NEW TECHNOLOGIES APPLIED IN PESTICIDE RESIDUE ANALYSIS

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INTRODUCTION

The development of new technologies and their implementation in the analysis of pesticides in food and the environment has greatly affected the way we perceive and use pesticides. In the 1940's and 1950's, pesticides were perceived as miracle chemicals that gave tremendous gains in crop yields, and they were used without adequate regard to health and the environment. At that time, thin-layer chromatography with semi-quantitative detection was the primary means of analysis. Gas-liquid chromatography (GLC, or simply GC) with packed columns became the method of choice as commercial instruments improved and selective quantitative detectors were developed in the late 1950's to mid-1960's. By the time of the publication of Rachel Carson's *Silent Spring* in 1962, GC was the predominant method of analysis. When the environmental and ecological impact of basically unregulated applications of pesticides became known, the perception of pesticides began to change. Laws that established regulatory controls on the use of pesticides and their presence in foods required residue analysis using state-of-the-art instrumentation.

With the development of improved capillary columns for GC in the 1970's, tremendous gains in separation power were achieved, and the capabilities of multiresidue methods improved accordingly. During the same time-frame, high-performance liquid chromatography (HPLC) was commercialized, and its implementation in pesticide residue analysis permitted detection of many compounds that were not easily analyzed previously. Through the complementary nature of GC and HPLC, a wide range of pesticides could be analyzed, and many environmentally safer pesticides were developed and registered through the use of these and other technologies. Just as these approaches greatly altered pesticide residue analysis in the past, several new technologies of today will impact the future of pesticide residue analysis.

Approaches to Pesticide Analysis

Despite the great advances made in chromatographic partitioning and detection of pesticides, many current methods of extraction remain essentially the same as those developed

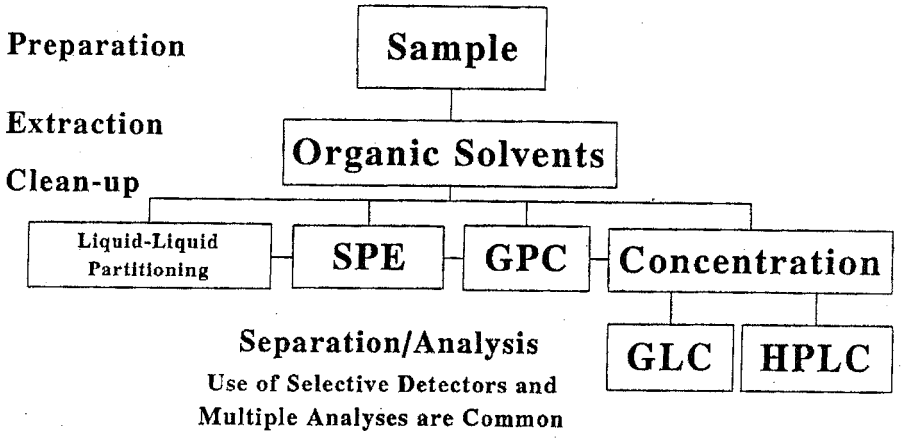

Figure 1
Traditional Approach for Multiresidue Analysis of Pesticides in Foods
(SPE = solid-phase extraction; GPC = gel-permeation chromatography; GLC = gas-liquid chromatography; HPLC = high-performance liquid chromatography)

in the early days of pesticide analysis. Traditionally, the way chemists have performed analysis is to extract many pesticides with a single initial step, then spend the majority of time and effort removing co-extracted interferants using a variety of clean-up approaches. GC and HPLC with selective detectors have been the two main options in analysis for most pesticides. Traditional methods are often time-consuming, labor-intensive, expensive, and require a lot of glassware, specialized equipment, and lab space. Another consideration, which was not much of a concern to chemists in the past, is that many classical methods of analysis expose workers and the environment to hazardous solvents and generate a great deal of hazardous waste. An inherent problem with these methods is the solvent evaporation and exchange steps that must be performed to achieve the desired limits of detection for analysis. Figure 1 exhibits the generalized traditional approach to multiresidue analysis.

Currently, despite the public perception that pesticides are a serious hazard in food, very few food samples exceed regulatory tolerance levels. Also, with the development of safer, more selective pesticides and improved farming practices, such as integrated pesticide management, pesticide residue analysis will not maintain such a high priority. In all likelihood, the regulation of pesticides in food will continue its important function in society, but how much are we willing to spend on this issue, especially in light of other social concerns facing us? Due to pressures to reduce the expense, time, labor, and waste in performing pesticide residue analysis, the time has come to reconsider the traditional approach of exhaustive extraction, intensive clean-up, and selective detection.

Fortunately, several new technologies have emerged that address the present heightened concerns. Figure 2 presents a possible new approach using advanced technologies to minimize the hazardous waste produced, and which also can be easily automated to reduce labor in routine

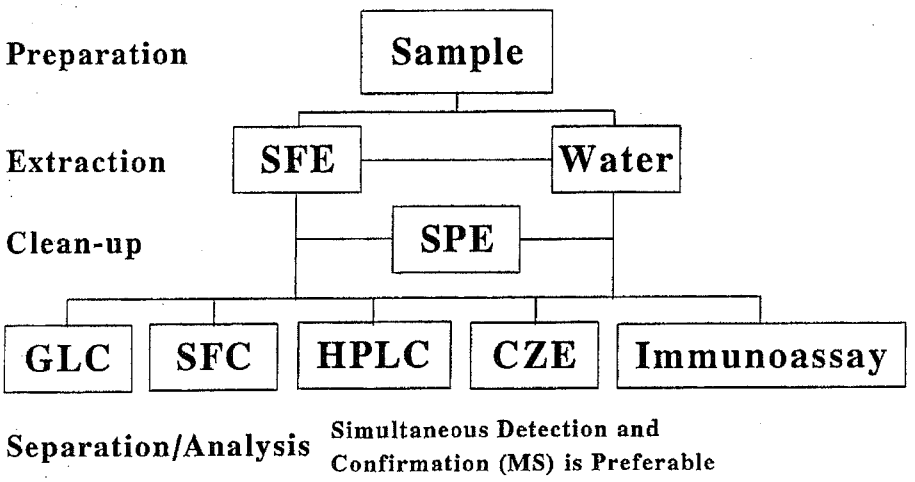

Figure 2
Potential New Approach for Multiresidue Analysis of Pesticides in Nonfatty Foods (SFE = supercritical fluid extraction; SFC = supercritical fluid chromatography; CZE = capillary zone electrophoresis)

analysis. In this approach, selectivity is emphasized in the extraction process, and fewer clean-up steps would be needed. Detection still relies on GC for the majority of pesticides, but a universally selective and confirmatory detector, such as mass spectrometry, would replace the multiple selective GC detectors. This and other aspects of the potential overall approach to pesticide analysis will be discussed in the following pages.

Selectivity

The degree of selectivity for the analyte(s) that the sample preparation procedure achieves is one of the two most important measures of an analytical method (the other is the limit of detection). An inherent difficulty with multiresidue methods, however, is that selectivity decreases as the polarity range of pesticides included in the method expands. Conversely, to increase selectivity involves reducing the number of pesticides that can be included in a method. Due the wide polarity range of pesticides, no existing method can detect all pesticides from all commodities. Therefore, a number of methods must be used in an overall analytical scheme for a variety of matrices. How the chemist divides the work to cover the entire range of pesticides should be designed to minimize cost and convenience without sacrifice of good quality assurance.

