{a}	0.00^{d}	161.85^{c}	267.58^{b}	458.04^{a}	26.69	0.599	<0.001	0.693	b
γ-terpinene	0.00^{d}	6.31^{c}	8.46^{b}	18.62^{a}	0.00	4.39^{c}	10.27^{b}	16.94^{a}	0.99	0.114	<0.001	<0.001	b
β-1-pinene	0.00^{d}	208.51^{c}	296.56^{b}	454.61^{a}	0.00^{d}	185.35^{c}	275.74^{b}	456.36^{a}	26.71	0.120	<0.001	0.035	b
β-myrcene	0.00^{d}	147.87^{c}	279.56^{b}	395.95^{a}	0.00^{d}	146.61^{c}	259.38^{b}	378.88^{a}	23.25	0.158	<0.001	0.609	b
β-2-pinene	0.00^{d}	4.18^{c}	6.47^{b}	8.83^{a}	0.00^{d}	3.54^{c}	5.67^{b}	8.30^{a}	0.51	0.005	<0.001	0.363	b
β-terpinene	0.00^{d}	5.60^{c}	8.04^{b}	11.22^{a}	0.00^{d}	4.74^{c}	7.34^{b}	11.28^{a}	0.66	0.085	<0.001	0.309	b
α-terpinene	0.00^{d}	1.64^{c}	5.29^{b}	7.08^{a}	0.00^{d}	1.64^{c}	5.23^{b}	7.55^{a}	0.47	0.513	<0.001	0.590	b
I-limonene	1.95^{d}	374.92^{c}	575.34^{b}	803.06^{a}	0.78^{d}	344.86^{c}	641.33^{b}	819.86^{a}	49.01	0.428	<0.001	0.214	b

	Iberian				white					*p*-value[2]			
Compound	Control	T#150	T#300	T#600	Control	T#150	T#300	T#600	SEM[1]	O	R	OxR	rel[3]
1,8-cineole	0.00^{d}	1661.06^{c}	2120.30^{b}	2816.01^{a}	0.00^{d}	1487.34^{c}	2038.41^{b}	2901.22^{a}	169.65	0.083	<0.001	0.005	b
(*E*)-ocimene	0.00^{d}	2.96^{c}	6.30^{b}	8.66^{a}	0.00^{d}	2.31^{c}	6.27^{b}	8.69^{a}	0.49	0.201	<0.001	0.160	b
β-ocimene	0.00^{d}	24.06^{c}	40.11^{b}	56.11^{a}	0.00^{d}	21.87^{c}	36.89^{b}	54.39^{a}	3.29	0.075	<0.001	0.700	b
α-terpinolene	0.00^{d}	15.00^{c}	27.08^{b}	36.70^{a}	0.00^{d}	15.18^{c}	24.77^{b}	34.89^{a}	2.16	0.193	<0.001	0.544	b
linalool	1.59^{d}	75.00^{c}	152.34^{b}	231.22^{a}	0.00^{d}	69.72^{c}	164.00^{b}	217.08^{a}	13.72	0.601	<0.001	0.242	b
p-menth-3-en-1-ol	0.00^{d}	3.80^{c}	6.72^{b}	10.39^{a}	0.00^{d}	3.31^{c}	6.95^{b}	9.64^{a}	0.63	0.102	<0.001	0.050	b
camphor	0.00^{d}	583.68^{c}	1058.47^{b}	1642.70^{a}	0.00^{d}	563.56^{c}	996.20^{b}	1641.45^{a}	96.39	0.115	<0.001	0.127	b
α-terpineol	0.00^{d}	8.69^{c}	25.22^{b}	38.04^{a}	0.00^{d}	9.41^{c}	23.61^{b}	35.51^{a}	2.18	0.109	<0.001	0.225	b
endo borneol	0.00^{d}	18.78^{c}	58.78^{b}	156.28^{a}	0.00^{d}	14.84^{c}	50.97^{b}	158.00^{a}	11.12	0.116	<0.001	0.018	b
linalyl propionate	0.00^{d}	69.24^{c}	150.92^{b}	273.40^{a}	0.00^{d}	63.28^{c}	156.11^{b}	281.00^{a}	25.35	0.460	<0.001	0.230	b
isoterpinolene	0.00^{d}	16.29^{c}	29.23^{b}	43.17^{a}	0.00	14.33^{c}	27.49^{b}	41.76^{a}	2.38	0.245	<0.001	0.212	b
linalyl acetate	0.00^{d}	10.10^{c}	15.67^{b}	27.59^{a}	0.00^{d}	8.64^{c}	14.19^{b}	28.64^{a}	1.57	0.116	<0.001	0.314	b
endobornyl acetate	0.00^{d}	83.76^{c}	134.20^{b}	214.02^{a}	0.00^{d}	67.42^{c}	142.58^{b}	203.12^{a}	12.52	0.294	<0.001	0.216	b
α-cubebene	0.00^{d}	1.09^{c}	2.03^{b}	3.34^{a}	0.00^{d}	0.81^{c}	2.12^{b}	3.36^{a}	0.20	0.364	<0.001	0.035	b
α-copaene	0.00^{e}	0.94^{c}	1.61^{b}	2.48^{a}	0.00^{e}	0.70^{d}	1.77^{b}	2.50^{a}	0.15	0.644	<0.001	0.002	b
geranyl propionate	0.00^{e}	2.86^{d}	5.25^{c}	9.06^{a}	0.00^{e}	2.54^{d}	6.05^{b}	8.75^{a}	0.54	0.730	<0.001	0.007	b
(*Z*)-caryophyllene	0.00^{d}	4.72^{c}	7.15^{b}	11.76^{a}	0.00^{d}	4.20^{c}	7.30^{b}	12.05^{a}	0.66	0.500	<0.001	0.321	b
(*E*)-caryophyllene	0.00^{d}	40.72^{c}	78.15^{b}	133.28^{a}	0.00^{d}	34.34^{c}	83.97^{b}	123.00^{a}	7.52	0.125	<0.001	0.052	b
β-selinene	0.00^{e}	3.54^{d}	8.09^{c}	13.63^{a}	0.00^{e}	3.89^{d}	9.57^{b}	12.56^{a}	0.81	0.386	<0.001	0.002	b
α-elemene	0.00^{d}	1.29^{c}	3.08^{b}	4.46^{a}	0.00^{d}	1.30^{c}	2.80^{b}	4.08^{a}	0.26	0.034	<0.001	0.168	b
δ-cadinene	0.00^{d}	1.05^{c}	2.83^{b}	3.95^{a}	0.00^{d}	1.02^{c}	2.82^{b}	4.11^{a}	0.26	0.827	<0.001	0.950	b
Others													
1-methoxi-2-methylbenzene	0.00^{d}	0.41^{c}	1.12^{b}	1.71^{a}	0.00^{d}	0.45^{c}	1.08^{b}	1.75^{a}	0.11	0.738	<0.001	0.794	c

In the same line, means with different superscript, significantly differed in ANOVA test.
[1] Standard error of the mean. [2] Statistical significance. [3] Reliability of Identification, a: Mass spectrometry + Kovats Index + Coincidence of retention time with standard compound; b: Mass spectrometry + Kovats Index; c: Mass Spectrometry.

CHAPTER XI

Effectiveness of rosemary essential oil as inhibitor of lipid and protein oxidation: contradictory effects in different types of frankfurters[♣]

[♣] Accepted for publication in *Meat Science* (22 July, 2005)

XI.1. Abstract

The effect of increasing levels (150, 300 and 600 ppm) of rosemary essential oil on lipid and protein oxidation and the increase of non-heme iron (NHI) during refrigeration (+4ºC/60 days) of frankfurters produced with tissues from either Iberian pigs (IF) or white pigs (WF), was studied. Frankfurters with no added essential oil were used as controls. Iberian pigs were free-range reared and fed on acorns and grass whereas white pigs (Large-white x Landrance) were intensively reared and fed on a mixed diet. Large differences were detected between types of frankfurters (Iberian vs. white) in terms of fatty acid composition and tocopherols content due to the different feeding background of the animals. The effect of the addition of rosemary essential oil on the oxidative stability of frankfurters depended on the level of added essential oil and the characteristic of the frankfurter. In WF, 150 ppm rosemary essential oil showed an antioxidant effect, significantly reducing the generation of lipid and protein oxidation products. At higher levels (300 and 600 ppm) the essential oil had, in general, no effect on lipid oxidation while significantly enhancing the oxidation of proteins and the release of iron from myoglobin. The rosemary essential oil successfully inhibited the development of lipid and protein oxidation in IF with that antioxidant effect being more intense at higher concentrations of essential oil. The presence of certain amounts of tocopherols in the frankfurters could have influenced on the activity displayed by the added essential oil leading to antioxidant or prooxidants effects though the different fatty acid composition between frankfurters could also have had an effect.

XI.2. Keywords: Protein oxidation, lipid oxidation, frankfurters, non-heme iron, plant phenolics, tocopherol, fatty acids.

XI.3. Introduction

The oxidative deterioration of lipid and proteins is a major concern for food technologists due to the loss of quality associated with those processes. Lipid oxidation decreases nutritional and sensory properties of foods since involves

the loss of essential fatty acids and vitamins and the generation of toxic compounds, causing additionally, flavour, texture and colour deterioration (Morrissey *et al*., 1998). The oxidative deterioration of proteins has been considerably studied in biological systems but scarcely considered in muscle foods. Several studies have demonstrated that oxidative processes cause meat proteins polymerisation and degradation leading to a decrease of protein solubility and functionality and colour and texture changes in model systems (Pokorny *et al*., 1990; Howell *et al*., 2001). Recently, Rowe *et al*. (2004) and ourselves (Estévez & Cava, 2004) have suggested the influence of protein oxidation on certain quality deterioration associated to pigment degradation and colour and texture changes. However, quality changes in muscle foods as a result of the oxidative modification of proteins are still largely unknown.

In order to inhibit the development of oxidative reactions in meat products, natural and synthetic antioxidants have been commonly used in the meat industry. Materials derived from the plant kingdom such as dried herbs and essential oils have been successfully used to reduce lipid oxidation in meat products (Chen *et al*., 1999; McCarthy *et al*., 2001; Estévez *et al*., 2004a; Formanek *et al*., 2001). Conversely, the effect of the addition of antioxidants to muscle foods on the quality and functionality of muscle proteins has been poorly studied. The study carried out by Mercier *et al*. (1998) reported decreased protein carbonyls formation in muscle from turkey fed vitamin E. However, recent studies have described the complexity associated with the use of herbs or plant extracts as inhibitors of oxidative reactions (Kähkönen *et al*., 1999; Zheng & Wang, 2001). According to Kähkönen *et al*. (1999), Wong *et al*. (1995), Škerget *et al*. (2005) and Yen *et al*., (1997) the effect of plant phenolics on the oxidative stability of a food system could be unexpected since it is affected by the oxidation conditions and lipid characteristics of the system and the presence of tocopherols and other active substances leading to antioxidant or prooxidant effects. Most of the studies carried out in order to evaluate the activity of rosemary essential oil in meat products did not consider the effect of the compositional characteristics of the food even though it could be largely influential. Frankfurters from free-range reared Iberian pigs and

intensively reared white pigs are considerably different in terms of fatty acid composition and tocopherol contents which could affect the activity of added rosemary essential oil though this has never been studied. The aim of this work was to evaluate the effect of increasing levels of added rosemary essential oil on the oxidative stability of lipids and proteins in frankfurters from free-range reared Iberian pigs and intensively reared white pigs.

XI.4. Material and Methods

Raw material

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed were free-range reared and fed on natural resources (grass and acorns) following traditional livestock farming procedures. The animals were slaughtered at ~150 Kg and an age of 12 months. Seven white pigs (Large-white x Landrace) were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at ~85 kg live weight and at the age of 7 months. Iberian pigs and white pigs were slaughtered at the same slaughterhouse one week apart. After slaughter, meat and adipose tissues were removed from carcasses, vacuum packaged and stored at -80ºC until the manufacture of the frankfurter.

Manufacture of the frankfurters

The experimental frankfurters were manufactured in a pilot plant. Depending on the origin of the raw material two types of frankfurters were produced: frankfurters from free-range reared Iberian pigs (IF) and frankfurters from intensively reared white pigs (WF). Meat and adipose tissues from seven animals from each pig breed were used. The same formulation was used for all frankfurters except for the addition of a rosemary essential oil. The ingredients were as follows per 100g of elaborated product: 50 g meat, 10 g adipose tissue, 37 g distilled water, 2 g sodium caseinate, 1 g potato starch. Sodium chloride (2%), Sodium di- and tri-phosphates (0.5%) sodium

ascorbate (0.05%) and sodium nitrite (0.03%) (all from ANVISA, Madrid, Spain) were also added. Depending on the addition of rosemary essential oil (Soria Natural S.L., Soria, Spain) at 150 ppm (T#150), 300 ppm (T#300) and 600 ppm (T#600), four experimental groups within each pig breed were studied since a control (CON) group with no added essential oil was also considered. The eight set of frankfurters were independently produced in repeated manufacture processes. For the manufacture, the meat was firstly chopped into small cubes (1 cm^3) and mixed with the sodium chloride and the nitrification mixture (sodium nitrite and ascorbate) 2 hours before frankfurter's manufacture. Then, the meat was minced in a Foss Tecator Homogeniser (mod. 2094) for 2 minutes together with the starch and the 50% of the total amount of sodium caseinate which was previously dissolved in water (+75ºC). After that, the adipose tissue was added together with the remaining dissolved sodium caseinate and minced for 4 more minutes until a homogenous raw batter was obtained. Finally, the mixture was stuffed into 18 mm diameter cellulose casings, handlinked at 10 cm intervals and given the thermal treatment by immersion in a hot water bath (+80ºC/30'). After that, frankfurters (n=5 within each batch) were allowed to cool at +4ºC.

Frankfurters were stored at +4ºC for 60 days in the dark. Frankfurters were analysed at days 0, 20, 40, and 60 for TBA-RS number, hexanal, protein carbonyls and concentrations of NHI. At sampling times, samples were stored at -80ºC until the other analytical experiments were conducted.

Analytical methods

Compositional analysis of frankfurters

Moisture, total protein, and ash were determined using AOAC methods (AOAC, 2000a, b, c). The method of Bligh & Dyer (1959) was used for the extraction and quantification of the fat from frankfurters.

Tocopherols content

α- and γ-tocopherols were extracted from frankfurters according to the method described by Rey *et al*. (1997). The analysis was carried out by

reverse phase HPLC (HP 1050, with a UV detector, HPIB 10) (Hewlett-Packard, Waldbronn, Germany). Results are expressed as µg tocopherol/ g frankfurter.

Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a FFAP-TPA fused-silica column (Hewlett Packard 30m long, 0.53 mm internal diameter and 1.0 µm film thickness). The injector and the detector temperature were held at +230ºC. Oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma). The quantification of fatty acids was carried out by using C13 as an internal standard. Results are expressed as g fatty acid $100g^{-1}$ total fatty acid analysed.

TBA-RS measurement

Malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al*. (1996). Results are expressed as mg MDA/ kg frankfurter.

Hexanal analysis

The SPME fibre, coated with a divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30µm, was preconditioned prior analysis at +220ºC during 45 min. The HS sampling was performed following a method previously described (Estévez *et al*., 2004b). 1 g of frankfurter was placed in 2.5 mL vials and the SPME fibre was exposed to the headspace of the frankfurter while the sample equilibrated during 30 minutes immersed in water at +50ºC. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) coupled to a mass-selective detector

(Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm id., 1.0mm film thickness). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40 ^{o}C. The SPME fibre was desorbed and maintained in the injection port at 220 ^{o}C during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at +40ºC and then raised at the rate of +7ºC min^{-1} to 250 ^{o}C, and held for 5 min. The GC/MS transfer line temperature was +270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Hexanal was identified by comparing its retention time with that from the standard compound. Results from the hexanal analysis are provided in area units (AU).

Protein oxidation measurement

Protein oxidation as measured by the total carbonyl content was assessed following the 2,4-dinitrophenylhydrazine (DNPH) coupling method described by Oliver *et al* (1987). DNP hydrazones were quantified by measuring absorbance at 370 nm. Protein concentration was determined by spectrophotometry at 280 nm using bovine serum albumin (BSA) as standard. The amount of carbonyls was expressed as nM carbonyls/ mg protein.

Iron analysis

Nonheme iron (NHI) content was determined by spectrophotometry following the method described by Rhee *et al*. (1987). The amounts of iron were expressed as µg iron/g frankfurter.

Data analysis

Means and deviations from 5 measurements within each batch were obtained from all analytical experiments. Results from the experiments were used as variables and analysed by using an Analysis of Variance (ANOVA) from SPSS software in order to assess the effect of the addition of antioxidants and the

effect of refrigerated storage on liver pâtés. When statistically significant differences were found, Tukey's tests were performed. Statistical significance was set at p=0.05.

XI.5. Results

Proximate, tocopherol and fatty acid composition of frankfurters

No significant differences were detected between frankfurters concerning their proximate composition since they had similar moisture, fat, protein, and ash contents (Table XI.1.). The addition of rosemary essential oil did not affect the proximate composition of frankfurters (data not shown).

Large differences were found between types of frankfurters for most of the fatty acids analysed. Frankfurters from Iberian pigs (IF) had significantly smaller amounts of SFA (31.6 vs. 40.7 g/100g) and higher of MUFA (58.9 vs. 48.1 g/100g) than those from white pigs (WF). Compared to frankfurters from Iberian pigs, those from white pigs contained significantly larger amounts of PUFA (11.3 vs. 13.5 g/100g). Fatty acid composition of frankfurters and other composite meat products reflects the fatty acid composition of the ingredients, mainly meat and adipose tissue used for their elaboration (Estévez *et al*., 2004c). The differences reported between frankfurters from Iberian and white pigs are mainly explained by the different fatty acid composition of the feeds given to the animals during the fattening period, and therefore, meat and adipose tissues from Iberian pigs reflected the fatty acid composition of the acorns which had high levels of oleic acid. On the other hand, white pigs were fed on commercial mixed diets with relatively high amounts of PUFA which would explain the high levels of such fatty acids in their tissues and consequently, in the elaborated frankfurters. These results were profusely discussed in a previous paper (Estévez *et al*., submitted)

IF had higher levels of α- and γ-tocopherols compared to those from WF (3.7 vs. 1.3 μg/g and 0.23 vs. 0.05 μg/g, respectively), which is consistent with data reported in previous studies regarding tocopherol contents in the tissues (meat and adipose tissue) from free-range reared Iberian pigs and white pigs

reared indoors (Cava *et al*., 2000; Estévez *et al*., 2004c; Daza *et al*., 2005). The intake of grass by Iberian pigs during fattening outdoors has been postulated as the main reason explaining the high levels of tocopherols in their tissues and meat-based products (Estévez *et al*., 2004c; Daza *et al*., 2005).

Effect of rosemary essential oil on lipid oxidation

The evolution of the TBA-RS numbers during refrigerated storage of frankfurters from white pigs is shown in Figure XI.1. TBA-RS numbers increased from 0.49 to 1.12 mg MDA/kg frankfurter, from 0.36 to 0.86 mg MDA/kg frankfurter, from 0.42 to 1.06 mg MDA/kg frankfurter and from 0.50 to 1.24 mg MDA/kg frankfurter in control, T#150, T#300 and T#600 frankfurters, respectively. Consistently, hexanal counts significantly increased from day 0 to day 60 of refrigerated storage in control (from 22.1 to 40.7 AU), T#150 (from 11.0 to 15.3 AU), T#300 (from 18.1 to 33.1 AU) and T#600 (from 22.2 to 33.4 AU) frankfurters.

In both cases, the addition of rosemary essential oil had a significant effect on lipid oxidation, with this effect being different depending on the level of essential oil added. At 150 ppm, the rosemary essential oil successfully inhibited the development of the oxidative deterioration of frankfurters significantly reducing the generation of TBA-RS and hexanal during the whole refrigeration period. Unexpectedly, 300 and 600 ppm of rosemary essential oil had no effect on the lipid oxidative stability of frankfurters since TBA-RS and hexanal values in T#300 and T#600 frankfurters were similar to those in the control ones. However, T#600 frankfurters showed at days 30 and 60 of storage significantly higher amounts of TBA-RS suggesting an incipient prooxidant effect.

Results obtained regarding the effect of the rosemary essential oil on the oxidative stability of frankfurters from white pigs are in disagreement with those obtained from the study of the effect of the same essential oil on frankfurters from Iberian pigs (Figures XI.4a,b and XI.5a,b). The rosemary essential oil showed a protective role against lipid oxidation in frankfurters

from Iberian pigs, with this effect being more potent as the amount of added essential oil increased. It would be necessary, however, to add at least 300 ppm of rosemary essential oil to detect significant effects.

Effect of rosemary essential oil on protein oxidation

Results from the analysis of the oxidative deterioration of proteins during refrigerated storage of frankfurters from white pigs are shown in Figure XI.2. The amount of protein carbonyls significantly increased during refrigeration of control (from 5.5 to 6.5 nM carbonyls/mg protein), T#150 (from 4.6 to 6.2 nM carbonyls/mg protein), T#300 (from 6.2 to 8.1 nM carbonyls/mg protein) and T#600 (from 6.1 to 7.8 nM carbonyls/mg protein) frankfurters. At day 0, 150 ppm of rosemary essential oil significantly reduced the oxidation of proteins in WF though it showed no effect during the remaining days of study. The addition of 300 and 600 ppm of rosemary essential oil in WF enhanced the oxidative degradation of proteins since T#300 and T#600 frankfurters contained, at all days of storage, significantly higher amounts of protein carbonyls than control ones. As previously described for lipid oxidation, these results contrast with those obtained in a previous work (Estévez et al., in press) in which the effect of rosemary essential oil on frankfurters from Iberian pigs was studied (Figure XI.6a vs. XI.6b). In that work, the addition of the rosemary essential oil significantly inhibited the oxidation of proteins since T#300 and T#600 frankfurters had significantly smaller amount of carbonyls than the controls at days 0 and 60 of storage. In that case, the antioxidant effect was more intense when higher levels of essential oil were added which is in agreement with results previously described for lipid oxidation. In accordance, 150 ppm of rosemary essential oil showed no significant effect for what higher concentrations would be needed to significantly inhibit protein oxidation in IF.

In the present study, the amount of NHI gradually increased during refrigerated storage of frankfurters from white pigs (Figure XI.3.). In accordance to results from the protein oxidation, the addition of 150 ppm rosemary essential oil had no effect on the release of iron from the heme

molecule. At all days of storage, T#300 and T#600 WF contained significantly higher amounts of NHI than the control ones. Once again, the effect of the rosemary essential oil on the evolution of NHI during refrigerated storage of IF was different to that described for WF (Figure XI.7a vs. XI.7b). The addition of rosemary essential oil inhibited the release of iron from the heme molecule in IF. At day 60, this effect was dependent on the concentration of essential oil since higher concentrations showed a more intense effect.

XI.6. Discussion

The oxidative deterioration of lipids from frankfurters as assessed by TBA-RS numbers could reflect noticeable sensory changes since Gray & Pearson (1987) reported that rancid flavour is initially detected in meat products with TBA-RS values between 0.5 and 2.0. Furthermore, Boles & Parrish (1990) reported that a warmed-over flavour (WOF) could be perceived in meat products at TBA-RS values above 1.0. Similarly, the hexanal is mainly generated as a consequence of the oxidative decomposition of PUFA and has been related to rancid odours and used as an indicator of lipid oxidation (Shahidi & Pegg, 1993).

Protein oxidation is considered to be linked to lipid oxidation. In fact, in the presence of oxidising lipids, protein oxidation is manifested by free radical chain reactions similar to those for lipid oxidation, which involve initiation, propagation, and termination stages (Gardner, 1979). In this sense, Mercier *et al*. (1995), Batifoulier *et al*. (2002) and ourselves (Estévez & Cava, 2004) reported a possible linkage between lipid and protein oxidation based on the significant correlation coefficients found between both processes. Accordingly, significant correlations (R^2=0.77; $p<0.01$) were found in the present study between lipid and protein oxidation as measured by TBA-RS and protein carbonyls, respectively.

In addition, the breakdown of the heme molecule and the subsequent release of iron from the porphyrin ring may have occured as a consequence of the oxidative deterioration of proteins in meats and cooked products (Miller *et al*., 1994; Estévez & Cava, 2004). Miller *et al*. (1994) suggested a relationship

between the iron release from heme and the disruption of the porphyrin ring during refrigerated storage of cooked meats. Recently, Purchas *et al*. (2004) reported similar conclusions studying the variations in the forms of iron during refrigerated storage of beef and lamb meat. The similarity between the evolution of protein carbonyls and NHI during refrigerated storage of frankfurters from white pigs is reflected on the significant correlations between both parameters (R^2=0.76; $p<0.01$) reasonably suggesting that the oxidative deterioration of some particular proteins such as the myoglobin could promote the degradation of the heme group and the subsequent release of iron.