The easiest way to reduce the amount of co-extracted interferants, and minimize solvent usage, is to simply use a smaller sample. Another way to increase selectivity is to use a selective extraction solvent that is free of impurities. Further clean-up steps can be averted if the initial extraction can achieve a high degree of selectivity. If necessary, however, there are a variety

of clean-up approaches involving liquid-liquid and liquid-solid partitioning after extraction to make further gains in selectivity. The ultimate approach for enhancing selectivity of a method is to use an analytical technique that can distinguish the analytes in the presence of a complex matrix.

Criteria for Acceptance of a New Technology

In general, it has taken a decade from when a new technology was first developed in the scientific community before its implementation in residue analysis. A new technology must overcome several hurdles, such as commercial availability and validated results, before it even will be considered by regulatory agencies. Good laboratory practices and quality assurance protocols that meet regulatory agency criteria must be followed in obtaining validated results, and a comparison of results of the new approach with existing methods for real samples should be performed. Assuming that basic criteria have been met, the new approach must be substantially advantageous to existing approaches. Furthermore, the economics of purchase and operation of the new technology must not exceed laboratory budgets. Ultimately, even if a technique has been proven to give reproducibly accurate results at the desired residue concentrations, and if commercial instrumentation at affordable cost is available, the technique may still not be implemented unless there exists a willingness for change. A great deal of expense, time, and effort are required to develop and implement new techniques of analysis, and in this era of rapidly changing technology, newer technologies are always on the horizon to potentially replace the previous approach.

Objectives

The goal of my research in the U.S. Department of Agriculture's Agricultural Research Service is to develop and help implement better methods for the analysis of chemical contaminants in agricultural and food products. In particular, investigations have concerned: 1) rapid, uncomplicated, and inexpensive analytical screening methods (immunochemical techniques); 2) multiresidue methods to extract, quantify, and confirm residues in nonfatty foods (supercritical fluid extraction and GC/ion-trap mass spectrometry); and 3) the determination of diverse, polar herbicides in soybeans with a single method (accelerated solvent extraction and capillary electrophoresis).

The main goal of this paper is to provide an overview of modern alternate technologies to the traditional approaches. The information provided is designed to show the potential of the new technologies for use in applications involving pesticide residue analysis. Due to the broad scope of this topic, only selected new approaches will be discussed, and only the most significant results and conclusions of investigations will be presented (including some previously unpublished work). The discussion of new methods will be divided into three categories that conform to the analytical process: 1) extraction, 2) clean-up, and 3) analysis.

EXTRACTION

Extraction is a fundamental process in pesticide residue analysis, at least until an *in situ* technique emerges that can adequately detect trace levels of pesticides in a complex matrix. For extraction, there are several alternatives to the traditional methods that use copious quantities of organic solvents. These alternate approaches include: supercritical fluid extraction (SFE) (1), pressurized liquid extraction (PLE) (2), aqueous extraction (3), microwave-assisted extraction (MAE) (4), solid-phase extraction (SPE) (5), solid-phase microextraction (SPME) (6), and matrix solid-phase dispersion (MSPD) (7). Only the first three techniques will be described in greater detail. With the development of new approaches, older approaches that have been dismissed can be re-investigated to determine if they can fill a niche in an advantageous overall analytical scheme involving the use of a new technique.

Supercritical Fluid Extraction (SFE)

SFE makes use of a supercritical fluid as the extraction solvent. Although the roots of SFE trace back several decades, the first commercial instruments did not appear until the late 1980's. Carbon dioxide is the most common solvent in SFE because it is safe, unreactive, readily available, relatively inexpensive, and has a low critical pressure and temperature point (74 atm and 31°C). A key property of supercritical fluids is that fluid density, which is related to solvating power in SFE, can be controlled by changing extraction temperature and pressure. This aspect is not easily managed with individual liquid solvents. Also, supercritical fluids have low viscosity, even at high fluid density, which allows good penetration into the matrix. In an inherent advantage over liquids, concentration of the analytes after extraction in SFE is convenient and fast because supercritical CO_2 becomes a gas after depressurization. SFE is also rapid, inexpensive to operate, generates no hazardous waste, takes up little lab space, uses no glassware, and lends itself to automation.

Disadvantages of SFE include the large capital cost of automated commercial instruments and the limited capabilities and questionable reliability of some instruments. Due to the many parameters of SFE, method development can be very time-consuming. With automated commercial instruments, sample size for extraction may be less than desirable to accurately represent the entire sample; therefore a reliable subsampling procedure must be used. Furthermore, gases may not be currently available to perform SFE in remote locations. A problem with SFE in certain cases is that the extraction fluid is too weak to break analyte-matrix interactions in the sample, but there are ways to break these interactions, such as adding solvent modifier, increasing temperature and/or pressure, or other means.

Method Development in SFE

The overall approach to method development in SFE can be divided into three aspects: 1) preparing the sample for SFE, 2) optimizing extraction conditions, and 3) collecting the analytes

Table 1
Parameters in SFE

Sample Preparation	Extraction	Collection
Sample Size	Extraction Fluid	Solvent or Sorbent?
Subsample Size	Temperature	Solvent or Sorbent Type
Subsampling Method	Pressure	Solvent Volume or Trap Size
Water Content	Modifier Type?	Collection Temperature
Drying Agent Type?	Modifier Amount	Trap Elution Solvent
Drying Agent Ratio	Static Time	Elution Volume
Vessel Size	Dynamic Time	Elution Temperature
Packing Density	Flow Rate	Elution Flow Rate

after SFE. Table 1 lists parameters from each category in SFE that should be optimized and controlled in method development studies. For most accurate results, real samples should be used in optimization studies when possible. In my opinion, method development should be performed in reverse to the SFE process. For example, collection parameters should be determined initially because inefficient collection undermines efficient extraction. Then, the extraction parameters should be determined by controlling each variable individually. Solvent modifiers should not be added unless CO_2 alone cannot perform the extraction (water is the strongest modifier). Depending on the application, method development can be a long process, especially when reiterations are required to determine the effect of one parameter on another.

Fortunately, a number of researchers have developed working SFE methods for a variety of pesticide residue applications. Table 2 presents the SFE conditions for extraction of multiple pesticides from foods developed by several different researchers. Reproducibly high recoveries for well over 100 pesticides in all have been achieved using these methods, and several studies have demonstrated comparable results of SFE and traditional methods for real samples. Based on these and other investigations of SFE, several important conclusions can be made about extraction of pesticide residues:

1) SFE is a viable approach to extract multiple and individual pesticide residues in foods.
2) SFE reduces operating costs, time, labor, space, waste, and glassware involved in routine analysis versus many traditional approaches.
3) Nonfatty foods often require no clean-up before analysis, but clean-up is frequently necessary for fatty foods after SFE.
4) Sample size reduction for SFE requires careful techniques and use of frozen samples to maintain sample homogeneity and to reduce pesticide losses (14).
5) Many current commercial instruments should be improved to achieve higher sample throughput, more versatility, and better reliability.
6) Water and salts in the sample can have strong effects on the SFE process.
7) SFE with CO_2 does not work well for certain pesticides (those that can be readily charged in particular) in some matrices.