The use of rosemary essential oil as an inhibitor of lipid oxidation in meat products has been profusely documented (McCarthy *et al*., 2001; Djenane *et al*., 2003; Estévez *et al*., 2004a, b; Sebranek *et al*., 2005). Recent studies also supported the effectiveness of natural antioxidants as inhibitors of protein oxidation (Viljanen *et al*., 2004; Estévez *et al*., 2004a). These results are in accordance with those reported in the present study on frankfurters from Iberian pigs and disagree with those obtained regarding frankfurters from white pigs. For the latter, the addition of rosemary essential oil had only antioxidant effects at the lower concentrations (150 ppm) while higher concentrations led to no effects or prooxidant effects. The unpredictable effect of plant essential oils on the oxidative stability of meat products was previously reported in liver pâté (Estévez *et al*., 2004b). In that work, rosemary and sage essential oils (1000 ppm) protected lipids and proteins in liver pâtés from Iberian pigs from oxidative reactions whereas the same essential oils enhanced the oxidative deterioration of such components in liver pâtés from white pigs. These results suggest that the activity of the rosemary essential oil was affected by some components of the food matrix. Food systems, and particularly comminuted meat products such as frankfurters or liver pâtés, are very complex in the number and the type of chemicals in the mixture, and a particular combination of these compounds might behave differently from the individual components. Accordingly, Yen *et al*. (1997) and Huang & Frankel (1997) reported that the effect of plant phenolics is

influenced by the compositional characteristics of the food system and the presence of other active substances such as tocopherols. In this regard, Wong *et al*. (1995) and Fang & Wada (1993) reported likely interactions between phenolic compounds from sage and rosemary essential oils and tocopherols, resulting in different activities depending on the individual amounts of these substances in the food system. The significantly higher amounts of tocopherols detected in the raw material (Estévez *et al*., submitted) and frankfurters from Iberian pigs compared to those from white pigs could have influenced on the activity of the added rosemary essential oil leading to antioxidant or pro-oxidant effects.

In addition, the activity of the rosemary essential oil could have been affected by the initial oxidation state of the frankfurter to which it was added. In systems with high oxidative instability, the activity of plant phenolics could be diminished since phenolic compounds can be oxidised and the oxidation products could act as prooxidants promoting oxidative reactions (Huang & Frankel, 1997). In this sense, the higher oxidative instability of WF compared to that of IF as suggested by the results obtained from both lipid and protein oxidation would explain also the prooxidant activity of the rosemary essential oil in frankfurters from white pigs.

The large differences in fatty acid composition between frankfurters from Iberian and white pigs could have affected also the activity of the rosemary essential oil according to findings by Huang & Frankel in model systems (1997). These authors reported that antioxidant or prooxidant activities of tea catechins depended on the lipid system used for the analysis (corn oil triglycerides vs. oil in water emulsions). Moreover, the prooxidant activity was stronger with higher concentrations, which is in agreement with the results from the present study. The different fatty acid composition between frankfurters affects the physical state of the lipids and the texture characteristics of the whole frankfurter that could have influenced the dispersion and antioxidant activity of the rosemary essential leading to different effects.

XI.7. Conclusions

Though the use of plant herbs and essential oils on meat and fat products is a common industrial practice, the effect of these additives can be unpredictable depending on the concentration of the substance and the characteristics of the meat product. In accordance with the present results, the decision to use a particular amount of plant extracts with antioxidant purposes on a meat product should be taken carefully considering the peculiar characteristics of each food. In the absence of the knowledge of precise mechanisms of interaction between the essential oil components and the meat product, systematic preliminary studies should be carried out to establish desirable effects at particular concentrations. Further experiments would be interesting to shed light on the specific interactions between plant extracts and meat components and to evaluate the influence of the chemical composition of meat in terms of fatty acids and tocopherols on the activity of these substances.

XI.8. Acknowledgements

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XI.9. References

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Table XI.1. Proximate, tocopherol and fatty acid composition of frankfurters from white and Iberian pigs.

	Iberian	White	SEM	p-value[1]
moisture[2]	63.44	62.33	0.39	0.161
fat[2]	18.38	18.69	0.19	0.444
protein[2]	11.43	10.88	0.17	0.096
ash[2]	1.28	1.36	0.05	0.448
α-tocopherol[3]	3.72	1.31	0.41	<0.001
γ-tocopherol[3]	0.23	0.05	0.03	<0.001
fatty acids[4]				
C14:0	1.27	1.39	0.02	0.038
C16:0	20.41	24.05	0.58	0.004
C18:0	9.17	14.17	0.81	<0.001
Σ SFA	31.56	40.66	1.46	<0.001
C16:1	2.63	2.68	0.01	0.358
C18:1	54.48	43.91	1.77	0.006
C20:1	1.43	1.08	0.06	0.017
Σ MUFA	58.85	48.14	1.80	0.010
C18:2	8.95	10.99	0.33	0.001
C18:3	0.72	0.73	0.00	0.559
C20:2	0.52	0.55	0.01	0.097
C20:4	0.42	0.45	0.01	0.108
Σ PUFA	11.29	13.51	0.35	0.002

[1] Statistical significance in a student's t-test for independent variables.
[2] g/100g of raw material.
[3] µg/g of raw material.
[4] mg fatty acid/100g total fatty acids analysed.

Figure XI.1. Evolution of TBA-RS numbers during refrigerated storage of WF treated with 150, 300 and 600 ppm rosemary essential oil. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

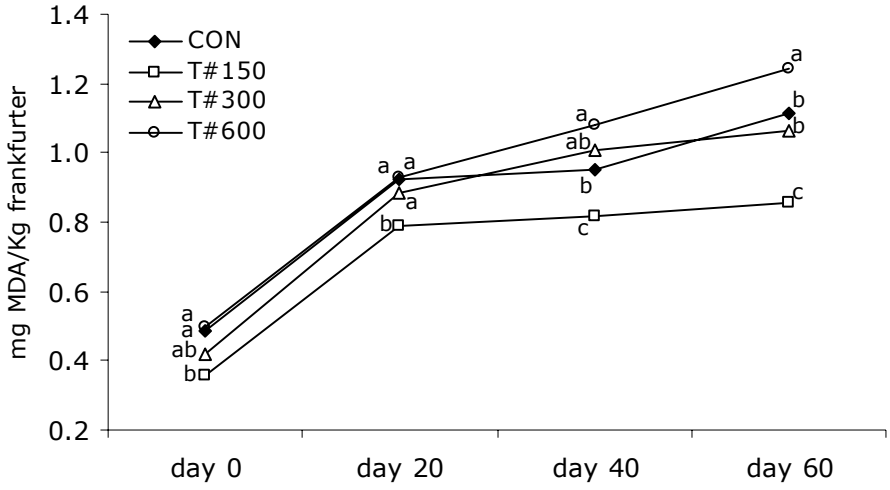


Figure XI.2. Evolution of protein carbonyls content during refrigerated storage of WF treated with 150, 300 and 600 ppm rosemary essential oil. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

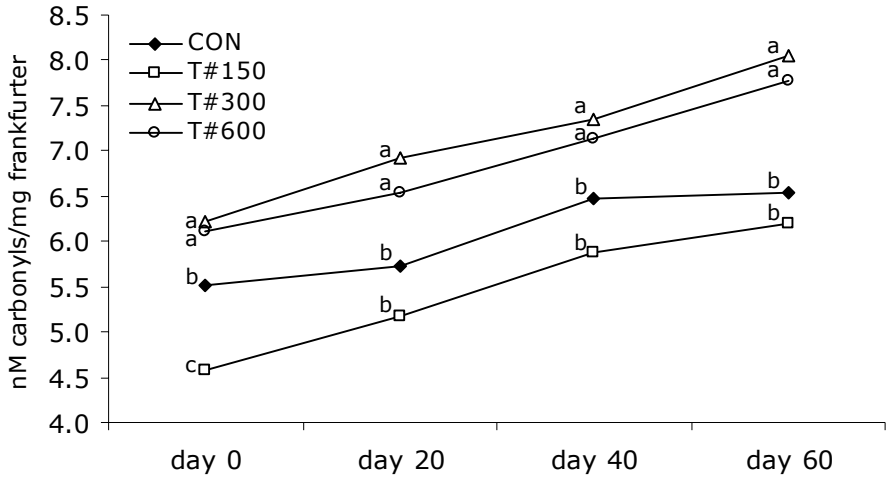


Figure XI.3. Evolution of NHI content during refrigerated storage of WF treated with 150, 300 and 600 ppm rosemary essential oil. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

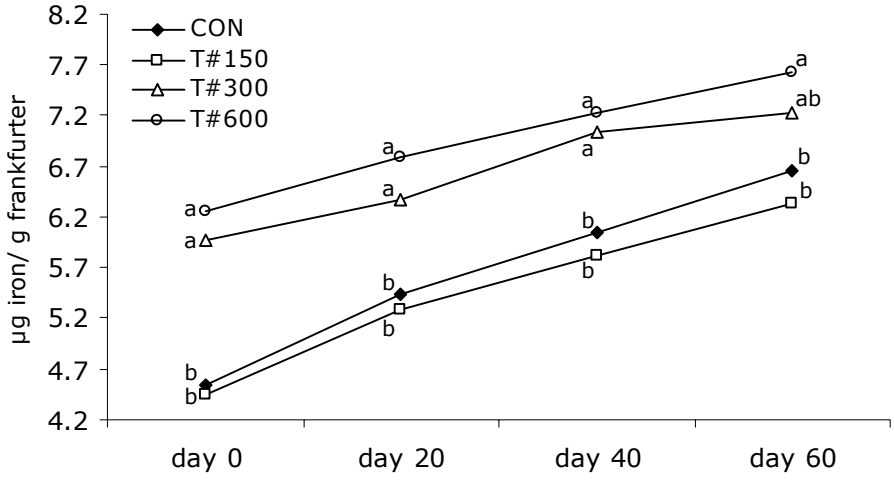


Figure XI.4. TBA-RS numbers in WF (A) and IF (B) treated with 150, 300 and 600 ppm rosemary essential oil. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

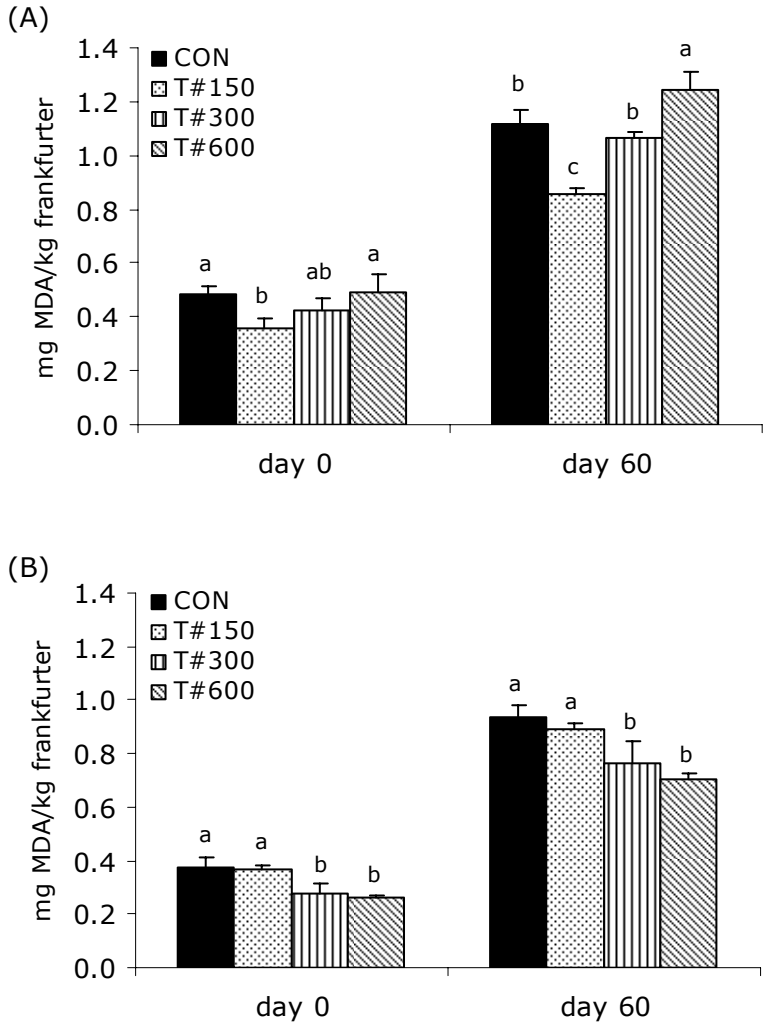


Figure XI.5. Hexanal counts in WF (A) and IF (B) treated with 150, 300 and 600 ppm rosemary essential oil. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

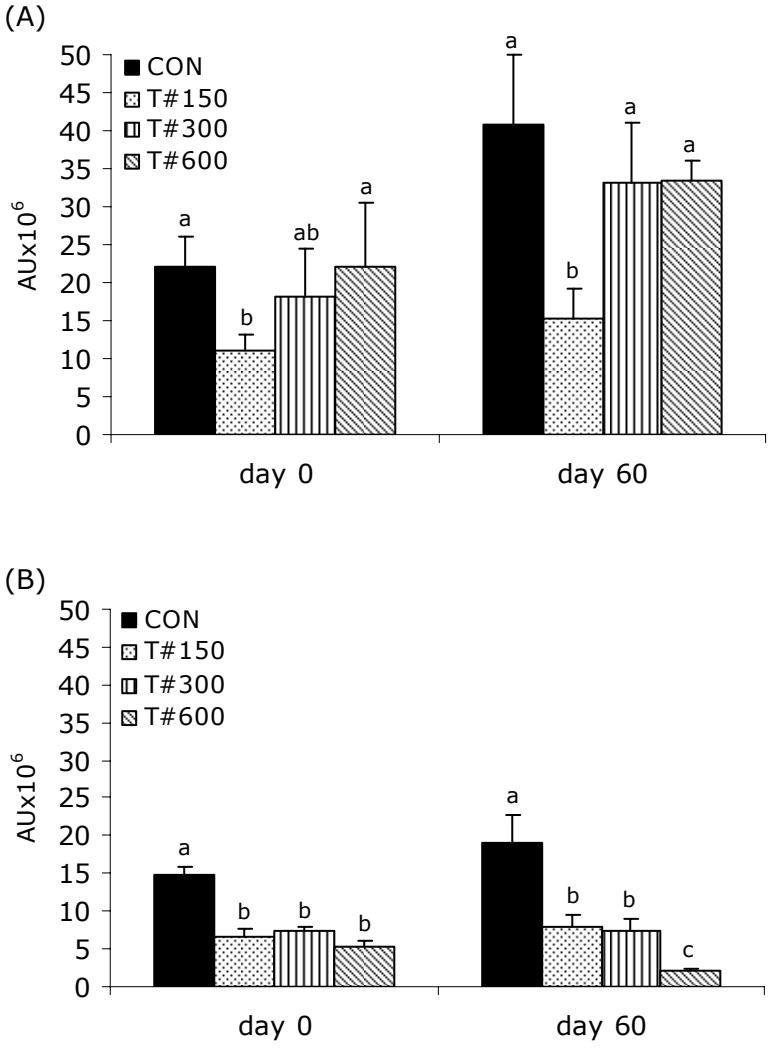


Figure XI.6. Protein carbonyls content in WF (A) and IF (B) (results taken from Estévez *et al.*, in press) treated with 150, 300 and 600 ppm rosemary essential oil. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

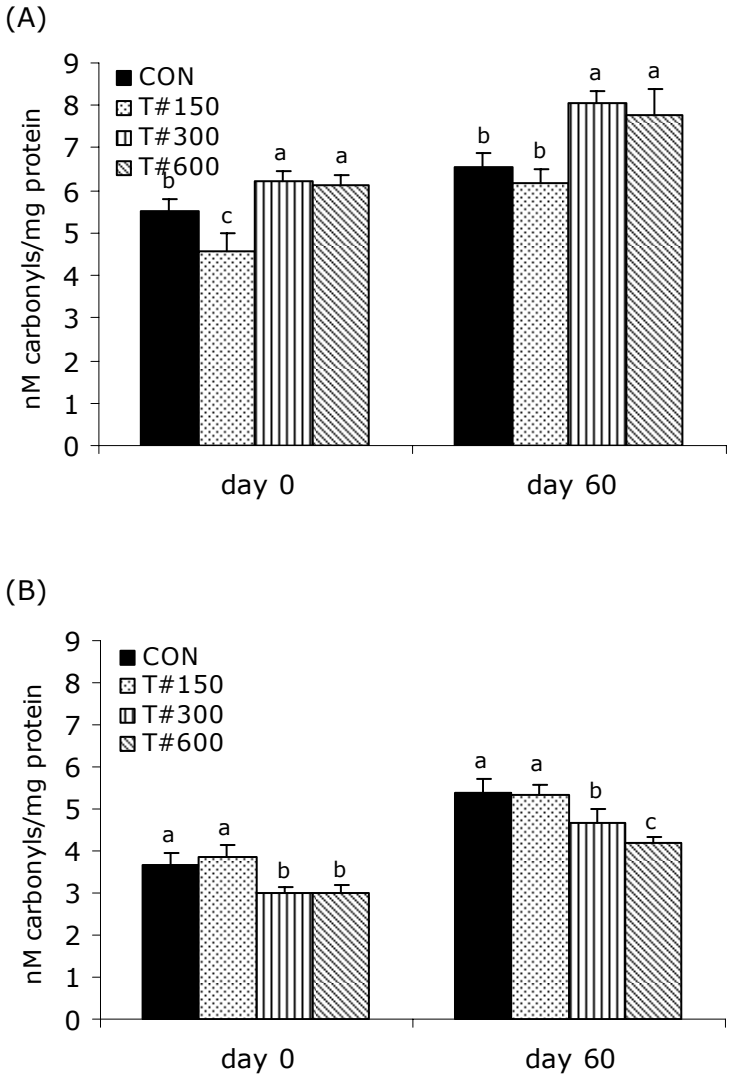
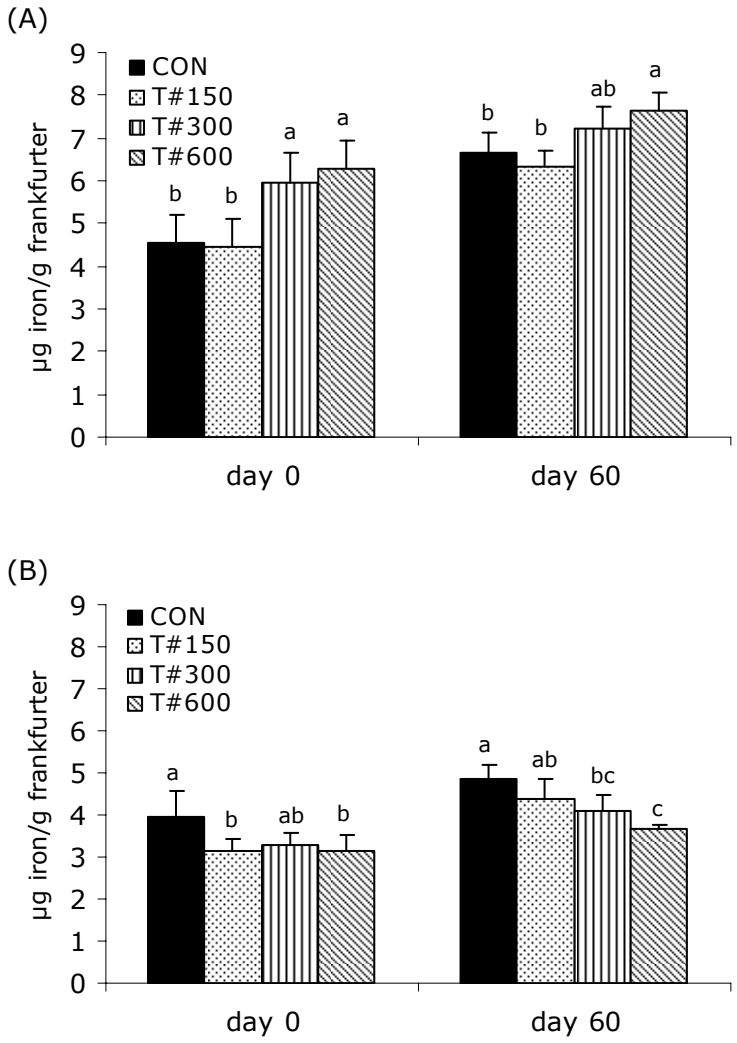


Figure XI.7. NHI content in WF (A) and IF (B) (results taken from Estévez *et al.*, in press) treated with 150, 300 and 600 ppm rosemary essential oil. (Significant differences, $p<0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).



General discusssion

I. EXTENSIVELY REARED IBERIAN PIGS VS INTENSIVELY REARED WHITE PIGS FOR THE MANUFACTURE OF LIVER PÂTÉS AND FRANKFURTERS.

I.A. Proximate composition

The chemical composition of a comminuted meat product is mainly influenced by the chemical composition of the raw materials used for its manufacture and the proportion of each ingredient in the recipe (Hughes *et al.*, 1998; Estévez *et al.*, 2005). The lack of significant differences between pâtés and frankfurters from Iberian and white pigs for their proximate composition was expected, considering that the same recipe and protocol of production was followed for all types of pâtés and frankfurters and that the raw material used for the manufacture of the cooked products from Iberian and white pigs showed similar chemical composition. The composition of the experimental pâtés from the present study is in accordance with that reported by other authors characterising porcine and fish liver pâtés (Perlo *et al.*, 1995; D'Arrigo *et al.*, 2004; Aquerreta *et al.*, 2004). Furthermore, the chemical composition of commercial porcine liver pâtés studied by Echarte *et al.* (2004) is, in general terms, similar to that showed by the liver pâtés studied in the present work. Similarly, the values obtained for the proximate composition in this work are within the range considered as acceptable in frankfurters (Matulis *et al.*, 1995). In fact, the chemical composition of the experimental frankfurters from the present study is similar to that reported by González-Viñas *et al.* (2004) for commercial frankfurters purchased in Spanish supermarkets.

I.B. Iron

Liver pâtés from both, Iberian and white pigs, contained high levels of iron, being those even higher than the iron contents reported by other authors in beef, ostrich meat, and certain fortified foods (Lombardi-Boccia *et al.*, 2005). The high levels of iron in pâtés are derived from the high levels of that metal in the liver (Mataix & Aranceta, 2002). No statistical differences were found between pâtés from Iberian and white pigs in agreement with the results obtained from the livers used for their manufacture. However, significant differences were detected considering the chemical forms of iron individually.

Pâtés from white pigs presented a significantly larger amount of NHI and smaller of HI than those from Iberian pigs. Consequently, liver pâtés from Iberian pigs (IP) presented a better iron profile than those from white pigs (WP) regarding the promotion of oxidative reactions.

In the case of the frankfurters, the meat (50% in the recipe) is the main source of iron for the manufactured product and, therefore, the large differences detected between meats from Iberian and white pigs regarding the iron levels, had a clear reflection in the frankfurters. Frankfurters from Iberian pigs (IF) had a significantly higher amount of iron compared with those from white pigs (WF). Consistently, the 'hybrid' frankfurters (HF), manufactured with meat from white pigs and adipose tissue from Iberian pigs, had similar iron contents than WF and smaller than IF. In agreement with the present results, Estévez *et al*. (2003a) and Forero (2003) reported significantly higher amounts of iron in meat and dry-cured hams from Iberian pigs than in those from industrial genotype pigs, respectively.

The variation in the iron levels is generally associated to different heme pigments concentrations due to the close relationship between both meat components. The high levels of heme pigments and iron in muscles from Iberian pigs is mainly explained by the peculiar genetic characteristics of the non-selected rustic pig breeds. It is known that muscles from lean pig breeds selected for fast growth contain higher content of fast fibres (glycolytic IIB) than muscles from rustic breeds (Swatland & Cassens, 1974; Weiler *et al*., 1995) which affects muscle heme pigments concentration and therefore, iron levels. Accordingly, Ruusunen & Puolanne (2004) and Lindahl *et al*. (2001) reported higher pigment concentrations in muscles from rustic pig breeds or wild pigs than in those from industrial genotype pigs. Some other extrinsic factors associated to the traditional procedures of Iberian pig's rearing system affect myoblogin and iron contents in muscles. In order to obtain heavy and fatty carcasses, Iberian pigs are traditionally slaughtered with 12-14 months of age whereas considerably shorter times are used for industrial genotype pigs (around 5 months). The concentrations of myoglobin and iron in muscles are known to increase with age (Lawrie, 1998). In addition, Pearson (1990)

described a beneficial impact of physical exercise on myoglobin content in muscles through the increase of the proportion of oxidative fibres (Petersen *et al.*, 1997). Dworschák *et al.*, (1995) reported higher amount of iron in muscles from free-range reared pigs than in those from intensively reared pigs. The positive effect of physical exercise on myoglobin and iron contents in muscles from Iberian pigs has been suggested in several papers (Mayoral *et al.*, 1999; Andrés *et al.*, 2000; Estévez *et al.*, 2003a).

I.C. Instrumental colour

Compared to WP, IP presented a darker colour with less redness and yellowness. Thus, WP presented larger values of chrome and smaller of hue that those from IP. The differences found between liver pâtés from Iberian and white pigs concerning their colour characteristics are principally explained by the different colour displayed by the raw material used in their manufacture. No clear differences were observed between groups for the colour exhibited by livers, and the influence of meat colour on pâtés is not expected because of the low proportion of meat in the recipe. Therefore, the colour characteristics of the adipose tissue could have been the main influence on the colour of pâtés. Actually, adipose tissue from white pigs presented higher a*- and b*- values than those from Iberian pigs. On the contrary, adipose tissues from Iberian pigs presented a higher L*-values whereas WP resulted paler than Iberian pigs' ones. Consequently, regardless of the small proportion of muscle in the recipe, the higher L*-values of muscles from white pigs compared to those from Iberian pigs could have increased the lightness in WP. In the absence of a sensory assessment and information concerning the influence of liver pâtés colour traits on consumer's preferences, the impact of the differences reported in the present study on consumers remains unknown. However, the different colour characteristics of IP could identify them from other sort of pâtés available in the market.

Concerning the frankfurters, IF exhibited a more intense, redder and darker colour compared to that from WF. The red colour in the frankfurters is caused by the presence of heme pigments from the meat, which is the main

ingredient. This explains the differences found between types of frankfurters since meat from Iberian pigs presented a higher a* and chroma values than those from white pigs. Though WF and HF presented similar colour characteristics, the latter were paler as a likely result of the colour traits of the raw material used for their manufacture. Meat from white pigs was paler than that from Iberian pigs and adipose tissues from Iberian pigs had also higher L*-values than those from white pigs.

The high concentrations of myoglobin in muscles from Iberian pigs have a direct impact on their colour traits due to the relationship established between those parameters (Warris *et al*., 1990; Cava *et al*., 2003). Therefore, the discussion described above regarding the influence of intrinsic (genetic) and extrinsic (environmental) factors affecting the concentration of iron and heme pigments in muscles from Iberian pigs would also be appropriate to explain the large differences detected regarding the colour characteristics of meat and frankfurters from Iberian and white pigs. The colour standards displayed by meat and meat products from Iberian pigs are preferred by consumers who appreciate intense red colours in fresh pig meat and frankfurters (Brewer *et al*., 1998, González-Viñas *et al*., 2004).

I.D. Fatty acid composition

Fatty acid composition of pâtés and frankfurters reflected the fatty acid composition of the raw materials used for their manufacture and, therefore, the large differences perceived between cooked products from Iberian and white pigs are consistent with the differences found in relation to their tissues (livers, muscles and adipose tissues). Particularly, fatty acid composition of pâtés mainly reflected the fatty acid composition of the adipose tissue as long as the proportion of that material in the recipe was the highest of all ingredients (40%). On the other hand, the fatty acid composition of the frankfurters was intermediate between those from the meat and the adipose tissue. Even though the proportion of adipose tissue in the frankfurters' recipe was relatively small (only 10%), the composition of that tissue was largely influence on the fatty acid composition of the manufactured product. This is

explained by the fact that 10% added adipose tissue containing between 75 and 83 g fat/ 100g, provides almost the same amount of total fat than 50% added meat which contained between 18 and 20 g fat/100 g.

Four fatty acids namely palmitic, stearic, oleic and linoleic acids comprised more than 90% of total fatty acids analysed in liver pâtés and frankfurters. WP presented higher proportions of SFA than IP. On the other hand, IP showed higher percentages of oleic and total MUFA than WP. The latter presented higher percentages of PUFA mainly due to the higher proportion of linoleic acid.

The differences between frankfurters from Iberian and white pigs concerning their fatty acid composition agreed with the differences previously reported on liver pâtés. IF had significantly smaller proportions of SFA than WF. The differences for MUFA are particularly remarkable since IF showed 10 percent of oleic acid higher than WF. The latter had higher percentages of PUFA. Replacing 10% adipose tissue from white pigs with adipose tissue from Iberian pigs in the HF significantly influenced their fatty acid profile, significantly reducing the proportion of SFA and PUFA and increasing the percentages of oleic acid and MUFA compared to WF.

The fatty acid composition of the animal tissues can be generally attributed to the compositional characteristics of the feeds given to the animals (Miller *et al.*, 1990; Enser *et al.*, 2000). Consistently, raw material from Iberian pigs reflected the fatty acid composition of acorns (with high levels of oleic acid). Contrarily, tissues from white pigs reflected the general composition of the mixed diet, with relative high proportion of linoleic acid and PUFA. According to previous works, these results represent the general pattern of fatty acid composition of different tissues from Iberian pigs fed extensively with natural resources (Cava *et al.*, 1997; Ruiz *et al.*, 1998; Timón *et al.*, 2001) and white pigs fattened intensively with mixed diets (Flachowsky *et al.*, 1997; Serra *et al.*, 1998).

Large proportions of oleic acid are considered one of the main characteristics of Iberian pigs' tissues and products including meat, livers, adipose tissues (Cava *et al.*, 1997; Ruiz *et al.*, 1998), dry-cured Iberian loins (Muriel *et al.*,

2004a) and dry-cured Iberian hams (Timón *et al*., 2001) and related to some of their high quality traits.

Focusing on nutritional and technological aspects, using raw material from extensively reared Iberian pigs for the production of liver pâtés and frankfurters led to cooked products with high-quality lipid characteristics. Contrarily to MUFA, PUFA are very prone to oxidation, leading to the generation of unpleasant odours and reducing nutritional value of meat and fat products (Morrissey *et al*., 1998). Thus, compared to SFA, MUFA are hypocholesterolemic, but, unlike PUFA, they do not decrease high-density lipoproteins (HDL) cholesterol which protects against coronary heart diseases (CHD) (Mattson & Grundy, 1985). The nutritional ratios between SFA hypercholesterolemic fatty acids (C12, C14, C16) and the unsaturated hypocholesterolemic ones (C18:1 n-9; C18:2 n-6) were significantly smaller in pâtés and frankfurters from Iberian pigs than in those from white pigs. Large importance has been given to long chain PUFA in meat products because of the role played by the ratio n-6/n-3 in the development of CHD (Okuyama & Ikemoto, 1999). The ratios n-6/n-3 were lower in Iberian pigs' products than in those from white pigs as a result of the higher content of C18:2 (n-6) in the latter. Using 10% adipose tissue from Iberian pigs for the manufacture of frankfurters significantly improved the fatty acid profile of frankfurters since HF presented better nutritional and n-6/n-3 ratios than those from WF. The content of long chain PUFA in tissues of pigs reared outdoors with access to pasture are thought to increase because of the intake of grass with high content of n-3 PUFA (Nilzén *et al*., 2001) though it was not generally detected in the present work. While in northern Europe the source of these essential fatty acids are mainly found in meat products (Enser *et al*., 1995), in Mediterranean countries, such as Spain, the high consumption of fish and fishery products make of them the main source of n-3 fatty acids. In spite of that, D'arrigo *et al*. (2004) have recently developed pâtés with increasing amounts of n-3 fatty acids and tocopherols in order to improve n-6/n-3 ratios without producing an arise of oxidative deterioration and off-flavors. Using natural sources of n-3 fatty acids (fishery by-products),

Aquerreta *et al*. (2002) have found better n-6/n-3 ratios in pâtés elaborated with mackerel fish and tuna liver, than those obtained by the aforementioned researchers. Nevertheless, the recommendations given by the World Health Organization (WHO, 2003), concerning the intake of n-3 and n-6 fatty acids (n-3/n-6 ratio value should be around 4) are referred to the whole diet and therefore, the reasonable way to fulfill this advice is to include the appropriate proportion of different foods in a well-balanced diet. Hence, the scientific efforts done to increase the levels of n-3 fatty acids in meats and meat products avoiding the unpleasant effect of lipid oxidation seem to be a helpless strategy from a practical point of view since the intake of foods with naturally high levels of n-3 fatty acids (i.e. fish and fish by-products) could be the easiest and also more recommendable way to increase the intake of those fatty acids and approach the nutritional recommendations.

I.E. Antioxidants

The raw material used for the manufacture of liver pâtés and frankfurters were analysed for the amount of certain substances with antioxidant activity such as tocopherols and phenolic compounds.

The raw material (meat, livers and adipose tissues) used for the manufacture of IP contained significantly higher amounts of α-tocopherol than those from white pigs.

In accordance, meat used for the production of IF contained higher amounts of α- and γ-tocopherol than those used for the production of WF. Consistently, higher amounts of α- and γ-tocopherol were detected in the adipose tissues used for the production of IF than those used for the production of WF.

The high content of tocopherols in tissues and meat products from free-range reared Iberian pigs has been profusely described in previous works, and considered as one of the most appreciated quality traits (Cava *et al*., 2000; Daza *et al*., 2005) as long as tocopherols enhance the oxidation stability of the meats and meat products, improving their nutritional and technological properties (Morrissey *et al*., 1998; Rey *et al*., 1998). The α- and γ-tocopherol contents in animal tissues reflect the tocopherol concentration of the diets

(Daza *et al*., 2005), and therefore, the high levels of tocopherols in the grass and acorns with which Iberian pigs were fed explain the high levels of such substances in their tissues. According to Cava *et al*., (2000) and Daza *et al*., (2005) meat from Iberian pigs fed on natural resources (grass and acorns) contained similar or even higher tocopherol levels than those fed with diets supplemented with α-tocopherol up to 200 mg/Kg. The traditional procedures used for Iberian pig's feeding are in absolute concordance with the current strategies carried out in order to enhance the oxidative stability of pig muscles. Moreover, Iberian pigs reared in confinement are usually fed using MUFA-enriched diets with α-tocopherol supplementation up to 200 mg/Kg in order to imitate the traditional free range feeding system. Furthermore, acorns have been shown to be important sources of γ-tocopherol for extensively reared pigs and the presence of such tocopherol isomer in pig muscles is almost restricted in tissues from pigs fed with that fruit (Rey *et al*., 1998; Daza *et al*., 2005) which is in agreement with results obtained in the present work.

Recent studies have considered the possibility that some other substances accumulated in tissues from Iberian pigs as a consequence of the intake of natural resources could also contribute to enhance their oxidative stability. González *et al*. (2004) have recently reported significantly higher amounts of total phenolic compounds in adipose tissue from Iberian pigs fed exclusively on grass and acorns than in those from pigs fed on a mixed diet. Accordingly, in the present study, meat and adipose tissues from Iberian pigs had significantly higher amounts of total phenolics than those from white pigs. The intake of grass and acorns by Iberian pigs could explain the higher amount of phenolic compounds in their tissues and elaborated frankfurters than in those from white pigs. In fact, Cantos *et al*. (2003) have recently reported elevated polyphenol levels in acorns which could explain the results obtained in the present study and those from González *et al* (2004). The information concerning the occurrence of phenolic compounds in animal tissues is extremely scarce since such compounds are widespread in plant kingdom and therefore, their presence in animal tissues is principally relegated to the

intake of plant materials and the subsequent accumulation in animal tissues. Amongst phenolic compounds, some particular polyphenols derived from plants, are substances with proven antioxidant activity and the presence of such compounds in the animal tissues could protect them and their transformed products from oxidative deterioration.

I.F. Oxidative stability of lipids

Regardless of microbial spoilage, lipid oxidation is the main factor reducing quality of meat and meat products (Morrissey *et al*., 1998).

As far as the Iberian pig's products is concerned, the oxidation of lipids has been evidenced during refrigerated storage of raw and cooked meat (Estévez *et al*., 2003b; Morcuende *et al*., 2003; Estévez *et al*., 2004) and ripening of dry-cured loins and hams (Cava *et al*., 1999; Ruiz *et al*., 1999; Andrés *et al*., 2004; Muriel *et al*., 2004b).

In the present study, the oxidative degradation of lipids during refrigerated storage of liver pâtés and frankfurters from Iberian and white pigs was assessed by quantifying the loss of PUFA and the generation of lipid-derived products such as TBA-RS and hexanal. The data obtained suggested that, regardless of the addition of substances with proven antioxidant activity such as nitrites and phosphates, considerably intense lipid oxidation processes affected liver pâtés and frankfurters during refrigeration. The intensity of the oxidative deterioration measured on refrigerated stored liver pâtés and frankfurters could have caused noticeable sensory changes. Based on findings by Gray & Pearson (1987), rancid flavor is initially detected in meat products with TBA-RS values between 0.5 and 2.0. Furthermore, Boles & Parrish (1990) reported that a warmed-over flavor (WOF) could be perceived in meat products at TBA-RS values above 1.0. On the other hand, the hexanal is mainly generated as a consequence of the oxidative decomposition of PUFA and has been related to rancid odors and used as an indicator of lipid oxidation (Shahidi & Pegg, 1993). Data revealed that liver pâtés suffered a more intense oxidative deterioration than frankfurters. Regardless of the origin of the product or the addition of essential oils, liver pâtés showed

considerably higher TBA-RS values at day 60 than frankfurters. This could be explained by the fact that liver pâtés contained much higher amounts of total lipids than frankfurters and therefore, higher amounts of substrate to undergo oxidative reactions. In addition, compared to frankfurters, liver pâtés contained considerably higher amounts of iron and particularly of non-heme iron which is considered one of the most important promoters of oxidative reactions in muscle foods (Kanner *et al*., 1994).

The decrease rate of PUFA contents as measured by the slopes of the calculated trend lines revealed that the loss of PUFA was more intense in WP than in the Iberian counterparts. After 90 days of refrigerated storage, WP lose 2.3 g PUFA/100g pâté as an average while those from Iberian pigs lose a significantly ($p<0.05$) smaller amount (1.5 g PUFA/100g pâté). The oxidative degradation of PUFA led to a gradually increase in the amount of TBA-RS and hexanal between day 0 and day 90 for liver pâtés from Iberian and white pigs. WP presented significantly ($p<0.05$) higher TBA-RS numbers and hexanal contents than IP at day 0 and day 90 which is in agreement with the results from the PUFA degradation.

The comparison of the lipid oxidative stability between frankfurters from Iberian and white pigs revealed similar results to those from the liver pâtés. Throughout the entire storage period, TBA-RS numbers in WF were significantly higher than in the IF. The chromatographic areas for hexanal were also significantly higher in WF than in the IF at days 0 and 60 of storage. The differences concerning the oxidative stability between cooked products from Iberian and white pigs agree with those obtained in previous works devoted to the study of the oxidation stability of raw and cooked meats from Iberian and white pigs (Estévez *et al*., 2003b; Estévez *et al*., 2004) and could be explained considering the balance between pro- and antioxidant factors. Particularly, the different fatty acid composition between products from Iberian and white pigs and the presence of certain amounts of substances with proven antioxidant activity plausibly affected on the results. Cooked products from white pigs presented higher proportions of PUFA which are more likely to be oxidised than MUFA or SFA. High levels PUFA in frankfurters

and other types of cooked meat products have been previously associated with high oxidative instability during storage (Bloukas & Paneras; 1993; Jeun-Horng *et al*., 2002). Moreover, significantly higher amounts of tocopherols were detected in the raw material from Iberian pigs used for the manufacture of pâtés and frankfurters when compared to those from white pigs. Relatively high tocopherols levels persisted in the cooked product after handling and manufacture process which allows the protection against lipid oxidation beyond cooking. Frankfurters from Iberian pigs contained significantly higher amounts of tocopherols than those from white pigs.

The relationship between the nutritional background (pasture- and mixed diet finishing) and the fatty acid profile and oxidative stability of liver, pork and their products is widely documented (Cava *et al*., 2000; López-Bote & Rey, 2001; Nilzén *et al*., 2001). The intake of pasture by animals increases the level of tocopherols in their tissues, enhancing their oxidative stability (Cava *et al.*, 2000; Nilzén *et al.*, 2001). On the other hand, pasture-grazing has been considered to increase the levels of *n-3* PUFA in meats as a result of the intake of grass (Nilzén *et al.*, 2001; Mercier *et al.*, 2004) and this could be regarded as a prooxidant factor. However, this general pattern was no detected in this study. The present results suggest that feeding on natural resources enhances the oxidative stability of liver pâtés and frankfurters as a result of the presence of high levels of tocopherols and MUFA in the tissues used for their manufacture. In agreement with these results, previous works have reported that meat from Iberian pigs fed on acorns and pasture shows a lower susceptibility to lipid oxidation than meat from pigs fed on non-supplemented mixed diets (Andrés *et al*., 2001) and similar oxidative stability than those supplemented with 200 mg/Kg α-tocopherol (Cava *et al*., 2000; Daza *et al*., 2005). The protective role of tocopherols against lipid oxidation has been reported also in other meat products such as dry-cured Iberian ham, significantly reducing the generation of lipid oxidation products during ripening and improving some particular sensory characteristics such as flavour and odour intensity (Cava *et al*., 1999).

In addition, the present results suggest that the high oxidative stability attributed to Iberian pigs' products could not only be explained by the presence of tocopherols in the tissues and products but also by the likely antioxidant activity of phenolic compounds. Amongst phenolic compounds, some particular polyphenols derived from plants, are substances with proven antioxidant activity and the presence of such compounds in the animal tissues could protect them and their transformed products from oxidative deterioration. Tissues from Iberian pigs contained significantly higher amounts of phenolic compounds than those from white pigs, and as well as for tocopherols, they also persisted in the frankfurters after cooking since IF had significantly higher amounts of phenolic compounds than WF and HF. The information concerning the occurrence of phenolic compounds in animal tissues and meat products is extremely scarce since such compounds are widespread in plant kingdom and as reported above; their presence in animal tissues is principally relegated to the intake of plant materials and the subsequent accumulation in animal tissues. The intake of grass and acorns by Iberian pigs could explain the higher amount of phenolic compounds in their tissues and elaborated frankfurters than in those from white pigs, which is in agreement with recently published data (González *et al.*, 2004; Cantos *et al.*, 2005). Therefore, if these data were supported by further results, the presence of plant phenolics in porcine tissues as a consequence of the intake of grass and other natural materials would have been described for the first time in Iberian pigs. Nevertheless, additional studies should be carried out to i) measure the proportion of plant polyphenols supposed to be contained in the heterogeneous group of phenolics compounds detected in the tissues from Iberian pigs, ii) describe the polyphenol profiles which could contribute to elucidate if those compounds are certainly accumulated in animal tissues as a result of the intake of natural resources and iii) evaluate if these compounds can work as inhibitors of oxidative reactions in meat and meat products.

Results from the present work show that including 10% adipose tissue from Iberian pigs in frankfurters elaborated with meat from white pigs (HF) improves the lipid oxidative stability of the product. Throughout the whole

refrigerated storage, the TBA-RS numbers and hexanal counts were significantly smaller in HF than in WF. This improvement was achieved by the modification of the fatty acid composition of the frankfurters, significantly increasing MUFA contents and reducing PUFA content. In fact, HF showed an intermediate fatty acid profile between IF and WF. In addition, the adipose tissue from Iberian pigs was a source of tocopherols and phenolics for HF, which likely enhanced their oxidative stability. Surprisingly, HF had even smaller TBA-RS values than IF. This could be due to the different iron content between types of frankfurters. Iron is considered a potent oxidation promoter (Kanner *et al*., 1991) and high levels of that metal in meat from Iberian pigs could increase its oxidative instability. The manufacture of frankfurters with meat from white pigs and adipose tissue from Iberian pigs lead to a product with improved fatty acid composition and high levels of antioxidants without increasing the iron levels which explains its high lipid oxidative stability.

I.G. Oxidative stability of proteins

In the present work, the protein oxidation of liver pâtés and frankfurters was assessed by quantifying carbonyls generated as a consequence of the oxidative deterioration of proteins using the DNPH coupling method described by Oliver *et al*. (1987). The oxidation of proteins in foods has been poorly studied and, therefore, the results from the present work are useful to understand the occurrence of protein oxidation in meat products and to shed light of the effects of that on muscle food quality. The results obtained show that proteins from frankfurters and liver pâtés suffered oxidative deterioration during cooking and subsequent refrigeration. Compared to pâtés and frankfurters from Iberian pigs, cooked products from white pigs presented a higher amount of carbonyls at all days of study. These results agree with those obtained for lipid oxidation suggesting the possible relationship between lipid and protein oxidation. In fact, a statistically significant correlations were found between protein oxidation and TBA-RS and between protein oxidation and hexanal with those correlation coefficients being higher that those between TBA-RS and hexanal contents. Other authors (Mercier *et al*., 1995;

Viljanen *et al*., 2004a, b) have reported similar results in different food systems. The casualty relationship between lipid and protein oxidation is probable since primary and secondary lipid oxidation products can interact with proteins leading to protein radicals (Gardner, 1979). In this sense, the possible protective effect of tocopherols in cooked products from Iberian pigs against lipid oxidation and the large differences in the fatty acid composition between cooked products from Iberian and white pigs could explain the higher oxidative instability of proteins in the latter. Producing frankfurters with 10% adipose tissue from Iberian pigs improved the oxidative stability of their proteins since HF contained significantly smaller amounts of protein carbonyls than WF. The modification of the fatty acid profile of the frankfurters and the incorporation of antioxidants likely influenced the present results.

Closely associated to the development of oxidative reactions in meats and cooked products, the breakdown of the heme molecule and the subsequent release of iron from the porphyrin ring have been reported to occur as a consequence of the high temperatures reached during cooking (Schricker *et al*., 1982; Lombardi-Boccia *et al*., 2002). Moreover, Miller *et al*., (1994) established relationships between a gradual increase of NHI and the development of oxidative deterioration during refrigerated storage of meat and meat products. In the present study, the NHI content progressively increased in frankfurters and liver pâtés during refrigerated storage. WP contained significantly higher amounts of NHI than IP at all days of analysis. Though no differences were found between types of frankfurters at day 0 for the NHI content, at days 20-60 significantly higher amounts of NHI were detected in WF than in IF suggesting that the release of iron was more intense in the former.

Though the precise causes of the heme degradation have not been elucidated, the release of iron from heme molecule has been related to oxidative reactions. In fact, Miller *et al*. (1994) described the use of the NHI quantification during refrigerated storage of meats as a sensitive, reliable and consistent analysis for the evaluation of lipid oxidative changes. Accordingly, the aforementioned authors and ourselves have reported significant

correlations between NHI content and TBA-RS and between hexanal counts and NHI. In addition, a significant correlation was found between the carbonyl content derived from protein oxidation and the NHI in liver pâtés and frankfurters. Though relatively small, this correlation reasonably suggests that the oxidative deterioration of some particular proteins such as the myoglobin could promote the degradation of the heme group and the subsequent release of iron. Consistently, the liver pâtés and frankfurters from white pigs contained higher amounts of protein carbonyls and also higher increases of NHI during refrigeration. From a nutritional point of view, HI has a higher bioavailability than NHI and represents the primary source of iron in human's diet (Carpenter & Mahoney, 1992). Consequently, the degradation of heme iron would reduce the nutritional value of the frankfurters and liver pâtés in terms of iron bioavailability. IF contained, during the entire storage period, a significantly higher amount of HI than WF and HF which represents an important nutritional benefit. This difference is mainly explained by the higher amount of total iron in IF since the amount of NHI was similar among types of frankfurters. On the other hand, iron is considered as one of the most important oxidation promoters in meat systems (Kanner, 1994). The forms of NHI including ferritin, lactoferrin, cytosolic iron-dependant enzymes and low molecular weight (LMW) chelatable iron ions enhance lipid peroxidation in meat to a higher extent than HI (Kanner, 1994). Consequently, the increase of NHI content as a result of the release of the iron from the heme group would increase the oxidative instability of the cooked products promoting the formation of further TBA-RS, hexanal and carbonyls from proteins.