Table 2
Comparison of SFE methods for extraction of multiple pesticide residues in food

Ref.	Sample Preparation	Extraction	Collection/Clean-Up	Results
8	26 g (produce) or 50 g (meat) + 13 g Hydromatrix in 73 or 154 mL vessel	680 atm and 80°C (0.95 g/mL), 20 min dynamic at 10 mL/min (1.3-3 vessel volumes)	collection in empty boiling flask, traditional clean-up, GC analysis	comparable for 34 pesticides in meat, fat, produce, grain, and other foods vs. traditional
9	50 g frozen produce + 50 g Hydromatrix, 6 g mix (3 g sample) in 10 mL vessel	350 atm and 50°C (0.9 g/mL), 2 min static, dynamic at 2 mL/min (6 vessel volumes)	collect on 1 mL C-18 at 15°C, elute with 1-1.5 mL acetone, GC/ITD analysis	validated for 50+ pesticides in produce, low recovery of methamidophos
10	20 g sample+ 28 g $MgSO_4$; 8 g (3.3 g sample) in 10 mL vessel + 0.2 mL MeOH	300 atm and 50°C (0.88 g/mL), 1 min static, 15 min dynamic at ≈ 1 mL/min (1.5 vessel volumes)	bubble into 3-5 mL EtOAc, no clean-up, GC and HPLC analysis	high recovery of methamidophos and 15 others
11	homogenize 500 g frozen sample, 2.2 g + 1.8 g Hydromatrix in 10 mL vessel	320 atm and 60°C (0.85 g/mL), 22 min dynamic at 1.8 mL/min (4 vessel volumes)	bubble into 9:1 acetone:iso-octane, no clean-up, GC analysis	24 determinations for 12 pesticides in 5 commodities versus traditional method
12	2 g sample + 0.8 g celite + 2.5 g Na_2SO_4 in 10 mL vessel	189 bar and 45°C (0.8 g/mL), 1 min static, 20 min dynamic at 2.5 mL/min (7 vessel volumes)	collect on 1 mL C-18 at 45°C, elute with 1.5 mL 1:1 hexane:acetone; GC	high recoveries for 92 pesticides including methamidophos
13	grind and homogenize frozen sample, 6 g in 10 mL vessel +2 mL 4:1 MeCN:H_2O	340 atm and 60°C (0.87 g/mL), 10 min static, 25 min dynamic, 1.5 mL/min (3.75 vessel volumes)	bubble into 7 mL MeCN at 4°C, -NH_2 SPE clean-up, GC and HPLC analyses	validated and implemented for 41 pesticides in grains

Effect of Water and Salts

The role of water is very important in SFE. Water acts to improve recoveries of many pesticides, but too much water in the sample can form a barrier to the immiscible extraction fluid to water-insoluble pesticides. Furthermore, in the presence of excessive water, several polar pesticides prefer to remain in the water phase and give poor recoveries. A salt can sometimes effectively salt out these pesticides into the supercritical phase, but this may reduce recoveries of nonpolars.

Several organonitrogen pesticides (imides, amides, amines) have exhibited difficulties in extraction with supercritical CO_2, either due to hydrogen bonding with hydroxyl groups in the matrix, or ionization that renders them insoluble in supercritical CO_2. Diatomaceous earth drying agents contain hydroxyl groups that retain these type of pesticides (methamidophos, acephate, omethoate), but magnesium sulfate drying agent permits high recoveries of these pesticides (10). Other pesticides, such as imidazolinone, sulfonyl urea, and other polar herbicides, simply are not suited for good extraction using SFE. Derivatization is a potential approach to force the pesticides to be extractable by SFE, but other complementary approaches to SFE, such as aqueous-based PLE and MAE, are available that can more conveniently extract these type of pesticides.

Pressurized Liquid Extraction (PLE)

PLE, also known as accelerated solvent extraction, was developed by Dionex scientists within the last few years as a competing approach to SFE. The design of an PLE instrument (Figure 3) is very similar to the design of a SFE instrument except a liquid is used rather than a supercritical fluid for extraction. The PLE approach is also similar to SFE in that relatively small samples are loaded into vessels, which require a dispersant/drying agent to aid solvent flow, and extraction occurs at elevated temperature and pressure to improve recoveries. As in SFE, a static extraction mode is used to equilibrate the sample to the extraction conditions and dissolve the analytes in the fluid, but PLE does not have a dynamic mode as in SFE. In PLE, the vessel is flushed with solvent rapidly, rather than a controlled dynamic flow in SFE using a restrictor; thus, PLE is often faster than SFE. Vessel sizes in current PLE instruments are 11, 22, or 33 mL with 40 or 60 mL collection vials. Extraction temperature can be varied from room temperature to 40-200°C and a pressure of 1000-3000 psi can be set. Any liquid solvent can be used provided it can be pumped through the sample and its pH falls between 2-12. Typical extraction time is 10-20 min (not including water removal, clean-up, and solvent evaporation steps).

PLE reduces time and solvent volume for extraction by taking advantage of the increased analyte solubility and extraction kinetics at higher temperature versus traditional methods. Pressure has less influence on analyte recoveries than temperature in PLE, but high pressure 1) maintains the solvent in the liquid state at high temperatures; 2) helps the solvent enter small pores in the sample; and 3) forces flow through the sample and filter during the short dynamic mode. PLE also conveniently provides a filtered extract (2 μm). The choice of extraction solvent is the primary means of controlling the extent of selectivity in PLE. However, at increased temperature, more potential interferants from the matrix are co-extracted with the analytes and more clean-up may be necessary than with traditional methods.

Method development is simple in PLE due to the few parameters involved and adaptability of existing liquid-based methods. In an uncomplicated study, Lehotay and Lee adapted a current multiresidue approach developed by Fillion, *et al.* (15), to PLE, and we compared results for

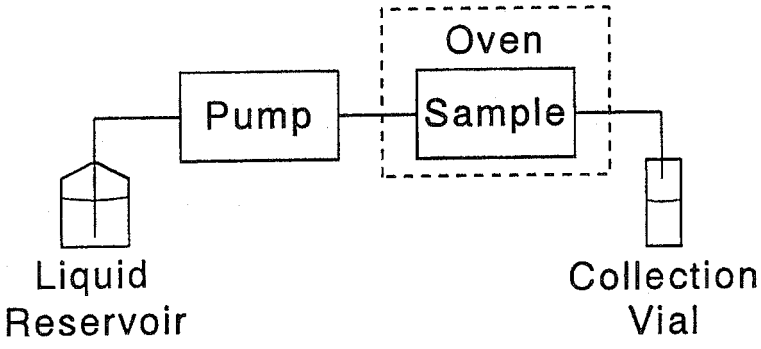

Figure 3
Instrument Arrangement of Pressurized Liquid Extraction

Table 3
Comparison of PLE and SFE recoveries and limits of detection (LOD) of several pesticides fortified in tomato

Pesticide	PLE		SFE	
	%recovery (%RSD)	LOD (ng/g)	%recovery (%RSD)	LOD (ng/g)
imazalil	109 (3)	220	41 (27)	770
thiabendazole	109 (5)	64	4 (77)	>1000
acephate	88 (1)	130	7 (2)	76
methamidophos	95 (8)	20	7 (12)	150
omethoate	89 (7)	66	14 (29)	820
dimethoate	98 (3)	23	99 (4)	23
dichlorvos	55 (5)	8	91 (5)	2
carbofuran	101 (2)	4	99 (3)	3
carbaryl	93 (3)	35	101 (3)	3
fenamiphos	105 (5)	18	84 (3)	4
myclobutanil	109 (1)	15	103 (2)	9
diazinon	98 (1)	1	94 (2)	1
atrazine	115 (1)	13	113 (8)	2
parathion	104 (1)	3	101 (4)	1
phosmet	85 (12)	12	103 (3)	2
azinphos-methyl	102 (2)	130	110 (6)	29
iprodione	102 (11)	28	104 (2)	6
lindane	104 (2)	82	101 (3)	32
vinclozolin	104 (0)	5	99 (1)	1
chlorpyrifos	102 (11)	9	85 (4)	1
dacthal	108 (5)	5	98 (5)	0.5
endosulfans	103 (1)	20	92 (6)	12
quintozene	92 (1)	9	92 (3)	3
pentachlorobenzene	69 (1)	4	70 (2)	2
trifluralin	94 (4)	0.5	66 (9)	0.3
aldrin	104 (3)	18	64 (5)	9
p,p'-DDE	112 (3)	3	61 (10)	3
o,p'-DDT	102 (3)	3	65 (8)	2
permethrins	103 (2)	3	57 (7)	1
fenvalerates	104 (2)	10	59 (8)	5

54 pesticides in tomato with SFE (16). The PLE procedure was: 1) mix produce sample 1:1 with drying agent and load 4 g in 11 mL vessel; 2) perform PLE using acetonitrile at 60°C, 2000 psi, 7 min static, ≈ 22 mL extraction volume (1 cycle and 60 s N_2 purge time); 3) add 1 g NaCl, shake, centrifuge, and pass upper layer through a short column of Na_2SO_4; 4) evaporate acetonitrile to 2 mL, add internal standard and inject in GC/ion trap MS detection (ITD). Normally, SPE clean-up is required for the extracts, but tomato is a relatively clean matrix and GC/ITD can still detect analytes in the presence of matrix components.

Table 3 lists results of this comparison study for selected pesticides which are listed in order of decreasing polarity. PLE gave much better recoveries of the polar pesticides that cause

difficulties in SFE with diatomaceous earth drying agent. In the case of nonpolars, PLE gave higher recoveries because the acetonitrile extraction solvent was miscible with water in the sample whereas water was a barrier to their extraction in SFE. Reducing water content in the sample improves recovery of nonpolars in SFE. All other pesticides, of moderate polarities, gave similar results in PLE and SFE. Although PLE gave higher recoveries for a wider range of pesticides, SFE gave lower limits of detection (LOD), despite the lower recoveries, due to fewer matrix interferants. Furthermore, the overall SFE procedure was easier to perform and faster than PLE.

Aqueous Extraction

Water can be used to extract pesticides that are not easily extracted using SFE. In this way, SFE and PLE with water can be complementary automated methods to extract nearly all pesticides. Water is a preferable extraction solvent because it is cheap, readily available, and safe. Selectivity of extraction can be controlled with pH, ionic strength, heat, and addition of detergents. To achieve temperatures > 100°C, pressure must be applied, which is possible in PLE or other means. In MAE, water is the best choice of solvent due to its high microwave absorption property. Concentration of water extracts can be done using SPE rather than with time-consuming and wasteful solvent evaporation steps commonly used for organic solvents. Water is also compatible with SPME, an interesting recent development that can permit direct GC or HPLC injection of extracts that partition onto a sorbent coated on a small probe (6).

A problem with water-based extraction of foods is the greater extent of co-extracted polar interferants which create difficulties with GC analysis. Also, some matrices are simply incompatible for water based extraction due to the inability to separate the liquid from the pulp. However, with the use of HPLC, capillary electrophoresis, and immunochemical methods of analysis, water becomes the desired solvent. These analytical techniques do not currently have the same multiresidue capabilities of GC, but for very polar, ionic, or thermally labile pesticides, GC is not an option.

PLE with Water

A PLE experiment was performed to determine which pesticides could be extracted with water from diatomaceous earth, and to determine the effect of temperature. As shown in Figure 4, semipolar pesticides such as atrazine and myclobutanil gave good recoveries from 25-200°C. For carbamates, such as carbofuran and propoxur, use of water at low temperature was better, and the pesticides either degraded or were not soluble in hot, pressurized water. For nonpolar organochlorine (OC) pesticides, such as aldrin and DDE, recoveries improved as temperature increased, but for the most nonpolar pesticides, such as pyrethroids, water could not extract the pesticides at any temperature tested. In the case of organophosphorus (OP) pesticides, which cover a wide polarity range, a more complex picture materialized. Water temperature greater than 150°C tended to decrease recoveries of all OPs, presumably due to