I.H. Volatile compounds profile

The study of volatiles in meat and meat products has reached high importance because of the interesting diversity of information given by this type of analysis. For instance, the deterioration of meat and meat products during storage or manipulation can be also evaluated analyzing volatiles generated as a result of enzymatic, microbial or biochemical alteration phenomena including lipid oxidation (Morrissey *et al.*, 1998). The results

obtained for the analysis of lipid-derived volatiles in cooked products agree with those above discussed regarding PUFA degradation and TBA-RS numbers. WP showed, compared to those from Iberian pigs, a higher number of lipid-derived volatiles since pentan-2-one, but-3-en-2-one, pentanal, hepta-(*E,E*)-2,4-dienal, hexa-2,4-dienal, deca-(*E,Z*)-2,4-dienal and deca-(*E,E*)-2,4-dienal were not detected in the HS of IP. Furthermore, WP presented significantly higher chromatographic areas of certain compounds closely related to lipid oxidation and off-flavors such as heptan-1-ol, oct-3-en-1-ol, octan-1-ol, hex-(*E*)-2-en-1-ol, heptanal, buten-2-enal, octanal, nonanal, oct-(*E*)-2-enal, nona-2,4-dienal, non-(*Z*)-2-enal and dec-(*E*)-2-enal. Differences between types of pâté are remarkably high on hexanal (white: 21.77 AU, Iberian: 2.66 AU; $p<0.05$) that has been widely used on meat products as an indicator of lipid oxidation (Shahidi & Pegg, 1993). Results obtained regarding frankfurters were in absolute agreement with the aforementioned: frankfurters from white pigs had, compared to those from Iberian pigs, a higher number of lipid-derived volatiles since hexanoic and heptanoic acids, hex-2-enal, dec-(*E*)-2-enal, 2-methylbut-(*E*)-2-enal, 2,5-dihydrofuran, hexane-2,4-dione and octan-2-one were not detected in the HS of IF. Furthermore, WF showed significantly ($p<0.05$) higher chromatographic areas of certain compounds closely related to lipid oxidation and off-flavors such as octanoic and nonanoic acids, pentanal and heptan-2-one. Differences between types of frankfurters were also significant on hexanal (white: 22.1 AU, Iberian: 14.9 AU; $p<0.05$). The significantly higher amount of iron in IF compared to that in WF, could have played a prooxidant role since that metal is considered the most potent oxidation promoter in muscle foods (Kanner *et al.*, 1991). The present results and those from previous studies suggest that other circumstances should be considered to fully comprehend the considerably high oxidative stability of meats from Iberian pigs. As discussed above when the oxidative stabilities of lipids and proteins from cooked products from Iberian and white pigs were compared, a higher proportion of MUFA and lower of PUFA (more prone to be oxidised) and the presence of significantly higher amounts of tocopherols in the tissues and elaborated

products from Iberian pigs, compared to those from white pigs, could partly explain the results obtained for the lipid-derived volatiles.

On the other hand, the large differences between types of cooked products from Iberian and white pigs in terms of fatty acid composition could affect their aromatic characteristics as long as the pathways for the generation of volatile compounds from lipid oxidation are fairly specific for each fatty acid. Oleic acid-derived volatiles are associated to pleasant notes, described as 'floral' and 'sweet' (Specht & Baltes, 1994), while the aromatic notes of linoleic and PUFA-derived volatiles have been described as intense 'grass-like' and related to rancidity in cooked meat and other food systems (Morrissey *et al*., 1998; Im *et al*., 2004). Consistently with results from fatty acid profiles, the ratio between oleic-derived volatiles (octanal, nonanal and octan-1-ol) and linoleic-derived volatiles (hexanal, oct-(*E*)-2-enal, and non-(*Z*)-2-enal) resulted significantly higher in IP (Iberian: 1.41, white: 0.50; $p<0.05$) suggesting a more pleasant aromatic profile in the latter. Similar results were obtained in frankfurters from Iberian and white pigs when the ratio between oleic-derived volatiles (octanal, nonanal and octan-1-ol) and linoleic-derived volatiles (hexanal, hex-2-enal and dec-(*E*)-2-enal) was calculated (Iberian: 3.28, white: 2.12; $p<0.05$). The high content of oleic acid and its oxidation-derived aldehydes in meat products from Iberian pigs has been related to essential quality traits (Ruiz *et al*., 1999; Cava *et al*., 2000).

In addition, significantly higher amounts of Strecker aldehydes (2- and 3-methylbutanal, benzaldehyde) and alcohols (2-methyl-propan-1-ol, 2-methyl-butan-1-ol, 3-methyl-butan-1-ol) were detected in IF compared to those in WF which could contribute also to define different aromatic profiles between types of frankfurters. Strecker volatiles have been described as quality indicators in Iberian dry-cured products in which they contribute with desirable 'almond-like', 'toasted' aroma notes (Ruiz *et al*., 1999; Carrapiso *et al*., 2002). Finally, IF contained also significantly higher amounts of certain aliphatic and aromatic hydrocarbons (heptane, 2-methylnonane, undecane, methylbenzene, 1,3-dimethylbenzene and 1-methyl-3(1-methylethyl)benzene) and volatile terpenes (α-pinene, I-limonene and

linalool). These compounds are likely to have been derived from the direct deposition in animal tissues from grass which would explain the significantly higher amounts in IF.

II. EFFECT OF THE ADDITION OF PLANT ESSENTIAL OILS ON THE OXIDATIVE STABILITY OF LIVER PÂTÉS AND FRANKFURTERS.

II.A. Essential oils as inhibitors of oxidative reactions

II.A.1. Antioxidant effect on lipids

Results obtained in the present work concerning the degradation of polyunsaturated fatty acids and the generation of lipid-oxidation products during the refrigerated storage of liver pâtés and frankfurters from Iberian pigs confirm data previously reported on different types of meat products. The addition of sage and rosemary essential oil (1000 ppm) is a successful strategy to inhibit the development of oxidative reactions in IP during refrigeration. Furthermore, the effect of plant essential oils was comparable to that shown by a synthetic antioxidant (BHT, 200 ppm). The decrease rate of PUFA content during refrigerated storage of liver pâtés as measured by the slopes of the calculated trend lines revealed that the rate of PUFA loss was higher in 'control' and 'BHT' pâtés than in those with added plant extracts. In liver pâtés, the oxidative degradation of PUFA caused a gradual increase of TBA-RS. Treated pâtés had significantly smaller TBA-RS numbers than control counterparts at days 60 and 90. Compared to those with added sage and rosemary essential oils, 'BHT' pâtés had significantly higher TBA-RS numbers at days 60 and 90. The data obtained from the analysis of lipid-derived volatiles was consistent with those previously reported. The addition of sage and rosemary oils in IP led to pâtés with smaller amounts of hexanal, nonanal and other lipid-derived volatiles in their HS than in that from the 'control' counterparts. Thus, the addition of plant essential oils inhibited the generation of lipid-derived volatiles during refrigeration of liver pâtés: at day 90, 'control' pâtés had a significantly higher amount of total lipid-derived volatiles such as hexanal, hexan-1-ol, oct-1-en-3-ol, 2-pentyl-furan, nonanal, but-2-enal, nona-(*E,E*)-2,4-dienal, non-(*E*)-2-enal, dec-(*E*)-2-enal, and deca-(*E,Z*)-2,4-dienal than those with added essential oils while 'BHT' pâtés presented an intermediate content.

The development of oxidative reactions during refrigerated storage of liver pâtés could have worsen the aroma characteristics of liver pâtés since most volatiles generated during refrigerated storage are closely related to WOF and rancid aromatic notes (Frankel, 1984; Im *et al*., 2004). In this sense, the addition of antioxidants might have reduced flavour deterioration through the inhibition of some lipid-derived volatiles generation. Furthermore, the addition of plant extracts greatly influences on the aromatic profile of the products in which they are added due to the presence of terpenes in rosemary and sage essential oils which might contribute to specific aromatic notes (Ibáñez *et al*., 1999; Chevance & Farmer, 1999). The addition of BHT, however, was not so efficient than the addition of sage and rosemary essential oils since compared to pâtés with added essential oils, 'BHT' pâtés presented significantly higher amounts of several volatiles such as but-2-enal, hept-(*Z*)-4-enal and nona-(*E,E*)-2,4-enal closely related to lipid oxidation and off-flavors in liver products (Im *et al*., 2004).

The analysis of the effect of the addition of rosemary essential oil on IF showed that the protective role of plant essential oils against lipid oxidation was dose-dependent. The addition of 150 ppm rosemary essential oil had no effect on TBA-RS numbers whereas 300 and 600 ppm significantly reduced the generation of TBA-RS. Consistently, the inhibitory effect of rosemary essential oil against the generation of hexanal was more intense at higher levels of essential oil, with the highest antioxidant effect detected at 600 ppm. The results obtained concerning other lipid-derived volatiles analysed with SPME-GC-MS were in agreement. Though the addition of 150 ppm of essential oil significantly inhibited the generation of certain lipid-derived volatiles such as octanoic and nonanoic acids, pentan-2-ol, octan-1-ol and pent-4-enal, higher antioxidant effects were achieved with higher rosemary levels with the highest antioxidant effect being detected at 600 ppm. Compared to the control ones, frankfurters with 600 ppm of rosemary essential oil had significantly smaller amounts of octanoic and nonanoic acids, pentan-2-ol, oct-1-en-3-ol, octan-1-ol, hexanal, pent-4-enal, but-(*E*)-2-enal, heptanal, octanal and decanal.

The protective role exhibited by sage and rosemary essential oil against lipid oxidation in cooked products from Iberian pigs agree with results from other studies in which the antioxidant effects of plant phenolics on several types of muscle foods were reported (Chen *et al.*, 1999; McCarthy *et al.*, 2001; Formanek *et al.*, 2001; Yu *et al.*, 2002; Ahn *et al.*, 2002). Sebranek *et al.*, (2004) reported similar antioxidant activities of rosemary extracts (2500 ppm) and synthetic antioxidants such as BHT (200 ppm) regarding MDA generation in refrigerated sausages which is also in agreement with the present results.

II.A.2. Antioxidant effect on proteins

In the present work, the addition of plant essential oils resulted in a successful strategy to inhibit the generation of protein carbonyls during refrigerated storage of liver pâtés and frankfurters from Iberian pigs. At day 90 of refrigerated storage, 'control' pâtés had significantly higher amounts of carbonyls than treated pâtés. In this case, however, BHT was more effective than plant essential oils: the percent inhibition against protein oxidation was larger in pâtés with added BHT (75.18%) than in those with added sage and rosemary essential oils (59.66% and 51.28%, respectively). So far, the information available concerning the efficiency of antioxidant strategies against the oxidation of proteins from muscle foods and from other systems is scarce. Mercier *et al.* (1998) reported decreased protein carbonyls formation in muscle from turkey fed vitamin E-enriched diets whereas Viljanen *et al.* (2004b) showed the protective role of added berry phenolics against protein oxidation in liposomes. Since both lipid and protein oxidation are linked in their mechanisms and pathways (Gardner, 1979; Mercier *et al.*, 1995; Viljanen *et al.*, 2004a), it is plausible that they are affected by similar pro-and antioxidant factors.

The addition of antioxidants also affected the increase of NHI during refrigerated storage of IP. In agreement with results from the oxidation of proteins, the increase of NHI during refrigerated storage was significantly larger in 'control' pâtés when compared to those with added antioxidants.

Pâtés with added antioxidants had significantly smaller amounts of NHI at days 60 and 90. These results could be partly explained by a likely protective effect of antioxidants on the heme molecule through the inhibition of protein oxidation, reducing the release of iron. In consequence, the effect of the antioxidants on the release of iron would affect to both nutritional and technological properties of pâtés. The degradation of heme iron could decrease the nutritional value of the pâtés in terms of iron bioavailability, since HI is more available than NHI (Hunt & Roughead, 2000). In addition, iron achieves enhanced ability of promoting oxidation processes when it is released from heme molecule (Kanner *et al.*, 1991) and, therefore, pâtés with increasing amounts of NHI might also increase their oxidative instability.

In agreement with results from the lipid oxidation, the antioxidant effect of the rosemary essential oil on frankfurters was dose-dependent. The inhibition percents against protein oxidation at day 60 were 1.6%, 13.2% and 22.8% in frankfurters with added 150, 300 and 600 ppm rosemary essential oil, respectively. Results suggest that the rosemary essential oil should be used at 300 ppm or at higher concentrations to significantly inhibit the development of protein oxidation in refrigerated frankfurters. Accordingly, the addition of rosemary essential oil significantly reduced the release of iron from the heme molecule. At all days of storage, the addition 300 and 600 ppm rosemary essential oil significantly reduced the amount of NHI whereas the addition of 150 ppm did not affect the amount of NHI.

II.A.3. Effects on instrumental colour

Liver pâtés and frankfurters from Iberian pigs exhibited different colour characteristics depending on the addition of antioxidants. At day 0, pâtés with added rosemary were redder than 'control' pâtés, and no differences in L*- and b*-values were found. 'BHT' pâtés showed higher a*- and b*-values than control pâtés. On the other hand, treated frankfurters showed smaller L*-values and higher b*-values than 'control' frankfurters.

Colour characteristics of cooked products changed during refrigerated storage. The modifications in instrumentally colour measurements can be considered

as noticeable visual changes since the total colour difference (ΔE) values were higher than 2 in most of the groups of liver pâtés (days 0-90) and frankfurters (days 0-60). In general, the evolution of the instrumental colour parameters followed similar trends in both types of cooked products: increases of lightness (L*-values) and loss of redness (a*-values) over time which is in agreement with changes reported in previous works on cooked products (Carballo *et al.*, 1991; Perlo *et al.*, 1995; Jo *et al.*, 2000; Fernández-Ginés *et al.*, 2003). The addition of antioxidants affected the evolution of the colour parameters in liver pâtés and frankfurters. As far as liver pâtés is concerned, higher L*-values were measured at day 90 in pâtés with added antioxidants than in 'control' ones. In addition, the decrease of a*-values was considerably more intense in liver pâtés with added antioxidants than in the 'control' ones. It has been suggested that colour deterioration during refrigerated storage of cooked meats is explained by the degradation of certain nitrosopigments caused by oxidative processes, though no precise mechanisms were reported (Fernández-Ginés *et al.*, 2003). Some other authors linked the discolouration of cooked products with lipid oxidation (Akamittath *et al.*, 1990; Jo *et al.*, 1999). Nevertheless, colour changes reported in the present work seemed not to be directly related to oxidation processes since pâtés with the higher oxidative stability (those with added antioxidants) suffered more colour changes when compared to 'control' pâtés. In fact, total colour change between day 0 and day 90 as measured by $\Delta E_{0\text{-}90}$, was significantly higher in treated pâtés ('BHT': 5.45; 'sage': 5.34; 'rosemary': 4.49) than in the 'control' ones (3.38). Some compositional or physical changes not directly related to oxidative processes and not considered in the present study could have affected colour traits of liver pâtés much more than protein oxidation did.

Opposite results were obtained studying the effect of rosemary essential oil on the discolouration of IF during refrigeration. At day 60, higher L*-values were measured in control frankfurters than in the treated ones. The redness (a*-values) of frankfurters decreased during refrigerated storage with this decrease being more intense in the control frankfurters than in the treated

counterparts. At day 60, frankfurters with added 600 ppm rosemary essential oil showed higher a*-values than those from the other groups of frankfurters. Accordingly, total colour differences (ΔE_{0-60}) in frankfurters with added 300 and 600 ppm rosemary essential oil were significantly smaller than in those with added 150 ppm and 'control' frankfurters. It is reasonable that the colour changes in frankfurters were caused by oxidative reactions since the addition of substances with proven antioxidant activity inhibit to some extent their discolouration which is in agreement with previous observations (Cava *et al.*, 2004; Sebranek *et al.*, 2005). The modification of the pigment structure, suggested in the present work by the degradation of the heme molecule and the release of iron, might affect the colour displayed by frankfurters. In fact, the significant correlations between the NHI content and the colour parameters L* (R^2= 0.74; $p<0.01$) and a* (R^2= -0.74; $p<0.01$) suggest a possible relationship between the degradation of the heme molecule and the discolouration of the frankfurters causing the loss of colour intensity and increasing hue values. Furthermore, significant correlations were also found between protein oxidation and L* (R^2= 0.73; $p<0.01$) and a* (R^2= -0.84; $p<0.01$) suggesting that the development of protein oxidation in frankfurters affected their colour characteristics through the degradation of the heme molecule and the release of iron. Therefore, the protective role of the rosemary essential oil on frankfurter proteins and particularly on the heme protein would explain why treated frankfurters (particularly those with added 300 and 600 ppm) showed a more stable colour during refrigerated storage than the 'control' ones.

II.A.4. Effects on instrumental texture

Texture characteristics of liver pâtés and frankfurters from Iberian pigs were affected by the addition of antioxidants.

Hardness significantly increased in liver pâtés during refrigerated storage. The addition of BHT and sage essential oil significantly affected the texture characteristics of liver pâté, reducing hardness at day 30 of refrigerated storage. The effect of rosemary essential was even more intense since pâtés

with added rosemary showed, at days 60 and 90, significantly lower values of hardness than those from the other sets of pâté. In accordance with results obtained on liver pâtés, hardness of frankfurters also increased significantly after 60 days of storage in all groups, with this increase being significantly higher in the 'control' frankfurters than in the treated ones. At all days of storage, 'control' frankfurters were harder than those with added essential oil.

Though loss of moisture during storage could explain the increase of hardness in cooked products, it is not applicable in the present study since liver pâtés and frankfurters showed similar proximate compositions during the whole refrigerated storage. Furthermore, the addition of antioxidants did not affect the proximate compositions of cooked products and regardless of the presence of antioxidants, all groups of pâtés and frankfurters had similar moisture contents during the whole refrigerated storage. Hardness increase during refrigerated storage of frankfurters and other food emulsions has been previously described and related to the process of emulsion destabilization due to water and fat separation from the protein matrix (Fernández-Ginés *et al.*, 2003; Fernández-López *et al.*, 2004). To form a stable emulsion, proteins must surround the finely chopped fat particles before cooking and, therefore, protein functionality is essential to yield stable products (Smith, 1988). Protein oxidation is believed to affect protein functionality and their emulsification ability (Xiong, 2000). In addition, the oxidative damage of proteins has an impact in protein solubility, leading to the aggregation and complex formation due to crossed links (Karel *et al.*, 1975). It is plausible that the protein oxidation caused an increase of hardness in frankfurters and liver pâtés through the loss of protein functionality and the formation of crossed links between proteins since the addition of the antioxidants significantly reduced the hardness in frankfurters.

In frankfurters, the secondary parameters, gumminess and chewiness behaved similarly to hardness, on which they are dependent. Consistently, significant correlations were found between protein carbonyls and hardness (R^2= 0.56; $p<0.01$) and gumminess (R^2= 0.42; $p<0.01$).

II.B. Contradictory role of added essential oils on cooked products: antioxidant versus prooxidant effects

Results reported concerning the effect of the added essential oils on liver pâtés and frankfurters from Iberian pigs contrast with those obtained in cooked products from white pigs. The disparity of results affects to those obtained from both lipid and protein oxidation experiments. The addition of sage and rosemary essential oil enhanced the generation of TBA-RS in WP at day 90 whereas significantly reduced TBA-RS numbers in IP. In WP, the essential oils had no effect on the hexanal counts while greatly influenced on IP significantly reducing the generation of hexanal. Similar results were obtained on protein oxidation since no effect of essential oils was observed in WP and the same essential oils significantly inhibited the generation of protein carbonyls in IP at day 90. This contradictory effect was also observed regarding the release of iron from the heme molecule since control and treated pâtés from white pigs contained similar amounts of NHI and the addition of sage and rosemary oils in IP significantly reduced the release of iron from the heme molecule. The effect of the synthetic antioxidant (BHT) was not affected by the characteristics of the liver pâté since exhibited an antioxidant effect in both pâtés from Iberian and white pigs.

The results obtained from the evaluation of the rosemary essential oil in frankfurters from Iberian and white pigs were consistent with those obtained on liver pâtés. In IF, the effect of rosemary essential oil against the generation of TBA-RS, hexanal and protein carbonyls was more intense at higher essential oil levels, with the highest antioxidant effect found at 600 ppm. Accordingly, the rosemary essential oil also inhibited the release of iron from the heme molecule with this effect being more intense at higher concentrations.

On the contrary, 150 ppm of the rosemary essential oil successfully inhibited the development of the oxidative deterioration of WF whereas 300 and 600 ppm had no effect. Moreover, 600 ppm of essential oil in WF showed significantly promoted the generation of TBA-RS at day 60 of storage suggesting an incipient prooxidant effect. The effect of the rosemary essential

oil concerning the protein oxidation and the amounts of NHI in WF was similar: 150 ppm was effective to inhibit the generation of protein carbonyls at day 0 and no effect was detected on NHI contents whereas 300 and 600 ppm significantly increased the generation of protein carbonyls and promoted the release of iron from the heme molecule.

The results from the present study suggest that the activity of the plant essential oils was affected by some components of the food matrix. Food systems, and particularly comminuted meat products such as frankfurters or liver pâtés, are very complex in the number and the type of chemicals in the mixture, and a particular combination of these compounds might behave differently from the individual components. Accordingly, Yen *et al*. (1997) and Huang & Frankel (1997) reported that the effect of plant phenolics is influenced by the compositional characteristics of the food system and the presence of other active substances such as tocopherols. In this sense, Wong *et al*. (1995) and Fang & Wada (1993) reported likely interactions between phenolic compounds from sage and rosemary essential oils and tocopherols, resulting in different activities depending on the individual amounts of these substances in the food system. The significantly higher amounts of tocopherols detected in the raw material and frankfurters from Iberian pigs compared to those from white pigs could have influenced on the activity of the added plant essential oils leading to antioxidant or pro-oxidant effects.

In addition, the activity of the essential oils could have been affected by the initial oxidation state of the product in which it was added. In systems with high oxidative instability, the activity of plant phenolics could be diminished since phenolic compounds can be oxidised and the oxidation products could act as prooxidants promoting oxidative reactions (Huang & Frankel, 1997). In this sense, the higher oxidative instability of cooked products from white pigs compared to those from Iberian pigs as suggested by the results obtained from both lipid and protein oxidation could also partly explain the prooxidant activity of the essential oils in products from white pigs.

The large differences concerning the fatty acid composition between cooked products from Iberian and white pigs could have affected also the activity of

the plant essential oils according to findings by Huang & Frankel (1997). These authors reported whether antioxidant or prooxidamt activities of tea chatechins depending on the lipid system used for the analysis (corn oil triglycerides vs oil in water emulsions). Moreover, the prooxidant activity was stronger with higher concentrations, which is in agreement with the results from the present study. The different fatty acid composition between products affects the physical state of the lipids and the texture characteristics of the whole products that could have influenced on the dispersion and antioxidant activity of the plant essential oils leading to different effects.

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Conclusiones/conclusions

1. Los patés de hígado y salchichas cocidas elaboradas con tejidos de cerdo Ibérico presentan unas características nutricionales superiores a los productos elaborados con tejidos de cerdo blanco. Los productos de cerdo Ibérico presentaron un mayor contenido en hierro hemínico y un perfil de ácidos grasos más saludable, caracterizado por un alto contenido en ácidos grasos monoinsaturados (AGMI) y unos menores valores de las proporciones entre ácidos grasos n-6/n-3 y entre ácidos grasos hiper- e hipocolesterolemiantes.

2. Los patés y salchichas de cerdo Ibérico presentan, en comparación con los de cerdo blanco, una mayor estabilidad oxidativa durante su almacenamiento en refrigeración como consecuencia de un mayor contenido en antioxidantes (tocoferoles y compuestos fenólicos) y un menor contenido en ácidos grasos poliinsaturados.

3. La sustitución en la fórmula de las salchichas de cerdo blanco del tejido adiposo de dicho animal por el de cerdo Ibérico, incrementa significativamente en éstas los niveles de AGMI y antioxidantes, mejorando sus características nutricionales y tecnológicas.

4. Los productos cocidos de cerdo ibérico poseen, en comparación con los de cerdo blanco, un perfil de compuestos volátiles más favorable en lo referente a características aromáticas y estabilidad oxidativa. La presencia en el espacio de cabeza de los productos de cerdo ibérico de un alto contenido de compuestos volátiles derivados del ácido oleico, frente a los derivados del ácido linoleico, sugieren una más agradable percepción sensorial de éstos frente a los obtenidos a partir de cerdo blanco.

5. El deterioro oxidativo de las proteínas en los productos cocidos influye sobre determinados parámetros de calidad, provocando i) la decoloración de las salchichas cocidas durante su refrigeración debido a la degradación de pigmentos hemímicos, y ii) el deterioro de la textura de patés y salchichas

debido a la posible generación de enlaces cruzados entre proteínas y la pérdida de su funcionalidad.

6. El efecto de la adición de los aceites esenciales de salvia y romero sobre la estabilidad oxidativa de los productos cocidos es dependiente de la dosis añadida y de las características de composición de la materia prima utilizada para su elaboración. La actividad de los compuestos fenólicos presentes en los aceites esenciales es modificada por ciertos componentes presentes en los tejidos animales resultando en efectos globales antioxidantes o prooxidantes.

7. La adición de aceites esenciales mejora la estabilidad oxidativa de patés y salchichas de cerdo Ibérico, disminuyendo la intensidad de los procesos de oxidación de lípidos y proteínas y retrasando el deterioro de textura y color durante el almacenamiento a refrigeración. En patés de cerdo ibérico, el efecto antioxidante de los aceites esenciales a niveles de 1000 ppm es equivalente o incluso superior al efecto del BHT a niveles de 200 ppm, mostrándose como interesantes alternativas al uso de antioxidantes sintéticos.

8. La adición de aceites esenciales de salvia y romero influye sobre el perfil de compuestos volátiles de los productos de cerdo Ibérico, reduciendo la generación de compuestos volátiles derivados de la oxidación de ácidos grasos y contribuyendo al perfil con terpenos volátiles que aportarían a los productos características aromáticas adicionales.

1. Liver pâtés and frankfurters from Iberian pigs show better nutritional characteristics than those from white pigs, since the former contain higher heme-iron contents and show healthier fatty acid profiles with high levels of MUFA and lower ratios between n-6/n-3 and hyper- and hypocholesterolemic fatty acids.