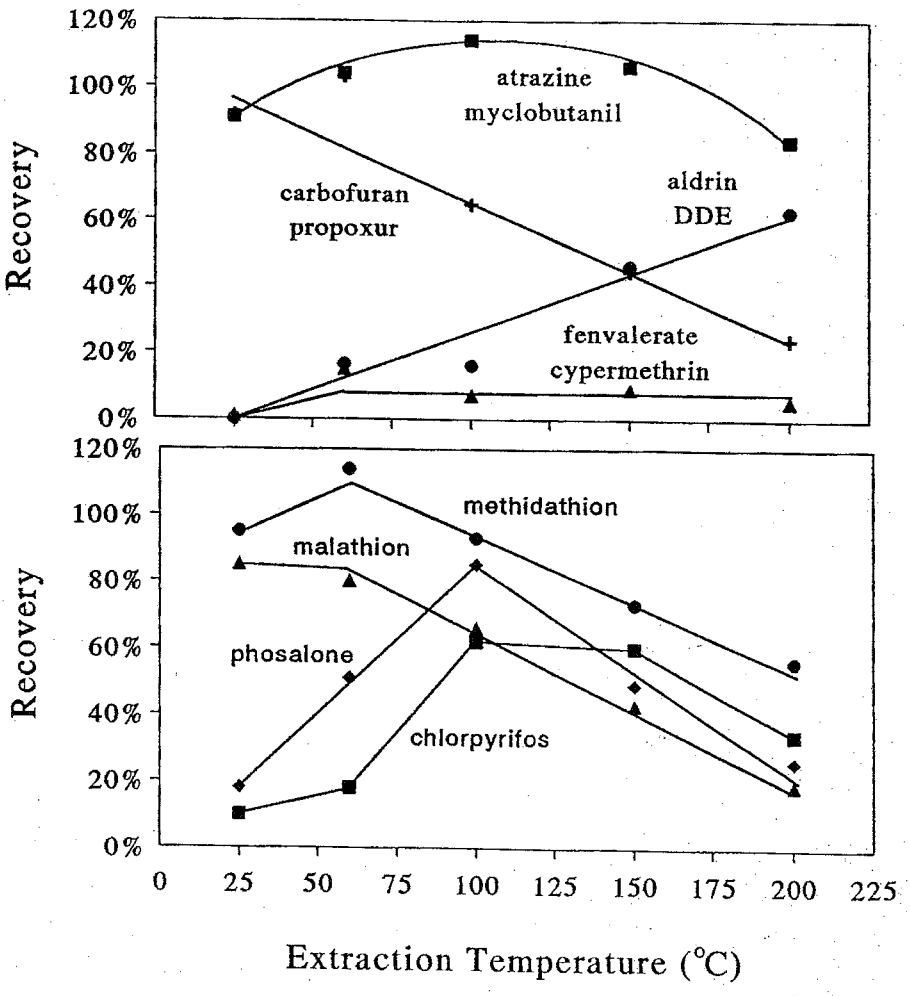

Figure 4
Recoveries of Pesticides in PLE with Water at Different Temperatures

degradation. The recoveries for individual pesticides varied versus temperature; some pesticide recoveries peaked at 60°C while others peaked at 100°C or 150°C.

In summary, water can be a useful extraction solvent for relatively nonpolar pesticides at high temperatures, but SFE or organic solvents for extraction are better methods for those pesticides. High recoveries of semipolar pesticides can be achieved with water at moderate temperatures, but recoveries often decrease as temperature is increased. SFE or organic solvents typically work well for these pesticides, too. For the most polar or ionic pesticides that do not work well in SFE, such as sulfonyl urea and similar herbicides, water is often a very good medium for extraction. Typically, low temperature should be used to avoid degradation and proper selection of pH is essential.

CLEAN-UP

With the development of more selective extraction techniques and analytical methods that are less affected by co-extracted matrix components, clean-up becomes less important than with many current methods. However, clean-up will continue to be necessary in many applications for years to come, especially for residue analysis of fatty foods. Liquid-liquid and liquid-solid (*e.g.*, SPE) partitioning, in which separation is based on polarity, are the primary means of clean-up in residue analysis. For separation based on molecular weight, gel-permeation or size-exclusion chromatography (GPC or SEC) is a common clean-up technique. Biologists commonly use precipitation, centrifugation, ultrafiltration, dialysis, immunochemical techniques and/or other means of separations in sample preparation methods, but few of these approaches have been widely applied in residue analysis. Immunoaffinity chromatography in particular appears to be an applicable technique of the future for selective separation of analytes from complex matrices.

Automation

The recent heightened concerns in labor reduction and ease of use have led to a push for automation of clean-up techniques. Automation has always been a desirable trait, but automated devices for traditional methods are cumbersome and expensive. With smaller samples in the new type of approach, automated clean-up instruments can often be combined in series with automated SFE, PLE, and/or analytical instruments. In an automated extraction/clean-up/analysis instrument arrangement, off-line automation (independent instrument operation) is often preferable to in-line automation for several reasons: 1) the extract is recovered for re-analysis if necessary, rather than having to repeat the entire process; 2) sample throughput is increased by having the extraction and analytical process occur simultaneously; 3) for in-line set-ups, when one part of the instrument is broken, the entire set-up is broken; and 4) each instrument can be used individually in off-line set-ups, whereas in-line arrangements require undoing the connections.

Solid-Phase Extraction (SPE)

The solid-phase cartridge format was developed soon after HPLC when high quality sorbent materials became available. The cartridges are used for extraction of water samples, but a large part of their use has been for clean-up of extracts. The mechanism of separation in SPE is the same as in HPLC: the type of solvent and solid-phase material can be varied in an attempt to achieve the desired separation. Essentially, options for phases in SPE are the same as in ion-exchange, and reversed or normal-phase liquid chromatographic methods. Particle sizes are larger in SPE, and some common SPE stationary phases, such as, florisil, alumina, and carbon, are not so common in HPLC. As new stationary phases are developed for HPLC, these sorbents often become available in SPE. Recent developments include polymer-based phases, which do not retain certain compounds that were problematic with silica-based phases, and an SPE disk format. Although the disk format can become clogged more easily than cartridges, the disks allow faster throughput for large sample volumes.

Another fairly recent development is MSPD extraction which incorporates the solid sorbent in SPE with the sample, and clean-up is performed during the extraction. Although MSPD is not commonly mentioned by name, this technique is commonly done in SFE and PLE to retain water or unwanted matrix components. Conversely, choice of conditions can be made to retain the analyte and remove the interferants with one solvent followed by elution of the analyte with a different set of conditions.

A drawback with the use of certain sorbents in solid-phase techniques is the cost. In most methods that use any SPE approach, the sorbents are often the most expensive materials in the procedures. If the sorbents can be regenerated and re-used, costs are reduced dramatically, but most cartridges are designed to be discarded after a single use. SFE-SPE studies have shown that sorbent traps can be flushed and re-used hundreds of times, and other automated instruments also make use of this aspect.

Gel-Permeation Chromatography (GPC)

GPC was first developed in the late 1960's and early 1970's, and began to be used in pesticide regulatory methods in the late 1970's and early 1980's. GPC, also known as size-exclusion chromatography (SEC), separates compounds with large molecular weight, such as lipids and proteins, from smaller analytes, such as pesticides using a porous stationary phase material. Recent developments in GPC include investigations of uncommon gels and solvents for separation, and the introduction of more densely-packed, smaller columns. New instruments have incorporated solvent reduction, evaporation, and recycling, and automation into the designs. GPC can be automated in series with SFE (17), and studies have been performed that utilize supercritical CO_2 mixed with liquids as the mobile phase in GPC.

ANALYSIS

Analytical methods and instrumentation have improved immensely over the years. Even long established approaches such as GC and HPLC are still undergoing significant improvements in techniques and instrument designs many years after their initial development. Advances in computer technologies has led to tremendous gains in instrument control, data collection, and analytical determinations in recent years, and this trend will continue (state-of-the-art instruments today may be obsolete in 10 years).

In HPLC, coupling of electrospray ionization mass spectrometric (MS) detection has enabled detection and confirmation of many polar pesticides at trace concentrations. HPLC/MS with atmospheric pressure ionization techniques are expected to greatly enhance analytical capabilities for polar and ionic pesticides. The first appearance of a commercial, benchtop HPLC/ion-trap tandem mass spectrometric (MS-MS) detector has just recently occurred. This instrument is still expensive, but costs much less than triple stage quadrupole MS-MS instruments. Other developments in HPLC are the use of narrow-bore columns to improve separations and reduce solvent usage, and introduction of polymer-based stationary phases.

Investigations of capillary electrophoresis as a possible replacement for HPLC has only just begun in residue analysis, and perhaps in the future, HPLC will not be as prominent as it is currently. Also, supercritical fluid chromatography (SFC) can perform separations of pesticides that have been traditionally done using HPLC, as well as those done by GC. A recent trend in SFC is to use HPLC columns as the stationary phase, and supercritical CO_2 with novel modifier combinations as the mobile phase. Detection in SFC has been problematic, but when detection capabilities improve, SFC and CE may be able to cover the entire range of pesticides for separation and analysis.

Gas-Liquid Chromatography (GC)

In GC analysis, recent developments include coupling to several new and improved detectors, such as atomic emission spectrometry, ITD and MS-MS, pulsed flame photometry, and radiationless electrochemical detection. New GC instruments have 1) automatic pressure control to provide highly reproducible separations and peak shapes, 2) high volume injectors which effectively decrease detection limits, and 3) fast GC capabilities (1-3 min separations) using narrower capillaries. New stationary phases are continually being developed, and the latest features include low-bleed polar phases. Modern analytical software enables excellent control of data, and can generate reports automatically.

GC/MS was first commercialized in the late 1970's and only recently has it been given serious consideration by many regulatory agencies to possibly replace selective detectors. The LOD using GC/MS with quadrupole devices was lacking in the past, but the most recent generation of quadrupole MS detectors in full-scan operation with electron-impact ionization, approach the LOD capabilities of selective detectors for a wider range of pesticides. The use of selected ion monitoring (SIM) further reduces the LOD, but confirmation of pesticides is a little more problematic with this approach. GC/ion-trap MS makes gains in efficiency of

detection versus quadrupole devices, and permits MS-MS to further avoid co-eluted matrix components. Interestingly, the cost of a GC/MS-MS instrument is not much higher than that of many other GC systems. The main advantage in the use of mass spectrometry for detection versus traditional detectors is that quantitation and confirmation of a wide range of pesticides in a complex extract can be done with a single injection. Unfortunately, MS using the same conditions cannot adequately detect all pesticides at this time, and chemical ionization or other techniques are required to achieve the desired results for certain pesticides (18). Also, precautions in quantitation and careful review of the results should still be performed by the analyst, despite the excellent software capabilities.

Like GC/MS, GC/atomic emission spectrometric detection (AED) is a universally selective method of detection (19). Unlike GC/MS in the full scan mode, though, the information AED provides is not adequate in itself to meet pesticide confirmation criteria. AED provides the elemental composition of a compound, which is very helpful in compound identification, but not conclusive. In combination with carefully determined and preprogramed relative retention ratios, the software can identify and quantify the analyte with confidence, but legal cases may not accept this as confirmatory. GC/AED monitors atomic emission wavelengths for selected elements in an inductively-coupled plasma. Several elemental channels can be monitored simultaneously to provide information which can help identify unknown compounds. Typically, GC/MS is used to look for a known set of analytes, but the combination of GC/MS+AED can provide an excellent way to detect and identify unknown contaminants or metabolites. Other aspects of GC/AED include 1) accurate single-point calibration for any element (pesticide calibration standards not needed), 2) no matrix effects in detection, and 3) greater cost of GC/AED than competing instrumental techniques.

Capillary Electrophoresis (CE)

CE is a technique developed in biological and biochemical applications during the early 1980's, and it is just now slowly making its way into pesticide residue applications (20). The main problem with CE has been the low sensitivity of detection for small molecules in a narrow capillary. With the development of a z-cell or bubble cell, and use of other detection systems, CE analysis is becoming more useful. Figure 5 shows a simplified set-up of a CE instrument and the mechanism of operation. CE works by applying a potential difference to a buffer solution placed at both ends of a capillary. A very small amount of sample is typically sucked or forced into the capillary by pneumatic pressure, then the voltage is applied. Separation of ions occurs based on the mobility of the ions, which is a function of size and charge. The smaller cations in the buffer have higher mobility and force overall flow toward the anode. The neutral molecules are carried with this flow in the electro-osmotic front (E.O.F.) while the charged molecule migrate faster or slower through the capillary based on their charge and mobility. Plug flow occurs in CE because flow originates along the negatively charged capillary walls, unlike the laminar flow profile of chromatographic techniques. This means that peaks will be sharper in CE and better separations can be achieved.

Several modes of operation have been developed in CE: 1) free solution or capillary zone electrophoresis (CZE), which is described above; 2) micellar electrokinetic chromatography

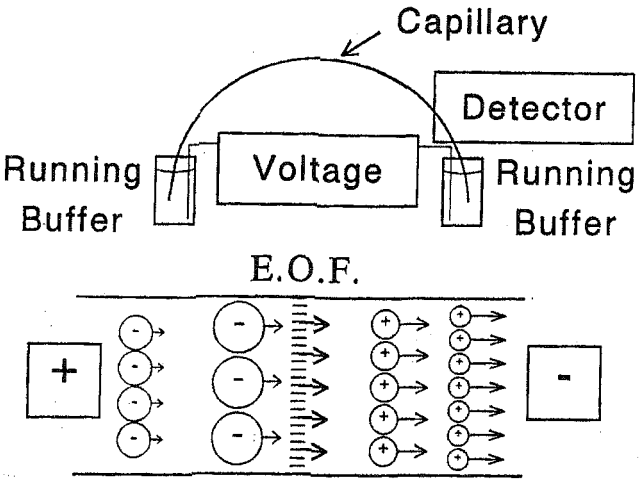

Figure 5
Instrument Arrangement and Mechanism of Separation in Capillary Electrophoresis

(MEKC), which allows separation of neutral analytes by adding micelles or cyclodextrins to the buffer; 3) capillary electrophoresis chromatography (CEC), which is a combination of HPLC and CE by adding a stationary phase to the capillary; and 4) capillary gel electrophoresis (CGE), in which the capillary is filled with a gel and separation is based on molecular weight as in gel electrophoresis. Many other techniques can be used in CE to achieve desired results: 1) coating capillaries to reduce surface interactions with analytes; 2) field amplified injection to increase injection volumes; 3) stacking and focusing to form narrower injection bands and reduce potential matrix effects; 4) use of several detector types, such as absorbance, fluorescence, conductivity, and/or electrospray MS; and 5) derivatization to increase detection or separation capabilities.

CE is typically an aqueous-based system, and very low volumes are used, which means that no hazardous waste is generated and very little sample is required. Due to the number of options, method development can be time-consuming and difficult, but no more so than HPLC. Detection limits are high in CE due to the very small injection volumes and narrow capillaries unless techniques mentioned above are used. A major concern with CE is the inconsistent migration times for peaks as the buffer pH and ionic strength change after multiple injections. The use of a migration time marker, to which all peaks give highly reproducible relative migration times, should be used to identify peaks of interest. Also, as in HPLC, it is often difficult to separate a diverse range of pesticides with the same set of CE conditions.