2. Pâtés and frankfurters from Iberian pigs exhibit, compared to those from white pigs, a higher oxidative stability during refrigerated storage as a consequence of the higher amounts of antioxidants (tocopherols and phenolic compounds) and smaller amounts of polyunsaturated fatty acids.

3. The replacement of the adipose tissue from white pigs by that from Iberian pigs in frankfurters from white pigs significantly increases their levels of MUFA and antioxidants, improving their nutricional and technological properties.

4. Compared to cooked products from white pigs, those from Iberian pigs show a more pleasant volatiles profile as far as the oxidative stability and the aromatic characteristics is concerned. The presence of high levels of oleic acid-derived volatiles in the HS of the products from Iberian pigs in contrast to those derived from linoleic acid, suggests a more pleasant sensory perception in these compared to those from white pigs.

5. The oxidative damage of proteins in cooked products influences on certain quality parameters causing: i) the discolouration of frankfurters during refrigeration due to the degradation of heme pigments and ii) the texture deterioration of liver pâtés and frankfurters due to the loss of functionality and the likely generation of cross-linkings between proteins.

6. The effect of the addition of sage and rosemary essential oils on the oxidative stability of the cooked products is dependent on the level of added essential oil and the compositional characteristics of the raw material used for the manufacture. The activity of the phenolic compounds from the essential

oils is modified by certain components of the porcine tissues leading to global antioxidant or prooxidant effects.

7. The addition of essential oils improves the oxidative stability of pâtés and frankfurters from Iberian pigs, reducing the intensity of the oxidative deterioration of lipids and proteins and decreasing the colour and texture deterioration during refrigerated storage. The antioxidant effect of the essential oils at 1000 ppm on pâtés from Iberian pigs is similar or even higher than that exhibited by the BHT at 200 ppm, suggesting that the natural antioxidants could be used as alternatives to the synthetic ones.

8. The addition of sage and rosemary essential oils influences on the volatiles profile of cooked products from Iberian pigs, reducing the generation of lipid-derived volatiles and contributing with volatile terpenes which likely provide additional aromatic notes.

Attached papers

PAPER I

Physico-chemical properties and oxidative stability of liver pâté as affected by fat content[♣]

Mario Estévez, Sonia Ventanas & Ramón Cava

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I.1. Abstract

This study provides data on the physico-chemical characteristics and technological properties of liver pâtés with different fat content. Pâtés with high fat content (HFC) presented a smaller cooking yield than pâtés with medium and low fat contents (MFC and LFC, respectively) ($p<0.05$) mainly due to a higher loss of lipids. Fat content was closely related to the caloric value of pâtés being more calorific those with higher fat content. Pâtés with LFC were darker (lower L* value; $p<0.05$), redder (higher a* value; $p<0.05$) and harder (higher hardness value; $p<0.05$) than those with HFC. Oxidation stability of pâtés was affected by lipid content. HFC pâtés presented higher lipid and protein oxidation than LFC ones ($p<0.05$) as measured by TBA-RS and carbonyls content, respectively. Generation and release of lipid-derived volatiles might be affected by fat content as long as increases from 20 to 26% of fat in pâtés revealed higher amounts of volatiles, while a higher increase (from 26 to 31%) resulted in a decrease of total volatiles detected.

I.2. Keywords: Liver pâté, fat content, cooking yield, instrumental colour, oxidative stability.

I.3. Introduction

Foodstuff obtained from animals have recently played the role of being injurious for human's health because of the high content of fat, the presence of large proportion of saturated fatty acids and cholesterol (Jiménez-Colmenero *et al.*, 2001). In spite of that, meat and meat products are essential components of human's diet and provide, among other elements, high quality protein, vitamins and large amounts of essential metals such as iron. However, consumer's concern about the relationship between health and nutrition, challenge food technologists to develop new meat and fat-based products with enhanced characteristics. In order to fulfil the demand of consumers, a large number of low-fat products have been developed in order to reduce the presence of lipids and several lipid oxidation products considered as risks for human health, such as free-radicals, malondialdehyde

(MDA) and cholesterol oxidation products (COPs) (Khegal *et al*., 1987; Troutt *et al*., 1992; Sylvia *et al*., 1994). On the other hand, the reduction of fat in meat products might affect on their sensory characteristics (*revised by* Jiménez-Colmenero, 2000), mainly in those with a particular high content of this component, such as patties, cooked sausages or liver pâté. Among several sensory traits, fat has been demonstrated to influence on palatability and aroma characteristics of meat and fat products (Berry & Leddy, 1984; Troutt *et al*., 1992, Jo *et al*., 1999; Chevance & Farmer, 1999). The reduction of fat in foodstuffs is thought to modify their aromatic profile since large amounts of volatiles are generated from lipid oxidation and their interaction with other food components (Mottram, 1998). Moreover, lipids influence on physical and chemical stability of flavours as long as a reduction of fat content will result in flavours losses due to an increase of aroma compounds volatility (De Ross & Graff, 1995). The equilibrium between generation and release of volatile compounds from the food matrix might have a decisive impact on odour sensation since flavour is generally understood as the perception of volatile compounds released from food while eating (Lubbers *et al*., 1998). Nevertheless, limited information is available on the release of volatile compounds from meat and fat products with different lipid content.

On the whole, the level of fat in foods is closely related to numerous quality traits and in finely comminuted mixtures such as liver pâté, the producer determines the fat content, and this decision may affect the nutritional, technological and sensory characteristics of the manufactured product (Jiménez-Colmenero, 2000). As far as we know, few studies concerning the physico-chemical characteristics of pork liver pâté has been accomplished (Rosmini *et al*., 1996; Estévez *et al*., in press) and the effect of different levels of fat on the physicochemical and oxidative stability characteristics of liver pâtés remains unknown. This study was undertaken to gain more information on the characteristics of pork liver pâtés and to go deeply in the knowledge of the effect of fat content on the physicochemical and nutritional characteristics and oxidative stability of this product.

I.4. Material and Methods

Animals, feed and sampling

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed selection schemes were free-range reared and fed on natural resources (grass and acorns) following the traditional livestock farming for Iberian pigs. The animals were slaughtered at ~150 Kg live weight and an age of 12 months. After slaughter, back fat, muscle *quadriceps femoris*, and liver were removed from carcasses, vacuum packaged and stored at -80ºC until the manufacture of the experimental pâtés.

Manufacture of the liver pâté

The experimental pâtés were manufactured in a pilot plant. The formulation of the experimental pâtés is presented in Table I.1. For all pâtés, the sum of the contents of back fat and meat in their formulation represented the 50% of the total of the ingredients. Depending on the content of back fat used for the manufacture of pâtés, three different formulations were considered: Low, Medium and High Fat Content (LFC, MFC or HFC; n=5 for each) with 35, 40 and 45 % of fat respectively. LFC, MFC and HFC pâtés presented 5, 10 and 15% of meat in their composition, respectively. The range of fat contents was chosen, based on the diversity generally found in the Spanish market. The other ingredients were as follows per 100 g of elaborated product: 33 g liver, 11.5 g distilled water, 2 g milk powder, 2 g sodium chloride, 1 g caseinates. Sodium di- and tri-phosphates (0.5%) sodium ascorbate (0.025%) and sodium nitrite (0.05%) (ANVISA, Madrid, Spain) were also added. The day of the manufacture, the adipose tissue, livers and muscles from Iberian pigs were chopped into small cubes (1.5 cm^3). The livers and meat were mixed during mincing in a cutter (Foss Tecator Homogeniser, mod. 2094) during 3 minutes. During this period, the water, in form of small cubes of ice, was added to the bowl and mixed with the aforementioned ingredients in order to prevent the batter from temperatures above +15ºC. After that, the other

ingredients were added being the small cubes of fat the last ones in being added to the mixture in order to minimise their possible oxidation during mincing. The whole mixture was completely minced during 6 minutes until a homogenous raw batter was obtained. Finally, the mixture was packed in plastic containers and given the thermal treatment (+85ºC/30'). The packed liver pâtés were kept frozen (-80ºC) until required for analytical experiments.

Analytical methods

Cooking yield

Cooking yield was determined by assessing the value of exudation after thermal treatment. Each of the tubes was emptied on a sieve and drained. The exudative fluids (water and fat) were separated and considered in order to measure fat and water losses. The processing yield was given by the mean value of the weight difference before and after thermal treatment for the tubes:

$$\% \text{ cooking yield} = \frac{\text{drained pâté}}{\text{batter}} \times 100.$$

Compositional analysis and caloric value of liver pâtés

Moisture, total protein and ash were determined using official methods (AOAC, 2000). The method of Bligh & Dyer (1959) was used for determining fat content. Total iron was determined following the procedure described by Miller *et al*., (1994). Non-heme iron (NHI) content was determined following the method described by Rhee *et al*. (1987). The amount of heme iron (HI) was calculated by difference between total and NHI. The content of carbohydrates was obtained by subtracting to the 100%, the contents of fat, protein, moisture and ash. The caloric value was calculated by taking into account the appropriate conversion factors for protein and carbohydrates (4 Kcal/g) and fat (9 kcal/g).

pH measurement

The pH was determined using a Crison pH meter (mod. 2001) following the method of AOAC (2000).

Instrumental texture

The penetration test was performed with a Universal TA-XT2i texture analyser (Stable Micro Systems, UK). Force in compression was measured with a 10 mm diameter cylinder prove using a 5 Kg load cell. Once the probe triggered on the surface it then proceeded to penetrate to a depth of 8mm within the sample, measuring the force value as the hardness (N) of the sample. Force-distance deformation curves were recorded at a crosshead speed of 1.5 mm/s. Textural analyses were performed at ambient temperature.

Objective colour measurement

Instrumental colour (CIE L* a* b*; CIE, 1976) was measured in triplicate on the surface of liver pâtés using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ). Chroma (C) and Hue angle (Hº) values were obtained by using the following equations: $C= (a^{*2} + b^{*2})^{0.5}$; Hº= arctg b*/a* x (360/6.28).

Lipid oxidation

MDA and other thiobarbituric acid reactive substances (TBA-RS) were determined using the method described by Rosmini *et al*. (1996) for liver pâtés.

Protein oxidation

Protein oxidation as measured by the total carbonyl content was assessed following the method described by Oliver *et al*. (1987). Protein concentration was calculated by spectrophotometry using BSA as standard.

Lipid derived volatiles

The SPME fibre, coated with a divinyl-benzene-carboxen-polydimethylxilosane (DVB/CAR/PDMS) 50/30µm, was preconditioned prior analysis at +220ºC during 45 min. The headspace sampling was performed following a method previously described (Estévez *et al*., 2003) with minor modifications as follows: 1 g of pâté was placed in 2.5 mL vials and the SPME fibre was exposed to the headspace of the pâté while the sample equilibrated during 30 minutes immersed in water at +60ºC. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard) coupled to a mass-selective detector. Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (30m x 0.25mm id., 1.0mm film thickness; Restek). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40ºC. The SPME fibre was desorbed and maintained in the injection port at 220ºC during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at +40ºC and then raised at the rate of +7ºC min^{-1} to +250ºC, and held for 5 min. n-Alkanes (Sigma R-8769) were run under the same conditions to calculate the Kovats index (KI) values for the compounds. The GC-MS transfer line temperature was +270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Compounds were tentatively identified by comparing their mass spectra with those contained in the willey/NIST/EPA/NIH libraries and by comparison of Kovats index with those reviewed in scientific literature.

Data analysis

In order to find differences between groups, results of the experiments were used as variables and analysed using an Analysis of Variance (ANOVA) from SPSS software (SPSS, 1997). Tukey test was used to compare differences among mean values when ANOVA resulted significant. Statistical significance was defined at 0.05. Principal Component Analysis (PCA) from SPSS software

was carried out to establish relationships between variables measured and to discriminate among groups of pâté.

I.5. Results and Discussion

Effect of fat content on cooking yield

Experimental pâtés suffered losses of lipids and water after the thermal treatment (Table I.2.). Cooking yield was improved in pâtés with lower fat content from 86.74% (HFC) to 89.13% and 90.65% (LFC and MFC, respectively) ($p<0.05$). These results are in good agreement with those previously reported on beef patties (Troutt *et al.*, 1992; Garzon *et al.*, 2003) and cooked sausages (Hughes *et al.*, 1998; Pietrasik, 1999). Concerning loss of water after cooking, no statistical differences were found among groups (LFC: 4.52g water/100g pâté; MFC: 4.25g water/100g pâté; HFC: 3.60g water/100g pâté) ($p>0.05$). The differences in cooking yields among pâtés were mainly caused by losses of fat after cooking since pâtés with the highest lipid content (HFC) lost 11.46 g fat/100 g pâté while MFC and LFC pâtés lost lower amounts of fat (7.55 and 8.56 g fat/100g pâté, respectively; $p>0.05$).

Effect of fat content on chemical composition and nutritional value of liver pâtés

As expected, the manufacture of liver pâtés with increasing fat contents resulted in products with different chemical composition (Table I.3.). Parameters significantly affected by the fat content (moisture and total lipid content) presented an opposite behaviour. Moisture content followed the increasing order: LFC>MFC>HFC ($p<0.05$). In contrast, HFC presented the higher lipid content (31.19 g/100g pâté) compared to MFC (25.70 g/100g pâté) and LFC (20.49 g/100g pâté) ($p<0.05$). Other parameters measured in the pâtés such as protein (12.12-14.13 g/100g pâté), carbohydrates (1.10-2.10 g/100g pâté), ash (3.27-3.59 g/100g pâté) and iron contents (73.94-80.61 mg/100g pâté) did not show significantly differences among groups ($p>0.05$). In spite of that, pâtés manufactured with lower fat content tended

to present higher amounts of the aforementioned constituents, being all of those associated to the higher proportion of meat in the recipes. With reference to the chemical forms of iron, no differences between groups were found ($p>0.05$) either for heme, nor for non-heme iron. Consequently, the role of heme and non-heme iron in terms of bioavailability and promotion of lipid oxidation has to be considered to be similar in the samples studied. Mainly derived from the differences in the total lipid content, the pâtés presented significantly different caloric values, being higher in those with high fat content (HFC: 333.63 Kcal/100g pâté; MFC: 288.39 Kcal/100g pâté; LFC: 249.37 Kcal/100g pâté; $p<0.05$). Liver pâtés can be generally considered as a high-caloric product with large amounts of fat (Jiménez-Colmenero, 2000; Mataix & Aranceta, 2002) and therefore, its inclusion in the human diet might be restricted. However, previous considerations should be taken into account in order to consider the suitability of pâté to be included in human's diet in relation to its fat content. The frequency of consumption of pâté is relatively low as long as it can be considered as a high quality product with fairly high prizes. Thus, the study of the quality of fat should the considered quantitatively and qualitatively, and therefore, the compositional characteristics of the fat may make of the pâté a reasonably recommendable product or not. In the case of pâtés from Iberian pigs (as those manufactured for the present research), the large proportion of hypocholesterolemic fatty acids, such as oleic acid, reported in a previous work (Estévez *et al.*, in press) make of this product a high-quality propose compared to pâtés from white pigs. Moreover, liver pâté is considered as one of the most important sources of high bioavailability iron and therefore, highly recommended in order to prevent from iron's deficiency. In low-fat pâtés the iron content could be enhanced by increasing the meat proportion in the recipe as long as the proportion of liver should not exceed of the 30% because of the intense taste of the product.

Effect of fat content on physical characteristics of liver pâté

Pâtés elaborated with different fat content presented significant differences in most of the physical parameters assessed (Table I.4.). The pH values declined with increasing proportions of meat in the formulation of pâtés and followed the order: HFC>MFC>LFC. Fat content and hardness were inversely correlated (R^2: -0.70; $p<0.01$) and therefore, the presence of higher amounts of fat resulted in softer pâtés. HFC pâtés presented significant lower values for hardness than LFC pâtés (0.95 N *vs*. 1.55 N, respectively, $p<0.05$). The effect of fat on texture of meats and meat products has been largely studied and it is generally assumed that larger contents of fat are related to less firm and higher juicy products (Troutt *et al*., 1992; Sylvia *et al*., 1994; Hughes *et al*., 1998) that agree with results obtained in the present work. The fat content significantly affected the instrumental colour displayed by pâtés. These results were expected as long as the colour of pâtés is closely related to the colour characteristics of the raw material used for the manufacture (Estévez *et al*., in press) and therefore, changes in the proportion of the ingredients might lead to different colour characteristics. According to results from previous research on frankfurters and other meat products (Troutt *et al*., 1992; Hughes *et al*., 1998), the results in the present work, indicated that higher amounts of fat and lower of meat, increases lightness and reduces redness in the manufactured product. In fact, L* resulted significantly ($p<0.05$) correlated to fat content (R^2: 0.52). HFC pâtés resulted paler than LFC pâtés (L* values: 54.20 vs. 51.74 respectively; $p<0.05$), being the latter redder than the former (a* values: 15.45 vs. 13.85). Consequently, LFC pâtés presented a more intense colour (C values: 21.00 vs. 19.88) with lower values of hue (Hº values: 42.64 vs. 45.83) when compared to pâtés with higher fat content.

Effect of fat content on oxidation stability of liver pâtés

The oxidative stability of liver pâtés as measured by TBA-RS from lipid oxidation and carbonyls from protein oxidation is shown in Figure I.1. The lipid content significantly affected lipid oxidation since HFC pâtés presented significantly higher TBA-RS numbers, compared to pâtés with lower fat

content (5.56 vs. 2.87 mg MDA/kg pâté; $p<0.05$). These results were expected as long as TBA-RS are derived from lipid oxidation and in similar circumstances, pâtés with higher fat content would present a higher amount of oxidation products. Working on different types of meat, Jo *et al*. (1999), Sasaki *et al*., (2001) and ourselves (Estévez *et al*., 2003) found significant correlations between fat content and lipid oxidation, agreeing with results obtained in the present work (R^2: 0.52; $p<0.05$).

Pâtés with higher MDA content presented, in addition, higher amounts of carbonyls from protein oxidation (Figure I.1.). The amount of carbonyls was larger in pâtés with HFC (14.71 nM carbonyls/mg protein) as compared to those with medium (8.54 nM carbonyls/mg protein) and low-fat content (7.52 nM carbonyls/mg protein) ($p<0.05$). Reactive-oxygen species (ROS) and free radicals from lipid oxidation are believed to attack and damage proteins leading to a loss of functionality and a formation of residues such as carbonyls (Stadtman, 1990). This fact would link both degradation phenomena and may explain the results obtained. In the present work, the loss of protein functionality may have a reflection on the emulsion stability as measured by cooking yield and fluid losses. Pâtés with higher oxidation instability (HFC ones) presented a lower cooking yield and higher losses of lipids after cooking. Thus, significant correlations were found between protein oxidation and cooking yield (R^2: -0.53; $p<0.05$).

Effect of fat content on the generation and release of lipid-derived volatiles from liver pâtés

Nineteen lipid-derived volatiles were isolated from the headspace (HS) of the experimental pâtés (Table I.5.). Taking into account the higher variability commonly found on the analysis of volatiles using SPME (Estévez *et al*., 2003) and the relatively small size of the groups in the present work (n=5), it was unexpected to report so large differences among groups. The level of fat in pâtés significantly affected the amount of major volatile compounds detected. Increasing the fat content from 20% (LFC) to 26% (MFC), resulted in a significant larger amount of lipid-derived aldehydes such as hexanal (LFC:

272.7 AU, MFC: 506.4 AU; $p<0.05$), octanal (LFC: 17.6 AU; MFC: 27.9 AU; $p<0.05$) and decanal (LFC: 1.8 AU; MFC: 3.6 AU; $p<0.05$). The amounts of unsaturated aldehydes such as non-(*E*)-2-enal (LFC: 5.5 AU, MFC: 15.3 AU; $p<0.05$) dec-(*E*)-2-enal (LFC: 4.5 AU, MFC: 13.2 AU; $p<0.05$), dodec-(*E*)-2-enal (LFC: 3.2 AU, MFC: 10.7 AU; $p<0.05$) and 2,4 alkadienals such as hepta-(*E,E*)-2,4-dienal (LFC: 3.1 AU, MFC: 10.8 AU; $p<0.05$), nona-(*E,E*)-2,4-dienal (LFC: 3.9 AU, MFC: 18.0 AU; $p<0.05$), deca-(*E,E*)-2,4-dienal (LFC: 4.5 AU, MFC: 7.9 AU; $p<0.05$) and deca-(*E,Z*)-2,4-dienal (LFC: 3.3 AU, MFC: 10.7 AU; $p<0.05$) were significantly higher in MFC pâtés than in LFC ones. Other lipid-derived volatiles such as oct-1-en-3-ol (LFC: 13.1 AU, MFC: 30.1 AU; $p<0.05$) and octan-2-one (LFC: 1.5 AU, MFC: 16.1 AU; $p<0.05$) presented significant larger amounts in MFC when compared to LFC pâtés. In clear opposition to results described above, when compared the volatiles profile from MFC pâtés (~26% fat content) to that from HFC pâtés (~31% fat content), the amount of volatiles detected in the HS of liver pâtés, dramatically decreased. These differences were statistically significant for the most abundant compounds such as hexanal (MFC:506.4 AU, HFC: 318.3 AU; $p<0.05$), octanal (MFC:27.9 AU, HFC: 21.5 AU; $p<0.05$), dodec-(*E*)-2-enal (MFC:10.7 AU, HFC: 5.3 AU; $p<0.05$), oct-1-en-3-ol (MFC: 30.1 AU, HFC: 15.0 AU; $p<0.05$) and octan-2-one (MFC:16.1 AU, HFC: 3.5 AU; $p<0.05$). Higher amounts of other minority volatiles such as heptan-2-one (MFC: 2.4 AU, HFC: 1.2 AU; $p<0.05$) or 2-pentyl-furan (MFC: 5.5 AU, HFC: 2.8 AU; $p<0.05$) were detected in MFC pâtés as compared to those with higher fat content. The results obtained in the present work suggest a contradictory effect of fat on the volatiles profile of liver pâtés. The increasing amount of total volatiles in MFC compared to LFC pâtés was expected as long as these compounds are generated from lipid decomposition. As aforementioned for TBA-RS, foodstuffs with higher fat content, compared to those with lower fat content, are likely to present, under similar circumstances, larger amount of lipid-derived products. If this fact was true, pâtés with the highest lipid content (HFC pâtés), in which the largest lipid and protein oxidation indexes were found, might show the highest amount of lipid-derived volatiles. HFC

pâtés are supposed to have generated larger amount of volatiles but they might not be detected with SPME as long as they were not present in the HS. These results are in good agreement with results from other authors (Ahn *et al.*, 1998; Michaels & Istasse, 2002) and ourselves (Estévez *et al.*, 2003) who reported smaller amounts of lipid-derived volatiles in low-fat meat and meat products when compared to high-fat ones. In this sense, fat has been reported to reduce the release of volatiles from the food matrix to the HS (de Roos, 1997), avoiding their consequent detection with static or dynamic HS (Jo & Ahn, 1999; Chevance & Farmer, 1999). In agreement with aforementioned results, it is suggested a clear dissimilarity between TBA-RS and lipid-derived volatiles for the measurement of lipid oxidation so that the former analysis provide accurate information despite of the level of fat. In contrast to previous reports (Shahidi & Pegg, 1994), no significant correlations were found between the total amount of volatiles and TBA-RS (R^2: 0.06; $p>0.05$). Concerning the sensory assessment of flavour, the level of fat in pâtés might affect the perception of the aroma as long as this sensation is related to the detection of volatile compounds released from the matrix of the food (Lubbers *et al.*, 1998).

Principal Component Analysis

A Principal Component Analysis (PCA) was carried out to determine the relationships between the parameters studied and to discriminate liver pâtés based on their fat content. Figure I.2. shows the similarity map defined by the two first Principal Components (PC#1 and PC#2 respectively) that accounted for the 57.2% of the total variability. Agreeing with the aforementioned results, the groups of variables were associated in the map depending on the relationships established between them. The map on Figure I.3. showed that the PC allowed us to clearly discriminate the three formulas of pâté with different fat content. The pâtés with low fat content (LFC) are situated on the negative axis of PC#1, in the plane area corresponding to high values of moisture, protein, instrumental hardness and redness (Figure I.2.). Pâtés with medium fat content (MFC) that showed the highest amount of lipid-derived

volatiles are grouped on the positive axis associated nearby high amounts of volatiles such as hexanal, octanal, decanal, deca-(*E,Z*)-2,4-dienal, oct-3-en-1-ol and octan-2-one. Pâtés with high fat content (HFC) are mainly confined to the negative axis of the PC#2 and related to high oxidation numbers (from both protein and lipid oxidation), lightness and high lipid losses after thermal treatment.

I.6. Conclusions

Fat content affected the majority physico-chemical parameters measured. Some important quality traits such as texture, appearance and nutritional value would present a clear relationship with the total amount of fat. Liver pâtés with higher fat content are more prone to suffer lipid and protein oxidation and produce less stable emulsions. Measuring TBA-RS and carbonyls are appropriate methods to assess lipid and protein oxidation in liver pâtés while lipid-derived volatiles are not closely related to the aforementioned methods, being greatly affected by fat content. Fat seems to be a source of lipid oxidation products but influences on the release of these volatiles to the HS, avoiding their detection using SPME at high levels of fat. The equilibrium between generation and release of volatile compounds in liver pâtés may influence on the sensory perception of the aroma by consumers.

I.7. Acknowledgements

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Table I.1. Recipe (% of ingredients) used for liver pâtés with different fat content.

Ingredient (%)	LFC	MFC	HFC
Back Fat	35	40	45
Liver	33	33	33
Muscle	15	10	5
Water	11.5	11.5	11.5
Milk powder	2	2	2
Salt	2	2	2
Caseinates	1	1	1
Phosphates	0.5	0.5	0.5
Sodium nitrite	0.05	0.05	0.05
Sodium ascorbate	0.025	0.025	0.025

Table I.2. Cooking yield of pâtés and fluid losses (mean ± standard deviation) after thermal treatment of raw batter from pâtés with different fat content.

	LFC	MFC	HFC	p[1]
Cooking yield[2]	88.13[ab]±4.43	89.65[a]±1.40	86.74[b]±9.85	0.022
Water loss[3]	4.52±2.30	4.25±0.36	3.60±1.49	0.553
Lipid loss[4]	8.56±1.48	7.55±1.30	11.42±3.56	0.063

[1] Statistical significance.
[2] Expressed as percentage.
[3] g water/ 100 g raw batter.
[4] g lipid/ 100 g raw batter.

Table I.3. Chemical composition and caloric value (mean ± standard deviation) of liver pâtés from Iberian pigs with different fat content.

	LFC	MFC	HFC	p[1]
Moisture[2]	59.68[a]±1.18	56.98[b]±0.29	52.78[c]±1.49	<0.001
Fat[2]	20.49[c]±1.22	25.70[b]±1.83	31.19[a]±1.38	<0.001
Protein[2]	14.13±.069	12.75±2.07	12.12±1.53	0.151
Carbohydrates[2]	2.10±1.47	1.53±1.13	1.10±0.49	0.375
Ash[2]	3.59±0.44	3.40±0.39	3.27±0.34	0.358
Total iron[3]	80.61±9.23	78.11±12.59	73.94±6.64	0.469
Heme iron[3]	38.25±10.66	32.26±12.50	32.52±9.48	0.531
Non heme iron[3]	42.36±6.87	45.84±9.85	41.42±7.33	0.572
Caloric value[4]	249.37[c]±10.85	288.39[b]±2.39	333.63[a]±10.59	<0.001

[1] Statistical significance.
[2] g/100g pâté.
[3] µg/ g pâté.
[4] Kcal/100g.

Table I.4. Instrumental texture and colour characteristics, pH and lipid (TBA-RS) and protein oxidation (carbonyls) (mean ± standard deviation) of pâtés from Iberian pigs with different fat content.

	LFC	MFC	HFC	p[1]
Hardness[2]	1.55^{a}±0.26	1.29^{ab}±0.36	0.95^{b}±0.15	0.016
pH	6.34^{b}±0.01	6.36^{b}±0.01	6.39^{a}±0.01	<0.001
Cie L*	51.74^{b}±1.01	53.32^{ab}±1.62	54.20^{a}±1.19	0.018
Cie a*	15.45^{a}±0.09	14.74^{b}±0.25	13.85^{c}±0.31	<0.001
Cie b*	14.21±0.23	13.64±0.89	14.25±0.22	0.188
Chroma	21.00^{a}±0.19	20.09^{b}±0.43	19.88^{b}±1.96	0.002
Hue	42.64^{b}±0.43	42.78^{b}±1.96	45.83^{a}±0.97	0.003
TBA-RS[3]	2.87^{b}±0.40	3.76^{ab}±1.24	5.56^{a}±1.49	0.018
Carbonyls[4]	7.52^{b}±30.1	8.54^{b}±1.17	14.71^{a}±3.63	0.003

[1] Statistical significance.
[2] Newtons.
[3] mg MDA/kg pâté.
[4] nM carbonyls/mg protein.

Table I.5. Lipid-derived volatiles (mean ± stardard deviation) from the headspace of liver pâtés from Iberian pigs with different fat content as analysed using SPME.

Volatile Compounds	LFC	MFC	HFC	P[1]
Butanal[2,3]	1.4±0.2	3.4±1.3	2.4±1.8	0.091
Pentanal	7.8±1.8	11.2±4.5	8.6±6.7	0.722
Hexanal	272.8^{b}±51.7	506.4^{a}±109.7	318.3^{b}±112.8	0.008
Heptanal	7.6±0.5	11.3±1.6	9.6±4.9	0.203
Octanal	17.6±4.4	27.9±4.00	22.5±9.8	0.084
Nonanal	55.5±19.8	67.8±11.3	57.1±15.4	0.436
Decanal	1.8^{b}±0.5	3.6^{a}±0.8	3.1^{a}±1.1	0.009
Total saturated aldehydes	364.5^{b}±52.9	631.5^{a}±116.2	421.6^{b}±103.6	0.009
Hept-(*E*)-2-enal	9.6±4.1	21.8±10.3	12.9±7.6	0.121
Hepta-(*E,E*)-2,4-dienal	3.1^{b}±1.7	10.8^{a}±3.6	7.8^{a}±1.7	0.001
Non-(*E*)-2-enal	5.5^{b}±3.1	15.3^{a}±4.5	10.5^{ab}±3.6	0.004
Nona-(*E,E*)-2,4-dienal	3.9^{b}±1.0	18.0^{a}±4.6	14.3^{a}±5.2	<0.001
Dec-(*E*)-2-enal	4.5^{b}±2.6	13.2^{a}±1.2	10.4^{a}±4.2	0.001
Deca-(*E,E*)-2,4-dienal	4.5^{b}±1.5	7.9^{a}±1.8	5.4^{ab}±1.3	0.011
Deca-(*E,Z*)-2,4-dienal	3.3^{b}±2.2	10.7^{a}±1.5	9.1^{a}±2.9	<0.001
Dodec-(*E*)-2-enal	3.2^{b}±1.7	10.7^{a}±3.4	5.3^{b}±0.8	<0.001
Total unsaturated aldehydes	37.5^{b}±13.6	108.4^{a}±17.9	75.6^{ab}±15.2	0.001
Oct-1-en-3-ol	13.1^{b}±3.0	30.1^{a}±5.8	15.0^{b}±5.8	<0.001
Heptan-2-one	1.5^{b}±0.7	2.4^{a}±0.5	1.2^{b}±0.2	0.004
Octan-2-one	1.5^{b}±0.5	16.1^{a}±5.1	3.5^{b}±1.3	0.007
2-pentil-furan	2.2^{b}±0.4	5.5^{a}±1.0	2.8^{b}±1.4	<0.001
Total volatiles	420.2^{b}±53.0	794.0^{a}±154.6	519.8^{b}±129.3	0.002

[1] Statistical significance.
[2] UAA/10^{6}.
[3] Volatile compounds tentatively identified using MS, KI and Wiley libraries.

Figure I.1. Lipid and protein oxidation stability of liver pâtés with different fat content as assessed by TBA-RS (mg MDA/Kg pâté) and carbonyls (nM carbonyls/mg protein) content respectively (means ± standard deviation). Different letters indicate significant differences between groups in ANOVA test.

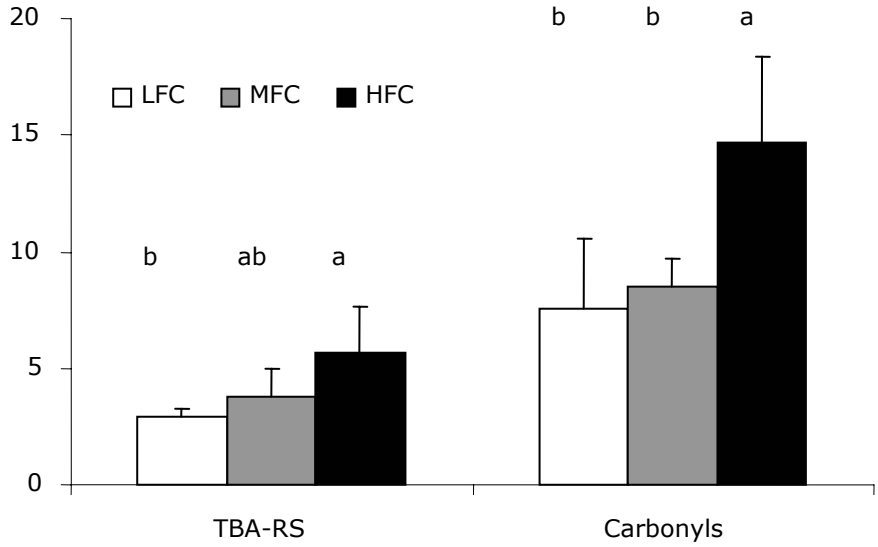
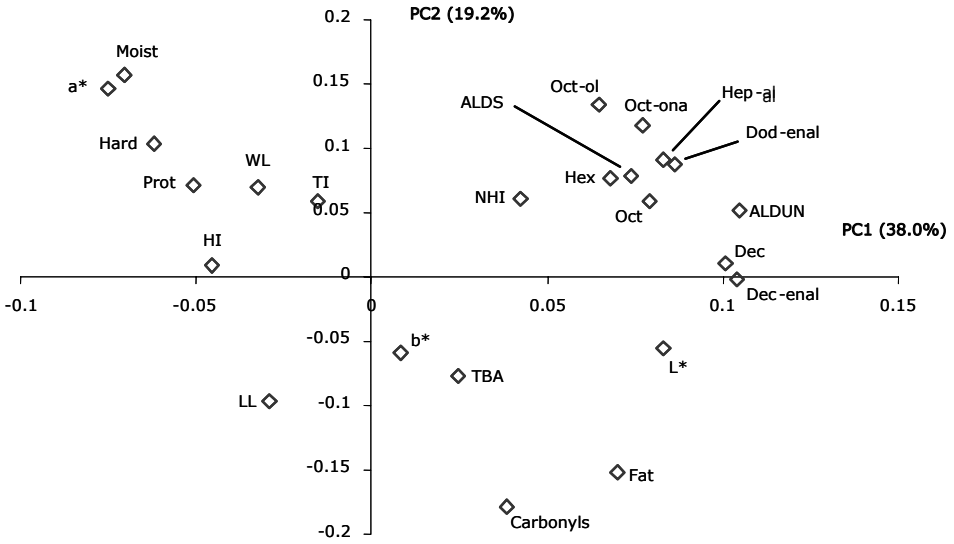
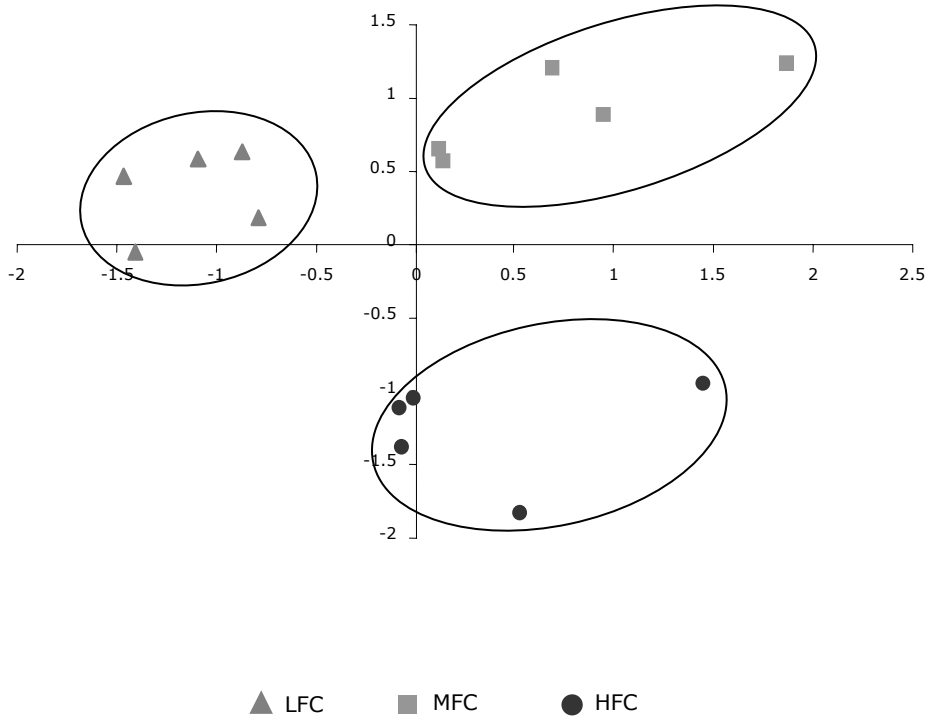


Figure I.2. Similarity map for the principal components (PC) 1 and 2 of the PC analysis performed on twenty-four physico-chemical variables of liver pâtés with different fat content.



Variables computed: Moisture (Moist), Fat content (Fat), Protein content (Prot), Total iron (TI), Non-heme iron (NHI), Heme iron (HI), CieL* (L*), a* (a*), b* (b*), Hardness (Hard), Water (WL) and Lipid losses (LL), Protein oxidation (carbonyls), TBA-RS (TBA), Hexanal (Hex), Octanal (Oct), Decanal (Dec), Hept-(*E*)-2-enal (Hep-al), Deca-(*E,Z*)-2,4-dienal (Dec-enal), Doc-(*E*)-2-enal (Doc-enal), Octan-2-one (Oct-one) and Oct-3-en-1-ol (Oct-ol).

Figure I.3. Similarity map for the PC 1 and 2 of the PC analysis performed on the samples of pâtés elaborated with different fat content. Spots are grouped denoting the discrimination between formulations with low, medium and high fat levels.



PAPER II

Characterisation of a traditional Finnish liver sausage and different types of Spanish liver pâtés: a comparative study[♣]

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II.1. Abstract

The physico-chemical characteristics of a traditional Finnish liver sausage (FLS) and different types of Spanish porcine liver pâtés (commercial, home-made and experimental) were studied. The characteristic recipes and different protocols of manufacture influenced on the chemical composition and oxidative stability of the liver products. FLS showed, amongst the liver products, the smallest percentage of monounsaturated fatty acids (MUFA) and the highest of polyunsaturated fatty acids (PUFA) acids, including long-chain PUFA. From a nutritional point of view, the FLS showed the smallest n-6/n-3 value whereas the experimental liver pâté (ELP) presented the smallest ratio between hyper- (lauric, myristic and palmitic acids) and hypocholesterolemic fatty acids (oleic and linoleic acids). A large variety of volatile compounds were isolated from the liver products including lipid-derived volatiles, Strecker aldehydes and alcohols, sulphur and nitrogen containing compounds and volatile terpenes, providing information from their oxidative stability and aroma characteristics. The FLS presented a balanced profile between lipid-derived volatiles and volatile terpenes from spices. The presence of potent odorants from added flavourings in the commercial liver pâté (CLP) could mask undesirable aroma odours from a large variety of lipid-derived volatiles. The home-made liver pâté (HMLP) also presented large amounts of lipid-derived volatiles as a likely consequence of strong thermal treatment while cooking, the presence of high amounts of iron and the absence of sodium nitrites and ascorbate in its composition. The ELP showed the simplest profile due to a considerably high oxidative stability and the absence of added spices.

II.2. Keywords: Liver sausage, liver pâté, fatty acids, volatile compounds, oxidation.

II.3. Introduction

The traditional Finnish liver sausage is popular with consumers. It is a typical cooked product manufactured using liver from pig or calf, porcine back-fat and some other characteristic ingredients such as grilled onions, raw

anchovies and herbs. The livers and back-fat are precooked, finely minced at high temperatures with the other ingredients, stuffed in synthetic casings and finally cooked in hot water.

In some other European countries, such as France or Spain, some other types of liver products such as the liver pâtés, are traditionally manufactured. Even when there is a large variety of liver pâtés in the Spanish markets, most of them are spreadable pastes made of liver, fat and pork or duck meat, and generally packed in glass containers. Nuts, fruits as well as spices and herbs can be included depending on the producer's customs.

Despite of the addition of nitrite, sodium ascorbate and some ingredients with proven antioxidant activity, the development of oxidative reactions during the refrigerated storage of liver pâté has been described as the major cause of liver pâté deterioration, leading to a loss of quality and generation of unpleasant aromas (Estévez *et al*., 2004a). According to studies carried out by Fernández-López *et al*. (2003) and ourselves (Estévez & Cava, 2004; Estévez *et al*., 2004a) lipids and proteins from liver pâtés are oxidised during refrigerated storage, causing the degradation of polyunsaturated fatty acids (PUFA), the instability of pigments and changes in texture and colour. Some particular factors, such as the amount of fat, fatty acid composition, the vitamin E and iron contents influence on the antioxidant status of the products (Miller *et al*., 1994; Morrissey *et al*., 1998; Estévez & Cava, 2004). The ingredients considered being included in the recipes and the protocols followed for the production of the different types of liver products influence on the aforementioned factors, modifying their quality traits and their oxidative stability.

The headspace (HS) analysis of volatile compounds in a liver product provides interesting information concerning their oxidative stability and aroma characteristics. Amongst the volatile compounds isolated from the HS of liver products, lipid-derived volatiles, Strecker aldehydes, nitrogen compounds and volatiles terpenes from particular spices, have been described (Ruiz *et al*., 2001; Im *et al*., 2004; Estévez *et al*., 2004a). Therefore, the aroma notes associated to a particular volatile compound and its threshold value provide

information about the aroma characteristics of the liver product containing such compound. On the other hand, the deterioration of a liver product during storage or handling could be evaluated analysing volatiles generated as a result of enzymatic, microbial or biochemical alteration phenomena (Vergnais *et al.*, 1998; Vinauskiene *et al.*, 2002; Estévez *et al.*, 2003).

As far as we know, only few papers devoted to the study of liver pâtés have been published and there is no information available concerning the characteristics of the traditional Finnish liver sausage.

The purpose of this study was to investigate the chemical characteristics and volatile components of FLS and compare the results with those obtained from the analysis of different types of Spanish porcine liver pâtés: a commercial liver pâté (CLP), a home-made liver pâté (HMLP) and an experimental liver pâté (ELP) in order to characterise different liver products from a nutritional point of view and shed light on the generation of volatiles as affected by different ingredients and protocols of manufacture.

II.4. Material and Methods

Production and sources of liver products

The ingredients and additives used for the manufacture of the different liver products are shown in Table II.1. The sources of the raw material and liver products and the protocols for the manufacture of each of them were as follows:

Finnish liver sausage (FLS). The Finnish liver sausages were produced in a pilot plant. The raw material (porcine back-fat and livers) was provided by a local slaughterhouse. The protocol followed a traditional style: the back-fat and livers were cut into pieces of ~30 cm^3 and precooked in a hot water bath (+90ºC) for 1 hour and 30 minutes, respectively. The onions were cooked until a desired golden colour was achieved. All the ingredients were gradually added, mixed and finely minced until a homogeneous raw batter was achieved (~10 min.). The water obtained from the precooking was also added for the production of the liver product. During mincing, the temperature of the raw

batter was always above +50ºC. Finally, the mixture was stuffed into 45 mm diameter synthetic casings, mechanically linked at 15 cm intervals and given the thermal treatment (+78ºC / 1 hour). After cooking, the sausages were kept in an ice/water bath (0-3ºC) for 1 hour.

Commercial liver pâtés (CLP). The porcine CLP were purchased from a local supermarket in Spain. The data in Table II.1. is according to the information provided by the producers.

Home-made liver pâtés (HMLP). The HMLP were produced by the Spanish catering company 'Anabel' using porcine back fat, meat and livers and following traditional recipes and procedures.

Experimental liver pâtés (ELP). The ELP were manufactured in a pilot plant following a protocol described elsewhere (Estévez *et al*., 2004b). Apart from the main ingredients (porcine back-fat, meat, liver and water) no species or additives were added, except those commonly found in the production of cooked meat products (sodium chloride, sodium nitrite, phosphates, sodium ascorbate and sodium caseinate) (Table II.1.).

All liver products were refrigerated and stored for 2 months at +4ºC. After that, they were kept frozen (-80ºC) until the analyses were carried out.

Analytical methods

Compositional analysis of liver products

Moisture, total protein, and ash were determined using official methods (AOAC, 2000). The method of Bligh & Dyer (1959) was used for isolating and quantifying fat from samples. Iron analysis was carried out according to the method described by Miller *et al*. (1994).

Fatty acid profile

Fatty acid methyl esters (FAMEs) were prepared by acidic esterification in presence of sulphuric acid, according to the method of López-Bote, Rey *et al*. (1997). FAMEs were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). FAMEs were separated on a semi-capillary column (Hewlett Packard FFAP-TPA fused-silica

column, 30m long, 0.53 mm internal diameter and 1.0 µm film thickness). The injector and the detector temperature were held at +230ºC. Column oven temperature was maintained at +220ºC. The flow rate of the carrier gas (N_2) was set at 1.8 mL/min. Identification of FAMEs was based on retention times of reference compounds (Sigma-Aldrich, Steinheim, Germany). Fatty acid composition was expressed as percent of total fatty acid methyl esters.

pH measurement

The pH was measured directly using an Ingold electrode connected to a Crison model 2001 pH-meter.

Objective colour measurement

Instrumental colour (CIE L* a* b*; CIE, 1976) was measured in triplicate on the surface of the liver products using a Minolta Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ) with illuminant D_{65} and 0º standard observer. CIELAB L*, a* and b* values were determined as indicators of lightness, redness and yellowness, respectively. Chroma (C) and Hue angle (Hº) values were obtained by using the following equations: $C= (a^{*2} + b^{*2})^{0.5}$; Hº= arctg b*/a* x (360/6.28).

Volatile compounds profile

The SPME fibre, coated with a divinylbenzene-carboxen-poly(dimethylxilosane) (DVB/CAR/PDMS) 50/30µm, was preconditioned prior analysis at +220ºC during 45 min. The HS sampling was performed according to the method previously described (Estévez *et al.*, 2004a). 1 g of liver product was placed in 2.5 mL vials and the SPME fibre was exposed to the headspace of the liver product while the sample equilibrated during 30 minutes immersed in water at +50ºC. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard) coupled to a mass-selective detector. Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (30m x 0.25mm id., 1.0mm film thickness; Restek). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 mL min^{-1} at 40ºC.

The SPME fiber was desorbed and maintained in the injection port at 220°C during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at +40ºC and then raised at the rate of +7ºC min^{-1} to +250°C, and held for 5 min. n-Alkanes (Sigma R-8769) were run under the same conditions to calculate the Kovats index (KI) values for the compounds. The GC-MS transfer line temperature was +270ºC. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650V and collecting data at a rate of 1 scan s^{-1} over a range of m/z 40 to 300. Compounds were tentatively identified by comparing their mass spectra with those contained in the Willey library and by comparison of their Kovats index with those reported by Kondjoyan & Berdagué (1996). Some compounds were identified by comparing their retention times with those from standard compounds (Sigma-Aldrich, Steinheim, Germany).

Data analysis

All experimental analyses were carried out in quindublicates. The results of the experiments were used as variables and analysed using an Analysis of Variance (ANOVA) (SPSS, 1997) in order to compare the physico-chemical characteristics and volatiles profiles of the four liver products. Statistical significance was predetermined at 0.05.

II.5. Results and Discussion

General composition, pH and colour characteristics of the liver products

The general composition of the FLS and the three different pork liver pâtés is shown in Table II.2. The liver products were constituted by water, fat and protein in significantly different proportions. The ELP presented the smallest amount of moisture and the highest amount of fat whereas the FLS and the CLP presented the highest amount of moisture and smallest of fat. The HMLP had an intermediate composition. The amount of protein ranged from 8.97 g/100 g (ELP) to 13.98 g/100 g (HMLP). The ash content was also different

amongst groups and ranged from 1.06 g/100 g in the FLS to 2.73 g/100 g in the HMLP. The amount of iron varied considerably among products as a consequence of the different recipes used for their production. The HMLP had a significantly higher amount of iron than the other liver products. The presence of a certain amount of liver in all of them led to products with considerably high levels of iron, with these levels being larger than those in other meat products (Lombardi-Boccia *et al.*, 2002) and fortified foods (Kosse *et al.*, 2001). Based on these data, the FLS and the liver pâtés can be considered as excellent sources of dietary iron for humans. Amongst the liver pâtés, the CLP was the one showing the most similar composition to that of FLS. In general, these results agree with those reported in previous works on porcine (D'Arrigo *et al.*, 2004; Echarte *et al.*, 2004) ostrich (Fernández-López *et al.*, 2003) and fish (Aquerreta *et al.*, 2002; Echarte *et al.*, 2004) liver pâtés.