In the study to determine diverse polar herbicides in soybeans, colleagues and I have developed CE separation conditions for six pertinent herbicides, consisting of: two sulfonyl ureas (chlorimuron-ethyl and thifensulfuron-methyl), an imidazolinone (imazaquin), a chlorophenoxy acid (2,4-D) and 2 others (acifluorfen and bentazon). Figure 6 presents an electropherogram of

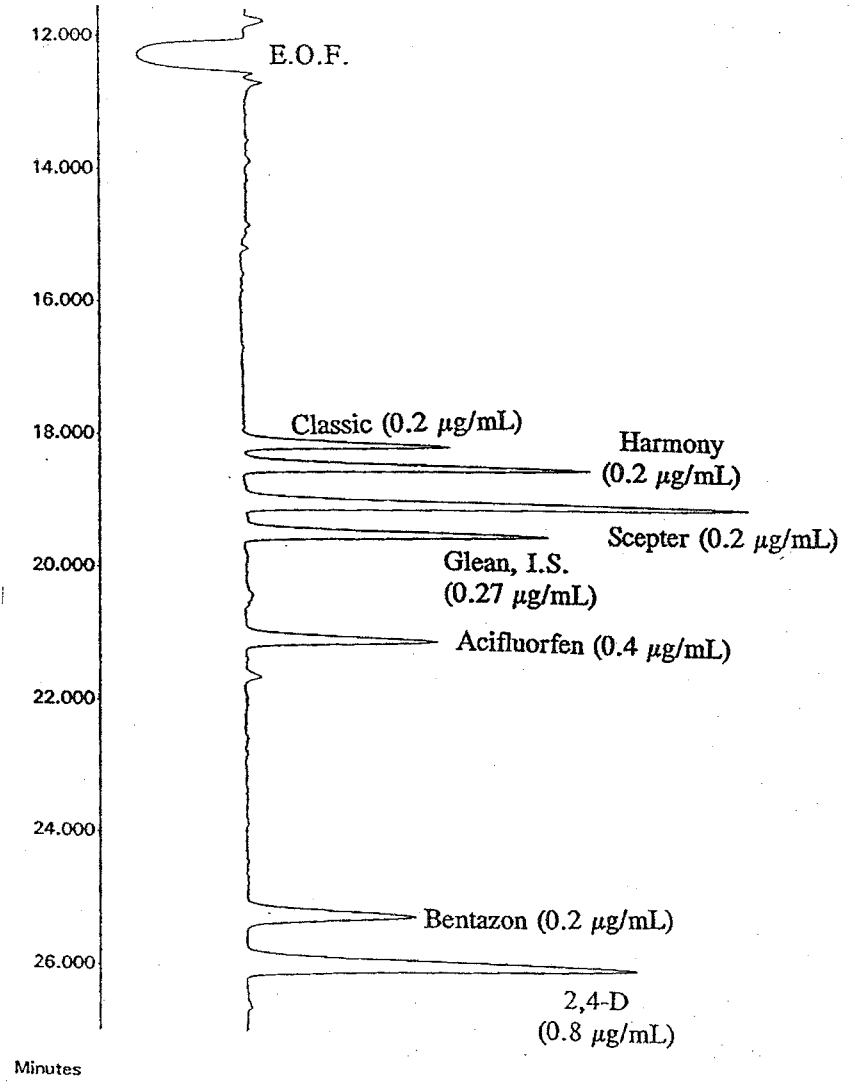

Figure 6
Separation of 7 Diverse Polar Herbicides in CZE (concentrations in parentheses) (Classic = chlorimuron-ethyl; Harmony = thifensulfuron-methyl; Scepter = imazaquin; Glean = chlorsulfuron)

their separation, plus chlorsulfuron as the migration time marker, using the following conditions: 50 mM ammonium acetate buffer, pH = 4.75; 75 μm i.d. capillary, 83 cm length (65 cm to detector); 17 kV voltage (27 μA current); 90 nL injection volume; 240 nm absorbance detection wavelength using a 3 mm high sensitivity optical cell. The LOD in water for the herbicides range from 2 ng/mL for imazaquin to 14 ng/mL for 2,4-D. In soybean, similar LOD were obtained in the final extracts, but a lengthy clean-up involving liquid-liquid partitioning, SPE, and semipreparative HPLC were required (less clean-up was needed for individual pesticide classes).

The ammonium acetate buffer was chosen so that MS detection would be possible with this set-up for confirmation; the CE/MS interface becomes clogged with buffers using buffer additives or metal salts. HPLC/MS can also likely be used for this analysis. Whether CE becomes a useful replacement for HPLC in the future looks promising, but not definite. The sharper peak shapes of CE often lead to higher signal/noise ratios, but the lower injection volume in CE may counteract gains made related to separation efficiencies. Use of narrow-bore HPLC may prove to be superior to both methods and achieve low LOD, good separations, and reduced solvent consumption.

Immunochemical Methods

Immunochemistry has been a tremendous area of research over the last several years. There are a host of techniques and formats that can be applied in residue analysis, especially for the purpose of rapid screening. Enzyme-linked immunosorbent assay (ELISA) is the only widely available commercial format for pesticide residue analysis at this time (21), and it will be discussed below, but other approaches include immunoaffinity, enzymatic, biosensing, flow immunosensing, optical fiber, and molecular imprinting methods. These and other immunochemical and biochemical approaches will probably replace ELISA in the future when the techniques improve and entrepreneurs venture into the pesticide residue applications.

ELISA

ELISA works by covalently bonding an antibody to a surface, either a plastic well, coated tube, or magnetic particle. A small volume of sample and enzyme-labeled antigen solution are added to the immobilized antibody, and a competitive reaction occurs in which the antigen (analyte) in the sample competes for antibody sites with the enzyme-labeled antigen. The more analyte there is in the solution, the fewer sites are available for the labeled antigens. After a fixed time, the solution is rinsed away. Then a color reagent is added; color change occurs at a rate dependent on the amount of enzyme-labeled antigen attached to the antibody. After another fixed time interval, the color change is halted with addition of an acid and measured photometrically or by other means. The more analyte that is originally present in the sample, the less intense is the resultant color. In ELISA, signal (relative to a blank control sample) is inversely proportional and semi-logarithmically related to analyte concentration, and analytical range for quantitation is rather limited (see Figure 9). For this reason, ELISA is not especially good for quantitative

analysis, especially in complex extracts, but its capabilities are potentially useful in pesticide screening applications.

ELISA kits are commercially available for a number of pesticides (≈ 25 at this time), and many antibodies have been developed for other pesticides in noncommercial applications. Depending on the format and costs of the antibody, ELISA kits typically cost $5-10 per assay. Assays, which are often performed in batches, can take from 10-90 minutes, depending on the incubation times for the competitive reaction and enzymatic color change. A batch can consist of 96 samples in the microtiter plate format, but usually fewer samples are analyzed in replicate. In the magnetic bead format, replicates are less necessary, and up to 60 samples can be analyzed in a batch. The procedure can be performed by an individual with minimal training and effort, and automated photometric readers can be used to determine results. ELISA can be performed in the field, in many cases, using tube formats with detection by eye or with portable tube photometers. Detection limits with ELISA kits are often very low (0.01-10 ng/mL), even in the presence of many matrix components in some cases.

Interestingly, many of the limitations with ELISA in residue analysis are simply an inherent condition of the advantages perceived with different applications in mind. Only a limited number of pesticides can be currently tested using ELISA, and high selectivity of detection, which can be an advantage in specific applications, is a disadvantage in multiresidue applications. The costs and labor of ELISA increases greatly if more than one kit is to be used to detect a number of pesticides. Also, the use of ELISA can be considered slow and tedious in many screening applications. The semi-quantitative and nonconfirmatory nature of ELISA also pose problems in several applications. The rate of false positives and false negatives in ELISA is often higher than with traditional approaches.