The liver products presented significantly different pH values depending of the characteristics of the raw material and of other additives in the recipes. The ELP had the highest pH probably due to the presence of sodium phosphates which considerably increase the pH values in liver and meat products (Puolanne *et al.*, 2001). The HMLP presented the lowest pH value whereas FLS and the CLP presented intermediate values.

In agreement with the results from the chemical composition, the FLS and the CLP presented similar colour characteristics, being significantly different to those from the HMLP and the ELP. The FLS and the CLP were paler (higher L* values) and redder (higher a* values) than the HMLP and the ELP. The former ones also showed a more intense colour (higher chroma values) and smaller hue values compared to the other two. These results are consistent with previous studies in which the relationships between the chemical composition and the colour characteristics of liver pâtés were established (Estévez *et al.*, in press). The high amount of fat in the ELP would explain the lower a* and chroma values in this product compared to those in FLS and the CLP since the amount of fat is inversely correlated with a* values in liver pâtés (Estévez *et al.*, in press). The absence of nitrites in the HMLP could have limited the

development of the colour in such product in comparison with the others, reducing their redness and intensity of colour.

Fatty acid composition of the liver products

The fatty acid composition of porcine liver products is shown in Table II.3. Significant differences were found among products for every single fatty acid analysed. Percentages of saturated (32.91- 39.07%), monounsaturated (44.59-57.28%) and polyunsaturated (9.81-19.3%) fatty acids varied considerably between different types of liver products. Compared to the liver pâtés, the FLS presented smaller percentages of MUFA (44.59%) and higher of PUFA (19.03%). The proportion of SFA (36.38%) in FLS was intermediate between that in ELP (32.91%) and in HMLP (39.07%). The liver products reflected the fatty acid composition of the raw materials, as previously reported (Estévez *et al.*, 2004b), and the fatty acid composition of the tissues from animals is influenced by feeding (Miller *et al.*, 1990; Gandemer, 1998). The results suggested that pigs from which the liver products were manufactured were fed with different materials. The large proportion of oleic acid and total MUFA in the ELP agrees with results previously reported on liver pâtés elaborated with back-fat and livers from Iberian pigs (Estévez *et al.*, 2004b). The intake of acorns during the fattening period of Iberian pigs in ever-green oak forests influences the fatty acid composition of their tissues reflecting the high amount of oleic acid from the acorns (Cava *et al.*, 1997). Therefore, the use of raw material from extensively reared Iberian pigs for the production of liver pâté allows the achievement of products with 10 percent points higher of MUFA than other types of liver products. The fatty acid composition of the liver products contributes to define their nutritional and technological characteristics. In contrast to MUFA, PUFA are very prone to oxidation, leading to the generation of unpleasant odours and to the reduction of the nutritional value of meat and fat products (Morrissey *et al.*, 1998). Thus, compared to SFA, MUFA are hypocholesterolemic, but, unlike PUFA, they do not decrease high-density lipoprotein (HDL) cholesterol which protects against coronary heart diseases (CHD) (Mattson & Grundy, 1985).

The nutritional ratio between SFA hypercholesterolemic fatty acids (C12, C14, C16) and the unsaturated hypocholesterolemic ones (C18:1 n-9; C18:2 n-6) was lower in the ELP (0.36 vs. 0.43; $p<0.05$), compared to the other liver products. The proportions of minor fatty acids such as C20 and C22 n-3 PUFA have been taken into account from a nutritional point of view because of the role played by the ratio n-6/n-3 in the development of coronary heart diseases (Okuyama & Ikemoto, 1999). The percentages of long chain PUFA were significantly higher in the FLS than in the liver pâtés. Furthermore, the FLS presented the smallest n-6/n-3 ratio, being those from the liver pâtés significantly higher. The addition of a fish product such as the anchovies in the FLS probably influenced on its fatty acid composition increasing the amount of n-3 fatty acids and improving, consequently, the health characteristics of the liver sausage through the modification of the n-6/n-3 ratio. Aquerreta *et al*. (2002) have reported even lower n-6/n-3 ratios in liver pâtés exclusively produced with fish livers and flesh. Enser *et al*. (2000) and D'Arrigo *et al*. (2004) reduced the n-6/n-3 ratio in porcine sausages and liver pâtés through the modification of the fatty acid composition of the animal tissues by dietary means using linseed oil with or without olive oil.

Volatiles profile of the liver products

143 volatile compounds were isolated from the HS of the liver products and tentatively identified. Table II.4. shows the volatile compounds categorised into eleven classes. The liver products are considerably complex systems and several can be the origins of the volatiles detected in their HS (Ruiz *et al*., 2001; Estévez *et al*., 2004a). Most of the volatile compounds are generated as a result of the development of chemical reactions between the components (fat, protein and carbohydrates) of the tissues used for the manufacture of the liver products. Amongst them, lipid-derived volatiles such as alcohols (pentan-1-ol, hexan-1-ol), aldehydes (hexanal, heptanal, hept-(*E*)-2-enal, octanal, nonanal, non-(*E*)-2-enal, dodecanal) and ketones (heptan-2-one, 4-octen-3-one, octan-2-one and nonan-2-one) and Strecker aldehydes (3-(methyltio)propanal, 2-methylbutanal, 3-methylbutanal, benzaldehyde) and

alcohols (2-methyl-butan-1-ol; 3-methyl-butan-1-ol), were detected in the present study. Compounds derived from the Maillard reaction such as nitrogen (pyrans, pyridines, pyrazines and furanones) and sulphur (thiophenes and thiazoles) compounds were also identified.

Another large group of volatile compounds is incorporated to the liver product through the addition of spices and herbs. Amongst them, the volatile terpenes are the most abundant and important compounds since they have low threshold values and defined aromatic notes which modify the aromatic characteristics of the product in which they are included (Chevance & Farmer, 1999; Paleari *et al.*, 2004). The SPME allowed the isolation of monoterpenes hydrocarbons (i.e. *α*-pinene, *β*-thujene, *β*-myrcene, 1-limonene), sesquiterpenes hydrocarbons (i.e. *α*-copaene, (E)-caryophyllene, *α*-humulene) and terpenoids (i.e. linalool, linalyl acetate, linalyl propionate) from the liver products.

Most of the volatiles analysed in the present work have been previously reported as volatile components of cooked pork, oxidised liver and canned liver sausages (Elmore *et al.*, 1999; Ruiz *et al.*, 2001; Im *et al.*, 2004). The four liver products showed different volatiles profiles since significant differences were found between groups for most of the volatile compounds detected. The patterns represented by the chromatograms obtained from the analysis of the samples were highly characteristic for each product and could be used to characterise each liver product (Figure II.1.). These patterns were defined by the oxidative stability of the samples and the presence of some particular ingredients, spices and additives which provide specific volatile compounds.

The results from the present study suggest that the FLS and the ELP exhibited the highest oxidative stabilities since they had the smallest amounts of hexanal and total lipid-derived aldehydes and ketones. The high oxidative stability of the ELP was reported in a previous study and explained by the high amounts of tocopherols and small of PUFA in their composition (Estévez *et al.*, 2004a). Unexpectedly, the FLS also presented small amounts of lipid-derived volatiles despite of its higher amount of PUFA. The addition of spices and

herbs likely contributed to increase the oxidative stability of the FLS since most of them contain phenolic compounds with antioxidant activity (Zankan *et al.*, 2002; Dorman *et al.*, 2003). The presence of some particular additives such as phosphates, sodium ascorbate and nitrite in the ELP and FLS are also influential in their oxidative stability (Walsh *et al.*, 1998; Morrissey *et al.*, 1998). The HMLP and the CLP presented the highest chromatographic areas for compounds generated from lipid oxidation suggesting a higher oxidative instability in these products compared to the FLS and the ELP. The HMLP had significantly higher amounts of hexanal, oct-2-(*E*)-enal and total amount of lipid-derived aldehydes than the other liver products. Compared to the other liver products, the HMLP also had significantly higher amounts of aliphatic and aromatic hydrocarbons. This product was elaborated following traditional recipes and procedures including a long precooking process of the tissues at high temperatures previous to mincing, cooking and packing. In addition, the HMLP had the highest amount of iron which is considered one of the most potent prooxidant in muscle foods (Kanner *et al.*, 1991). Furthermore, the absence of some particular additives with proven antioxidant activity (i.e. sodium ascorbate and sodium nitrite) (Walsh *et al.*, 1998; Morrissey *et al.*, 1998) in the HMLP probably allowed the development of oxidative reactions during cooking and the subsequent refrigeration. In fact, in this liver product, significantly higher amounts of volatiles used to evaluate the deterioration of refrigerated stored foods such as octan-2-one, 2-methyl-butan-1-ol and 3-methyl-butan-1-ol (Montel *et al.*, 1998; Estévez *et al.*, 2003) were detected.
The CLP also had significantly higher amounts of some particular lipid-derived volatiles such as non-2-(*E*)-enal, propan-2-one, butan-2-one, pentan-2-one, hexan-2-one, heptan-2-one, 1-octen-3-one and some others were exclusively detected in the CLP such as 2-ethyl-hex-2-enal, butan-2-one, hexan-2-one and hexadecanal. The present results suggest that intense oxidative reactions happened during production and/or storage of the CLP. Some the abovementioned compounds have been described as indicators of lipid decomposition and contributors to the overall off-flavour of oxidised liver (Im *et al.*, 2004). Non-(*Z*)-2-enal derives from the oxidation of linoleic and

arachidonic acids and has been related to a 'cardboard like' odour (Im *et al.*, 2004). Oct-2-(*E*)-enal is also generated from PUFA decomposition and contributes with 'tallowy' and 'stale' notes (Im *et al.*, 2004). 1-octen-3-one is the primarily responsible of the 'metallic' off-flavour and 'liver-like' odour in liver products (Im *et al.*, 2004). High importance has also been given to 2,4 alkadienals in relation to unpleasant aroma notes associated to 'rancid' and 'warmed-over' flavours (Chevance & Farmer, 1999; Im *et al.*, 2004). Therefore, all of them could contribute with undesirable flavour notes to the overall aroma characteristics of the CLP and the HMLP. However, the generation of volatiles as a result of a moderate lipid oxidation is necessary to achieve a complex and desirable flavour.

The aromatic profile of the liver products is also influenced by the presence of volatile compounds from added herbs, spices and others flavourings. The CLP showed small amounts of volatile terpenes though some spices such as black and white pepper were claimed to be added. The CLP had, however, large amounts of potent odorants such as those derived from the Strecker degradation and Maillard reactions (Hofmann & Schieberle, 2000) which are not commonly found in liver products at such levels. In fact, the amounts of Strecker aldehydes such as 2-methylpropanal, 3-(methyltio)propanal, 2-methylbutanal, 3-methylbutanal and benzaldehyde in the CLP were significantly higher than in the other liver products. The CLP also had a large variety of Maillard products such as sulphur compounds, pyridines, pyrazines, furanones and pyrrols (Shahidi, 1994) which was not detected in the other liver products and has not been previously reported in liver products (Ruiz *et al.*, 2001; Im *et al.*, 2004; Estévez *et al.*, 2004a). 2-methylbutanal and 3-methylbutanal are common components in cooked meat and dry cured products contributing to desirable 'almond-like', 'toasted' aroma notes (Elmore *et al.*, 1999; Carrapiso *et al.*, 2002; Estévez *et al.*, 2003). Other Maillard products such as methylpyrazine, ethylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2-methylthiophene and 2-acetyl-2-thiazoline have been also detected in cooked meat and meat products and contribute with 'roasted meat' flavours (Hofmann & Schieberle, 1997;

Chevance & Farmer, 1999; Elmore *et al*., 1999). These compounds, which were probably added as flavouring additives, provide intense and desirable flavours which could mask the rancid aroma notes from the lipid-derived volatiles (ketones, alkenals and 2,4 alkadienals) aforementioned.

The production of the FLS and the HMLP followed a different strategy to improve their aromatic profiles and fulfil consumer's expectations. Both had a large diversity of volatile terpenes from spices which contributed with specific aroma notes since some of them such as α-pinene, 1,8-cineole and linalool have been related to 'spices, pine needles', 'medicinal, cough syrup' and 'flowers, carnation' odours, respectively (Chevance & Farmer, 1999). The HMLP had the highest amount of volatile terpenes whereas the FLS presented the most complex profile since had a larger variety of terpenes. β-elemene, α-guaiene, AR-curcumene, α-zingibirene, β-bisabolene, patchoulane and γ-selinene were only detected in the HS of the FLS, with some of them being specific volatile components and aroma contributors of species added only in that product such as the marjoram and the ginger (Variyar *et al*., 1997; Gong *et al*., 2004).

As expected, a small amount of terpenes was isolated from the HS of the ELP since no extra species or herbs were added. The addition of spices and herbs in the ELP would be of interest in order to improve their aromatic characteristics so that they could be commercially accepted. In a previous study, we isolated a large variety of terpenes from the HS of spiced pâtés with rosemary and sage essential oils (Estévez *et al*., 2004a).

II.6. Conclusions

The Finnish liver sausage is a high quality liver product with favourable fatty acid profile, high oxidative stability and a complex and balanced volatiles profile. The ELP is characterised by a large amount of oleic acid and healthy fatty acid composition though several changes should be accomplished in order to make of it a commercially accepted product. Different strategies have been performed by producers to improve the aromatic profile of the liver products. The addition of spices with recognised desirable aromatic odours is

carried out in the HMLP and the FLS. The consumer's satisfaction is achieved in the CLP through the addition of flavourings and could mask off-flavours from lipid oxidation.

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Table II.1. Recipes of the liver products.

	Liver Products	FLS	CLP	HMLP	ELP
Ingredients[1]					
	Adipose tissue	42	40	50	40
	Liver	28	28	35	28
	Meat	-	5	10	5
	Water	26	25	15	23
	Grilled onion	0.6	-	-	-
	Anchovies	0.4	-	-	-
	Sodium caseinate/Milk powder	3	2	-	2
Additives[2]					
	Sodium chloride	1.4	n.a	3	2
	Sugar	0.4	n.a		-
	Sodium phosphates	-	-	-	0.3
	Sodium nitrite	0.008	n.a	-	0.03
	Sodium ascorbate	0.05	n.a	-	0.05
Spices[2]					
	Marjoram	0.07	-	-	-
	White pepper	0.05	n.a.	-	-
	Black pepper	0.05	n.a.	0.03	-
	Nutmeg	0.05	-	0.03	-
	Ginger	0.05	-	-	-
Others					
	Brandy	-	-	1	-
	Flavourings	No	Yes	No	No

[1] Expressed as g/100g of elaborated product.
[2] Expressed as percentage in base of the main ingredients.
n.a.: information not available.

Table II.2. Proximate composition, pH and instrumental colour of the liver products (means ± standard deviation).

	Liver Products				
	FLS	CLP	HMLP	ELP	p[1]
Moisture[2]	57.90^{a}±0.58	58.29^{a}±1.35	53.46^{b}±0.48	48.39^{c}±1.09	<0.001
Fat[2]	23.11^{c}±0.52	22.97^{c}±0.92	29.30^{b}±2.38	34.69^{a}±0.91	<0.001
Protein[2]	11.67^{b}±0.61	13.20^{a}±0.33	13.98^{a}±0.49	8.97^{c}±0.30	<0.001
Ash[2]	1.06^{c}±0.37	1.62^{b}±0.39	2.54^{a}±0.17	2.73^{a}±0.08	<0.001
Iron[3]	59.87^{b}±10.63	57.59^{b}±13.45	86.64^{a}±8.37	52.47^{b}±18.34	<0.001
pH	6.18^{c}±0.05	6.33^{b}±0.05	5.99^{d}±0.08	6.74^{a}±0.04	<0.001
CieL*	66.06^{a}±0.41	65.9^{a}±0.86	62.47^{c}±1.08	63.99^{b}±0.64	<0.001
Ciea*	11.19^{a}±0.25	11.78^{a}±0.59	8.89^{b}±0.35	9.29^{b}±0.58	<0.001
Cieb*	13.23^{ab}±0.33	13.80^{a}±0.59	13.49^{a}±0.29	12.73^{b}±0.37	0.006
Chroma	17.33^{a}±0.35	18.15^{a}±0.78	16.16^{b}±0.42	15.76^{b}±0.63	<0.001
Hue	49.81^{c}±0.71	49.55^{c}±0.89	56.66^{a}±0.57	53.91^{b}±1.02	<0.001

In the same line, means with different superscript, significantly differed in ANOVA test.
[1] Statistical significance.
[2] g/100g of liver product.
[3] µg iron/g of liver product.

Table II.3. Fatty acid profiles of the liver products (means ± standard deviation).

	Liver Products				
	FLS	CLP	HMLP	ELP	p[1]
C12	0.09^{a}±0.01	0.04^{c}±0.00	0.05^{b}±0.01	0.04^{c}±0.00	<0.001
C14	1.14^{a}±0.01	1.06^{b}±0.03	1.09^{b}±0.02	1.06^{b}±0.01	<0.001
C16	22.05^{a}±0.08	21.68^{b}±0.04	22.33^{a}±0.38	20.76^{c}±0.02	<0.001
C17	0.39^{b}±0.00	0.49^{a}±0.01	0.39^{b}±0.04	0.25^{c}±0.00	<0.001
C18	12.48^{b}±0.04	12.21^{b}±0.04	14.96^{a}±0.36	10.61^{c}±0.04	<0.001
C20	0.23^{a}±0.02	0.20^{bc}±0.02	0.23^{a}±0.03	0.19^{c}±0.01	0.016
Σ SFA	36.38^{b}±0.08	35.66^{c}±0.05	39.07^{a}±0.65	32.91^{d}±0.03	<0.001
C16:1 (n-7)	2.58^{b}±0.00	2.81^{a}±0.04	1.92^{c}±0.24	1.98^{c}±0.00	<0.001
C17:1 (n-7)	0.34^{b}±0.00	0.44^{a}±0.00	0.28^{bc}±0.07	0.25^{c}±0.00	<0.001
C18:1 (n-9)	40.71^{d}±0.07	42.98^{c}±0.13	44.34^{b}±0.64	53.22^{a}±0.05	<0.001
C20:1 (n-9)	0.88^{d}±0.02	1.03^{c}±0.02	1.14^{b}±0.01	1.80^{a}±0.01	<0.001
C22:1 (n-9)	0.09^{a}±0.00	0.06^{bc}±0.00	0.07^{ab}±0.00	0.04^{c}±0.02	<0.001
Σ MUFA	44.59^{c}±0.05	47.31^{b}±0.07	47.76^{b}±0.55	57.28^{a}±0.04	<0.001
C18:2 (n-6)	15.23^{a}±0.04	13.78^{b}±0.07	10.60^{c}±0.28	7.67^{d}±0.00	<0.001
C18:3 (n-6)	0.12^{b}±0.01	0.17^{a}±0.01	0.13^{b}±0.02	0.13^{b}±0.00	<0.001
C18:3 (n-3)	1.45^{a}±0.01	1.01^{b}±0.01	0.82^{c}±0.11	0.49^{d}±0.00	<0.001
C20:2 (n-6)	0.58^{a}±0.01	0.57^{ab}±0.01	0.46^{c}±0.02	0.55^{b}±0.00	<0.001
C20:3 (n-3)	0.07^{a}±0.00	0.08^{a}±0.01	0.06^{b}±0.00	0.04^{c}±0.00	<0.001
C20:3 (n-6)	0.14^{a}±0.00	0.14^{a}±0.00	0.10^{b}±0.00	0.08^{c}±0.00	<0.001
C20:4 (n-6)	0.97^{a}±0.04	0.94^{a}±0.01	0.70^{b}±0.08	0.54^{c}±0.01	<0.001
C20:5 (n-3)	0.17^{ab}±0.00	0.16^{b}±0.00	0.13^{c}±0.02	0.18^{a}±0.00	<0.001
C22:2 (n-6)	0.06^{a}±0.01	0.03^{b}±0.00	0.05^{a}±0.01	0.03^{b}±0.00	<0.001
C22:4 (n-6)	0.04^{a}±0.01	0.01^{b}±0.00	0.01^{b}±0.00	0.01^{b}±0.00	<0.001
C22:5 (n-3)	0.16^{a}±0.00	0.15^{a}±0.00	0.11^{b}±0.01	0.03^{c}±0.00	<0.001
C22:6 (n-3)	0.04^{b}±0.00	0.01^{c}±0.00	0.03^{b}±0.00	0.05^{a}±0.01	<0.001
Σ PUFA	19.03^{a}±0.09	17.04^{b}±0.07	13.19^{c}±0.31	9.81^{d}±0.01	<0.001
Σ LChPUFA	2.27^{a}±0.05	2.08^{b}±0.02	1.65^{c}±0.10	1.52^{d}±0.02	<0.001
n-6/n-3	9.07^{b}±0.06	11.07^{a}±0.11	10.64^{a}±1.15	11.40^{a}±0.04	<0.001
Nutritional ratio[2]	0.42^{a}±0.00	0.40^{b}±0.00	0.43^{a}±0.01	0.36^{c}±0.0	<0.001

Results expressed as percentage of total fatty acids analysed.
In the same line, mean with different superscript, significantly differed in ANOVA test.
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; LChPUFAs, long chain PUFA.
[1] Statistical significance.
[2] Nutritional ratio: (C12+C14+C16)/(C18:1+C18:2).

Table II.4. Volatile compounds isolated from the HS of the liver products with SPME.

		Liver products						
	Peak Nº	FLS	CLP	HMLP	ELP	sem[1]	p[2]	rel[3]
				Acids				
acetic acid	10	0.00^{b}	16.86^{a}	8.19^{ab}	0.00^{b}	1.97	0.001	a
butanoic acid	34	0.00^{b}	0.66^{a}	0.55^{a}	0.00^{b}	0.07	<0.001	b
pentanoic acid	41	0.00^{c}	0.94^{a}	0.47^{b}	0.00^{c}	0.09	<0.001	b
2-methyl hexanoic acid	42	0.00^{b}	0.87^{a}	0.50^{a}	0.00^{b}	0.10	<0.001	b
hexanoic acid	65	0.59^{b}	1.39^{a}	1.98^{a}	0.46^{b}	0.17	<0.001	b
octanoic acid	108	1.31^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.15	<0.001	b
Total acids		1.89^{b}	21.29^{a}	21.36^{a}	0.46^{b}	2.91	<0.001	
				Amines				
N,N-diethylethanamine	19	9.81^{a}	4.25^{b}	2.58^{b}	1.66^{b}	0.82	<0.001	b
N,N-dimethyl-1-dodecanamine	133	2.35	2.35	2.87	5.91	0.64	0.135	b
Total amines		12.16^{a}	6.60^{ab}	5.46^{c}	7.57^{ab}	0.91	0.033	
				Alcohols				
ethanol	3	25.35^{b}	4.04^{b}	2624.82^{a}	50.61^{b}	264.03	<0.001	a
propan-1-ol	6	0.00^{b}	0.00^{b}	6.11^{a}	0.00^{b}	0.63	<0.001	a
2-methylpropan-1-ol	14	0.00^{b}	0.00^{b}	3.41^{a}	0.00^{b}	0.35	<0.001	b
3-methyl-butan-1-ol	27	0.00^{b}	0.00^{b}	37.54^{a}	0.00^{b}	3.93	<0.001	b
2-methyl-butan-1-ol	28	0.00^{b}	0.00^{b}	8.01^{a}	0.00^{b}	0.86	<0.001	b
pentan-1-ol	31	0.46^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.05	<0.001	a
hexan-1-ol	48	1.57^{ab}	1.29^{b}	2.03^{a}	0.00^{c}	0.19	<0.001	a
3-methyl-1-butanol (nitrate)	58	0.59^{a}	0.00^{b}	0.00^{b}	0.70^{a}	0.08	<0.001	b
2-ethyl-hexan-1-ol	87	0.00^{b}	0.00^{b}	0.00^{b}	0.72^{a}	0.08	<0.001	b
4-methylphenol	94	0.29^{bc}	1.74^{a}	1.16^{ab}	0.00^{c}	0.20	0.001	b
2,6-dimethylciclohexanol	106	1.02^{b}	0.78^{b}	1.00^{b}	8.64^{a}	0.85	<0.001	b
benzeneethanol	107	0.00^{b}	0.00^{b}	2.91^{a}	0.00^{b}	0.34	<0.001	b
4-methyl-1(1-methylethyl)ciclohex-3-en-1-ol	112	59.78^{a}	0.00^{b}	63.55^{a}	0.00^{b}	7.16	<0.001	c
2-metoxi-4(1-propenyl)phenol	121	2.93^{a}	0.00^{b}	3.20^{a}	0.00^{b}	0.36	<0.001	c
Total alcohols		41.47^{b}	12.42^{b}	2701.14^{a}	67.56^{b}	270.36	<0.001	
				Aldehydes				
acetaldehyde	1	0.00^{b}	8.56^{a}	0.00^{b}	0.00^{b}	0.90	<0.001	a
2-methylpropanal	7	1.20^{b}	5.37^{a}	0.00^{c}	0.00^{c}	0.68	0.004	b
but-(E)-2-enal	15	0.00^{b}	0.00^{b}	0.00^{b}	0.31^{a}	0.03	<0.001	a
3-methylbutanal	16	1.25^{b}	30.74^{a}	1.80^{b}	0.96^{b}	2.96	<0.001	a
2-methylbutanal	17	0.93^{b}	9.24^{a}	1.07^{b}	0.48^{b}	0.93	<0.001	a
pentanal	20	2.66^{b}	1.43^{b}	9.07^{a}	1.87^{b}	0.75	<0.001	a
hexanal	37	20.76^{b}	34.64^{b}	164.68^{a}	30.45^{b}	14.02	<0.001	a
octa-(*E,E*)-(2,4)-dienal	50	0.00^{b}	0.32^{b}	1.02^{a}	0.00^{b}	0.11	<0.001	a

	Peak Nº	Liver products				sem[1]	p[2]	rel[3]
		FLS	CLP	HMLP	ELP			
heptanal	52	4.65[a]	4.57[a]	4.94[a]	1.85[b]	0.42	0.016	a
hept-(*E*)-2-enal	62	1.13[a]	1.15[a]	1.21[a]	0.00[b]	0.12	<0.001	a
benzaldehyde	66	8.34[b]	32.31[a]	7.91[b]	1.53[c]	2.77	<0.001	b
octanal	77	9.26[ab]	4.57[b]	10.95[a]	6.88[ab]	0.83	0.022	a
benzeneacetaldehyde	89	1.27[b]	8.31[a]	3.12[b]	1.54[b]	0.70	<0.001	b
oct-(*E*)-2-enal	90	0.98[c]	1.95[bc]	4.90[a]	2.82[b]	0.39	<0.001	a
2-ethyl-hex-2-enal	99	0.00[b]	1.46[a]	0.00[b]	0.00[b]	0.16	<0.001	a
nonanal	102	22.51[a]	11.86[b]	28.14[a]	27.42[a]	1.68	<0.001	a
non-(*E*)-2-enal	109	0.90[b]	3.04[a]	1.90[b]	1.07[b]	0.27	<0.001	a
decanal	114	1.00[b]	1.53[ab]	0.81[b]	2.07[a]	0.16	0.007	a
dodecanal	126	1.73	2.10	2.62	2.34	0.21	0.517	b
hexadecanal	143	0.00[b]	2.36[a]	0.00[b]	0.00[b]	0.25	<0.001	b
Total aldehydes		80.29[c]	182.33[b]	248.18[a]	82.19[c]	17.09	<0.001	
				Esters				
acetic acid ethyl ester	13	0.00[b]	0.00[b]	17.26[a]	1.94[b]	1.75	<0.001	b
propanoic acid ethyl ester	24	0.00[b]	0.00[b]	0.80[a]	1.11[a]	0.12	<0.001	b
2-hidroxi-propanoic acid ethyl ester	39	0.00[b]	0.00[b]	10.76[a]	0.00[b]	1.08	<0.001	c
2-methyl-butanoic acid ethyl ester	43	0.00[b]	0.00[b]	0.88[a]	1.07[a]	0.12	<0.001	c
butanoic acid ethyl ester	44	0.00[c]	0.00[c]	1.08[a]	0.51[b]	0.10	<0.001	b
hexanoic acid ethyl ester	74	0.00[c]	0.00[c]	12.35[b]	23.54[a]	2.45	<0.001	b
heptanoic acid ethyl ester	97	0.00[b]	0.00[b]	1.49[a]	2.57[a]	0.29	<0.001	b
butanedioic acid ethyl ester	110	0.00[b]	0.00[b]	9.67[a]	0.00[b]	1.11	<0.001	b
octanoic acid ethyl ester	111	0.00[b]	0.00[b]	2.12[b]	16.57[a]	1.63	<0.001	b
nonanoic acid ethyl ester	115	0.00[c]	0.00[c]	0.44[b]	2.01[a]	0.19	<0.001	b
decanoic acid ethyl ester	122	0.00[b]	0.00[b]	0.79[b]	9.89[a]	0.99	<0.001	b
dodecanoic acid ethyl ester	140	0.00[b]	0.00[b]	0.00[b]	1.83[a]	0.18	<0.001	b
tetradecanoic acid ethyl ester	142	0.00[b]	0.00[b]	0.00[b]	1.78[a]	0.18	<0.001	b
Total esters		0.00[b]	0.00[b]	57.63[a]	62.81[a]	7.22	<0.001	
				Aliphatic hydrocarbons				
2-methylpentane	8	2.85[a]	0.00[b]	0.00[b]	0.00[b]	0.31	<0.001	b
3-methylpentane	9	1.23[a]	0.00[b]	0.00[b]	0.00[b]	0.14	<0.001	b
heptane	21	2.02[b]	2.84[ab]	4.60[a]	1.07[b]	0.39	0.003	a
1,1-diethoxyethane	25	0.00[b]	0.00[b]	10.53[a]	0.62[b]	1.06	<0.001	b
decane	75	2.66[b]	0.00[c]	1.91[bc]	10.38[a]	0.93	<0.001	a
tetradecane	123	0.00[b]	0.00[b]	0.92[a]	0.00[b]	0.09	<0.001	a
Total aliphatic hydrocarbons		8.77[b]	2.84[c]	17.96[a]	12.07[b]	1.39	<0.001	
				Aromatic hydrocarbons				
methylbenzene	32	1.46	1.27	1.44	0.87	0.09	0.046	b
ethylbenzene	46	0.52[b]	0.76[a]	0.32[b]	0.00[c]	0.07	<0.001	b
1,3-dimethylbenzene	47	1.93[ab]	2.70[a]	0.94[c]	0.84[c]	0.23	0.003	b
1,2-dimethylbenzene	51	1.51[ab]	0.68[b]	2.92[a]	1.60[ab]	0.27	0.013	b

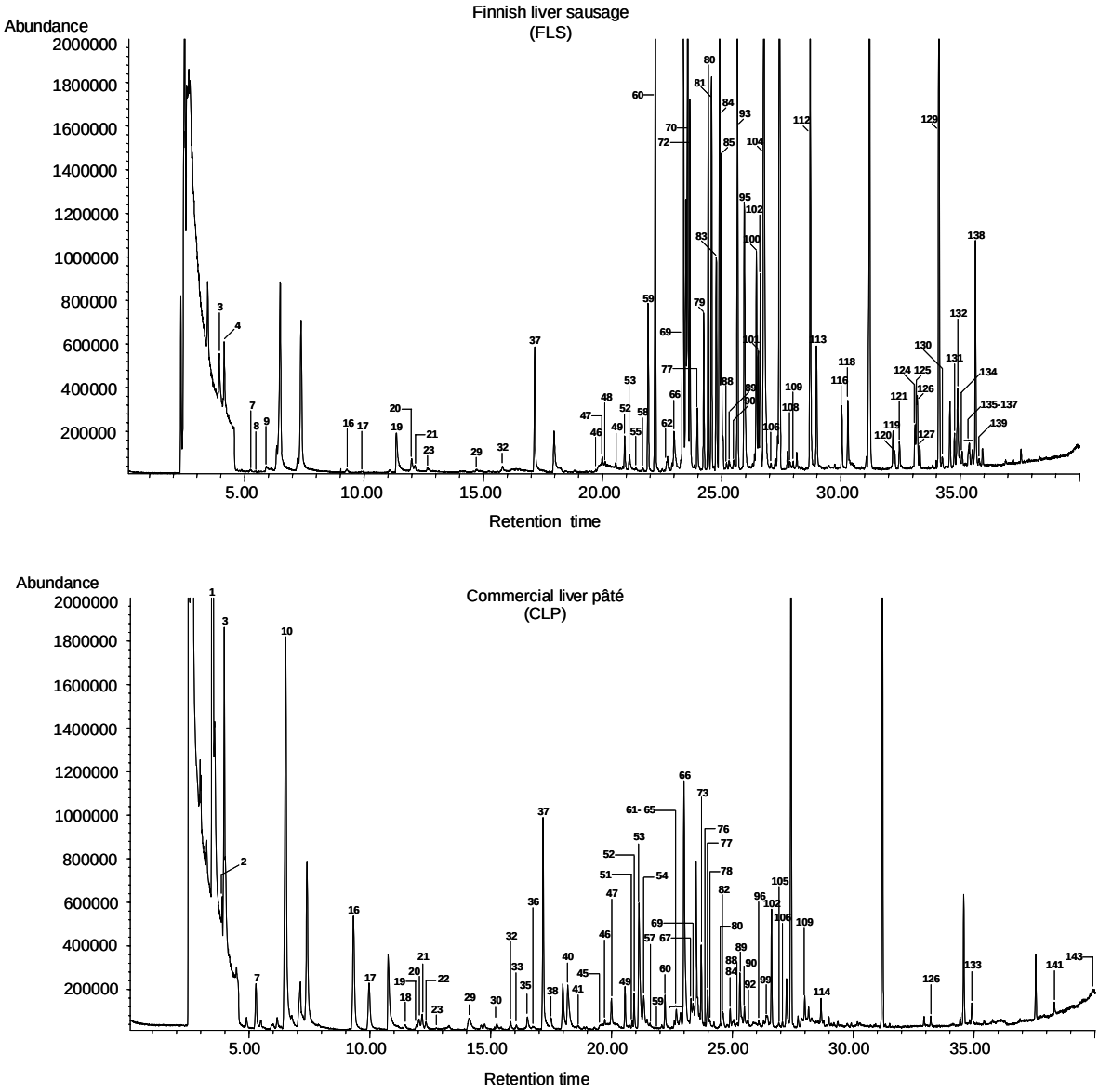
	Peak Nº	Liver products FLS	CLP	HMLP	ELP	sem[1]	p[2]	rel[3]
1-methyl-4(1-methyl-ethyl)benzene	83	24.32[b]	0.75[c]	32.10[a]	1.80[c]	3.22	<0.001	c
1,2-dimetoxi-4(2-propenyl)benzene	125	7.84[a]	0.00[b]	6.33[a]	0.00[b]	0.93	<0.001	c
Total aromatic hydrocarbons		37.58[a]	6.16[b]	44.05[a]	5.11[b]	4.14	<0.001	
				Ketones				
propan-2-one	4	13.35[b]	25.42[a]	0.00[c]	0.00[c]	2.50	<0.001	a
butan-2-one	11	0.00[b]	11.09[a]	0.00[b]	0.00[b]	1.20	<0.001	a
pentan-2-one	18	0.00[c]	1.29[a]	0.46[b]	0.00[c]	0.12	<0.001	b
3-hidroxi-butan-2-one	23	1.10[ab]	1.56[a]	0.00[b]	0.00[b]	0.20	0.002	a
hexan-2-one	36	0.00[b]	0.35[a]	0.00[b]	0.00[b]	0.04	<0.001	a
heptan-2-one	49	1.02[b]	5.43[a]	1.62[b]	0.71[b]	0.48	<0.001	a
6-methylheptan-2-one	61	0.00[d]	0.78[a]	0.46[b]	0.28[c]	0.07	<0.001	b
1-octen-3-one	67	1.16[b]	3.28[a]	1.88[b]	1.30[b]	0.23	<0.001	a
octane-2,3-dione	68	0.00[b]	0.00[b]	0.00[b]	6.26[a]	0.78	0.001	a
octan-2-one	71	0.76[b]	0.94[b]	2.14[a]	0.00[c]	0.18	<0.001	a
nonan-2-one	96	0.51[c]	0.90[a]	0.75[ab]	0.00[d]	0.09	<0.001	a
dodecan-2-one	141	0.00[b]	1.55[a]	1.37[a]	0.00[b]	0.17	<0.001	b
Total ketones		17.90[b]	52.58[a]	8.67[bc]	7.82[c]	4.33	<0.001	
				Nitrogen compounds				
2-methylfuran	12	0.00[b]	1.85[a]	0.00[b]	0.00[b]	0.19	<0.001	b
2-ethylfuran	22	0.00[b]	1.43[a]	0.00[b]	0.00[b]	0.16	<0.001	b
pyrazine	24	0.00[b]	3.66[a]	0.00[b]	0.00[b]	0.39	<0.001	b
pyridine	29	0.49[b]	0.84[a]	0.00[c]	0.00[c]	0.09	<0.001	b
1-H-Pyrrole	30	0.00[b]	0.92[a]	0.00[b]	0.00[b]	0.10	<0.001	b
3,4-dihydro-2H-pyran	35	0.00[b]	1.42[a]	0.00[b]	0.00[b]	0.15	<0.001	b
2-methyl-3-(2H)-dihydrofuranone	38	0.00[b]	1.66[a]	2.78[a]	0.00[b]	0.33	<0.001	b
methylpyrazine	40	0.00[b]	8.79[a]	0.00[b]	0.00[b]	0.92	<0.001	b
2-furanmethanol	45	0.00[b]	1.28[a]	0.00[b]	0.00[b]	0.15	<0.001	b
2,5-dimethylpyrazine	54	0.00[b]	4.75[a]	0.00[b]	0.00[b]	0.49	<0.001	b
2(3H)-dihydrofuranone	55	0.40[b]	0.00[c]	1.10[a]	0.00[c]	0.10	<0.001	b
ethylpyrazine	56	0.00[b]	1.01[a]	0.00[b]	0.00[b]	0.10	<0.001	b
2,3-dimethylpyrazine	57	0.00[b]	0.72[a]	0.00[b]	0.00[b]	0.07	<0.001	b
2-pentylfuran	73	1.25[b]	9.19[a]	1.58[b]	0.72[c]	0.83	<0.001	b
methyl-ethylpyrazine	76	0.00[b]	0.72[a]	0.00[b]	0.00[b]	0.08	<0.001	b
trimethylpyrazine	78	0.59[b]	1.02[a]	0.00[c]	0.00[c]	0.10	<0.001	b
tetrahydro-2H-pyran-2-one	64	0.00[b]	1.97[a]	0.00[b]	0.00[b]	0.20	<0.001	b
3-methylpyridine	92	0.00[b]	1.13[a]	0.00[b]	0.00[b]	0.12	<0.001	b
Total nitrogen compounds		2.73[b]	42.36[a]	5.46[b]	0.72[b]	4.10	<0.001	
				Sulphur compounds				
methanethiol	2	0.00[b]	3.00[a]	0.00[b]	0.00[b]	0.31	<0.001	b
tiobis methane	5	0.00[b]	1.89[a]	0.00[b]	0.00[b]	0.20	<0.001	b
2-methylthiophene	33	0.00[b]	0.73[a]	0.00[b]	0.00[b]	0.08	<0.001	b

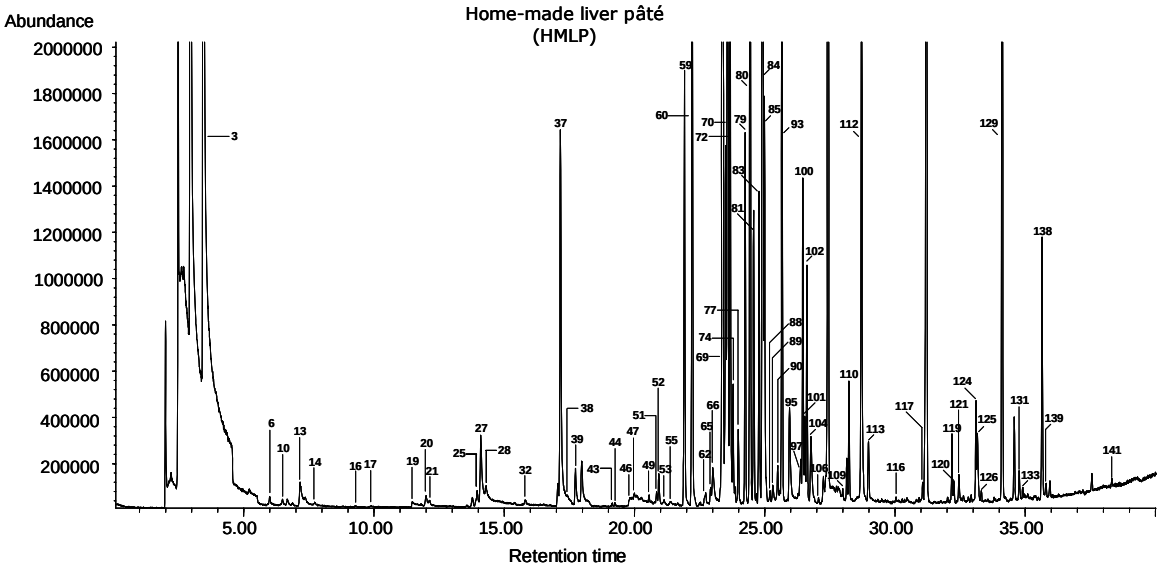
		Liver products						
	Peak Nº	FLS	CLP	HMLP	ELP	sem[1]	p[2]	rel[3]
3-(methylthio)propanal	53	2.64[b]	16.80[a]	1.14[b]	0.59[b]	1.67	<0.001	b
2-acetilthiazole	82	0.00[b]	2.74[a]	0.00[b]	0.00[b]	0.28	<0.001	b
2-acetyl-2-thiazoline	105	0.00[c]	0.92[a]	0.00[c]	0.00[c]	0.09	<0.001	b
Total sulphur compounds		2.64[b]	23.34[a]	1.14[b]	0.59[b]	2.30	<0.001	
				Terpenes				
α-thujene	59	22.33[a]	0.31[b]	26.05[a]	0.00[b]	2.87	<0.001	b
α-pinene	60	74.31[b]	4.75[c]	136.93[a]	1.97[c]	13.17	<0.001	b
camphene	63	1.92[a]	0.00[b]	2.12[a]	0.00[b]	0.23	<0.001	b
β-thujene	69	233.48[b]	4.04[c]	423.52[a]	0.00[c]	41.75	<0.001	b
β-2-pinene	70	82.65[b]	2.60[c]	129.32[a]	0.00[c]	12.93	<0.001	b
β-myrcene	72	37.99[b]	0.00[c]	90.23[a]	0.00[c]	8.65	<0.001	b
1-phellandrene	79	17.78[b]	0.00[c]	46.14[a]	0.00[c]	4.39	<0.001	b
δ-3-carene	80	48.72[b]	0.59[c]	145.44[a]	0.00[c]	13.75	<0.001	b
α-terpinene	81	43.51[a]	0.00[c]	24.43[b]	0.00[c]	4.34	<0.001	b
1-limonene	84	88.99[b]	2.37[c]	234.01[a]	2.05[c]	22.09	<0.001	b
sabinene	85	30.75[a]	0.00[b]	40.47[a]	0.00[b]	4.35	<0.001	b
1,8-cineole	86	3.63[a]	1.02[c]	2.62[b]	0.00d	0.34	<0.001	b
(*E*)-ocimene	88	0.93[b]	0.77[b]	2.00[a]	0.00[c]	0.16	<0.001	b
β-1-pinene	91	0.00[b]	0.00[b]	1.14[a]	0.00[b]	0.12	<0.001	b
γ-terpinene	93	69.05[a]	0.00[c]	48.81[b]	0.00[c]	7.15	<0.001	b
(*E*)-sabinene hydrate	95	40.49[a]	0.00[c]	18.38[b]	0.00[c]	3.85	<0.001	b
δ-4-carene	98	1.57[b]	0.00[c]	3.39[a]	0.00[c]	0.33	<0.001	b
α-terpinolene	100	22.00[b]	0.00[c]	27.46[a]	0.00[c]	2.94	<0.001	b
linalool	101	10.94[a]	0.00[c]	6.87[b]	0.00[c]	1.10	<0.001	b
(*Z*)-sabinene hydrate	104	104.78[a]	0.00[c]	15.01[b]	0.00[c]	10.09	<0.001	b
linalyl propionate	113	14.78[a]	0.00[c]	8.19[b]	0.00[c]	1.43	<0.001	b
linalyl acetate	116	7.17[a]	0.00[b]	0.86[b]	0.00[b]	0.70	<0.001	b
endobornyl acetate	117	1.31[b]	0.00[c]	2.04[a]	0.00[c]	0.20	<0.001	b
δ-elemene	119	3.26[b]	0.00[c]	7.03[a]	0.00[c]	0.68	<0.001	b
alloocimene	120	1.44[b]	0.00[c]	1.91[a]	0.00[c]	0.20	<0.001	b
α-copaene	124	3.63[b]	0.00[c]	8.79[a]	0.00[c]	0.83	<0.001	b
β-elemene	127	2.25[a]	0.00[b]	0.00[b]	0.00[b]	0.22	<0.001	b
calarene	128	0.00[b]	0.00[b]	1.56[a]	0.00[b]	0.16	<0.001	b
(*E*)-caryophillene	129	79.39[b]	0.00[c]	95.00[a]	0.00[c]	10.16	<0.001	b
α-guaiene	130	1.34[a]	0.00[b]	0.00[b]	0.00[b]	0.13	<0.001	b
α-humulene	131	3.44[a]	0.00[b]	3.21[a]	0.00[b]	0.38	<0.001	b
AR-curcumene	132	3.13[a]	0.00[b]	0.00[b]	0.00[b]	0.31	<0.001	b
α-zingibirene	134	1.60[a]	0.00[b]	0.00[b]	0.00[b]	0.16	<0.001	b
β-bisabolene	135	1.25[a]	0.00[b]	0.00[b]	0.00[b]	0.13	<0.001	b
patchoulane	136	2.21[a]	0.00[b]	0.00[b]	0.00[b]	0.22	<0.001	b
γ-selinene	137	1.50[a]	0.00[b]	0.00[b]	0.00[b]	0.15	<0.001	b
δ-cadinene	139	1.16[b]	0.00[c]	1.56[a]	0.00[c]	0.17	<0.001	b
Total terpenes		1053.72[b]	16.46[c]	1547.62[a]	4.02[c]	156.39	<0.001	

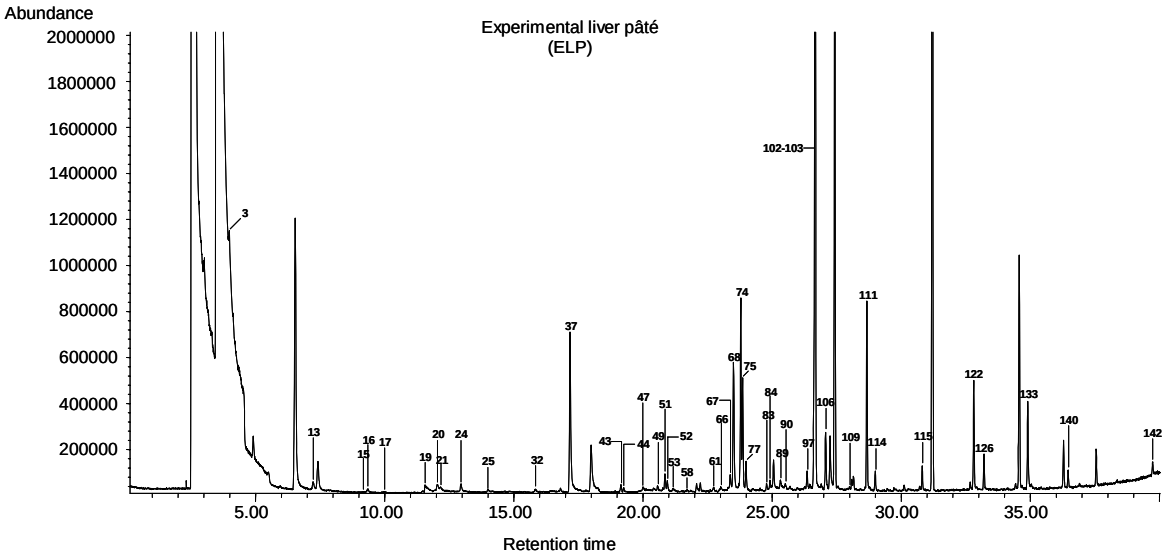
		Liver products						
	Peak Nº	FLS	CLP	HMLP	ELP	sem[1]	p[2]	rel[3]
				Others				
2-ethyl-hexanoate OTMS	103	0.00[b]	0.00[b]	0.00[b]	129.30[a]	13.18	<0.001	c
hexahydro-2H-azepin-2-one	118	3.55[a]	0.00[b]	0.00[b]	0.00[b]	0.36	<0.001	b
4-metoxi-6(2-propenyl)-1,3-benzodioxole	138	20.15[b]	0.00[b]	28.69[a]	0.00[b]	2.93	<0.001	c
Total others		23.69bc	0.00c	28.69b	129.30[a]	11.77	<0.001	

Results expressed as chromatographic area units (AU x 10^6).
In the same line, means with different superscript, significantly differed in ANOVA test.
[1] Standard error of the mean.
[2] Statistical significance.
[3] Reliability of Identification, a: Mass spectrometry + Kovats Index + Coincidence of retention time with standard compound; b: Mass spectrometry + Kovats Index; c: Mass Spectrometry.

Figure II.1. Total ion chromatograms from the liver products. (Number of peaks according to information given in Table 4).




Home-made liver pâté
(HMLP)
Abundance
Retention time


Experimental liver pâté
(ELP)
Abundance
Retention time