The capability of ELISA, and other immunochemical methods, is highly dependent on the antibody. Some antibodies are very rugged, while others are very sensitive to environmental factors, such as pH, temperature and exposure to salts and organic solvents. Some antibodies are prone to rapid degradation, while others may be rather stable. Some antibodies are specific to a single antigen, while others are selective to a chemical moiety, and still others can interact rather nonselectively with a variety of compounds. By altering incubation times, and antibody:sample:enzyme-antigen ratios, sensitivity of analysis can be tailored to achieve very low, or not so low, detection limits. In analysis of environmental water and soil samples, ELISA can be very useful, but residue analysis of foods often requires more precautions due to potencial protein, carbohydrate, and lipid interferences.

ELISA Studies

Figure 7 shows results from a simple study using a commercial ELISA tube kit for carbofuran. Several sample types were fortified at tolerance levels, blended with water, filtered, and then ELISA was performed. The signal, $\%B_0$, in this case is the absorbance result for the sample relative to the absorbance result of the water control sample. Milk contained an interferant, likely to be proteins and/or lipids, that caused a lower response, but the other matrices did not contain significant interferants.

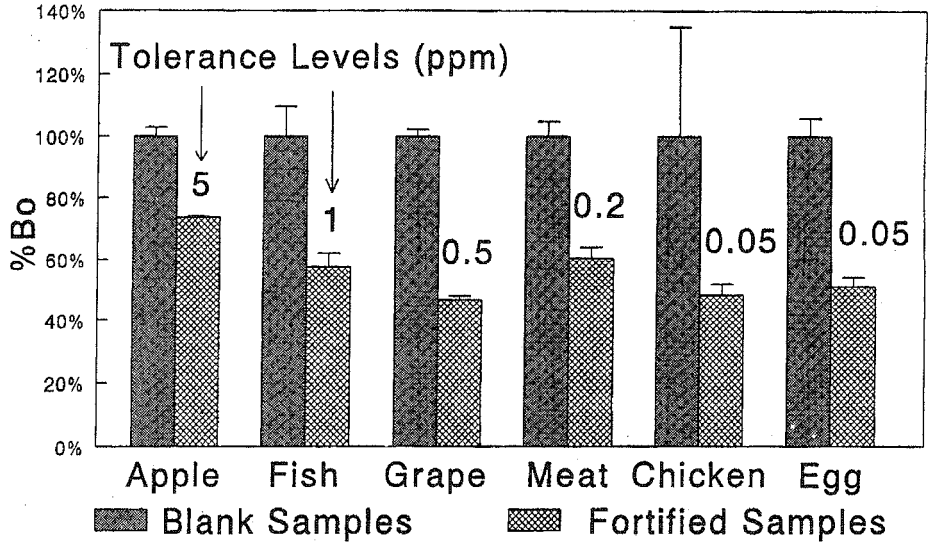

Figure 7
ELISA Results for Carbofuran Spiked at Tolerance Levels in Different Matrices

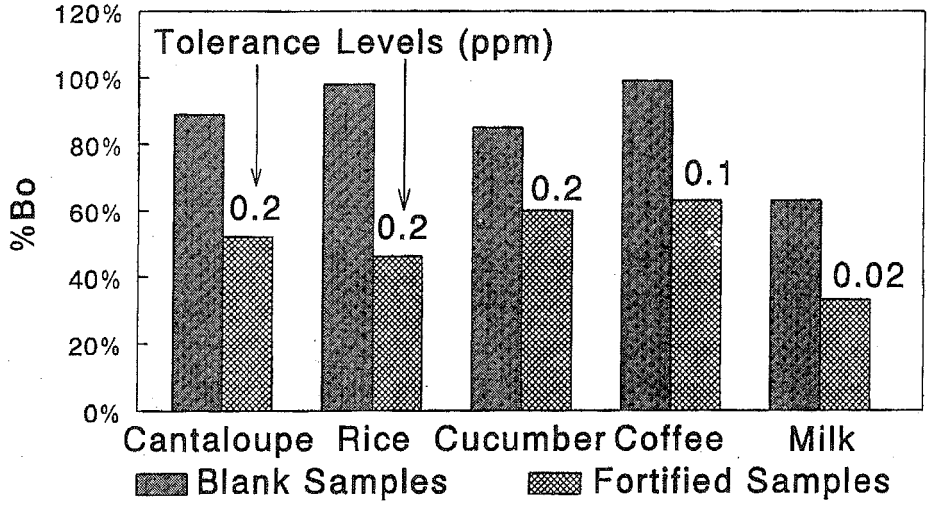

Figure 8
ELISA Results for 2,4-D Spiked at Tolerance Levels in Different Matrices

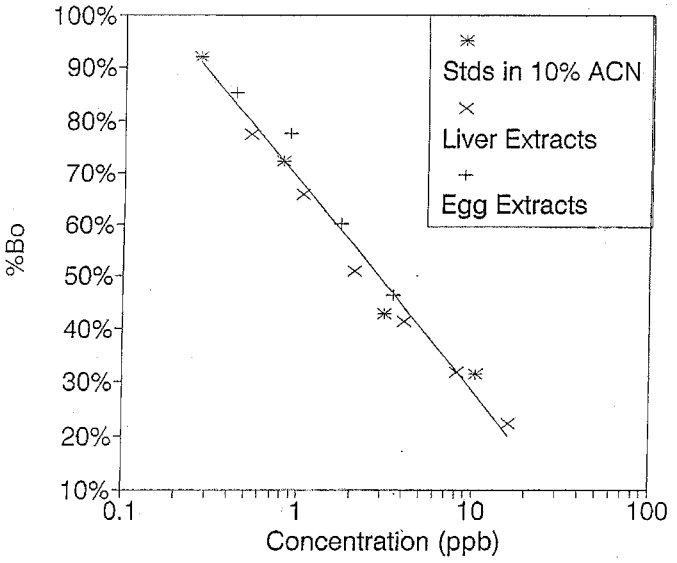

Figure 9
ELISA Calibration Plots of Alachlor in Different Matrices

Figure 8 presents results from an experiment to determine 2,4-D using a commercial ELISA kit. The extraction procedure used in this experiment was to simply blend a sample (fortified at tolerance levels) 1:1 with water, centrifuge the extract, dilute the supernate to give 25 ng/mL as the expected concentration, and take a 250 µL portion for ELISA (magnetic bead format). Although matrix interferants caused the signal to decrease versus water controls in a few cases (eggs in particular), calculation of $\%B_0$ with respect to blank matrix extracts gave significant differences in results. Provided that the variability of the matrix does not cause results that are more variable than allowable to reproducibly determine the pesticide at tolerance levels, then ELISA is useful for screening applications. In this instance, chicken gave an exceedingly variable result and clean-up should be performed before doing ELISA.

In the case of alachlor, the herbicide was not soluble enough in water at room temperature to enable high extraction efficiencies with water. Therefore, acetonitrile was chosen as the extraction solvent for liver and egg (blended 1:1). After filtering the extract, it was diluted 1:10 with phosphate buffer, pH=7.4. The kit could withstand 10% acetonitrile without problems and detection limits were still less than 1 ng/mL. Figure 9 shows how the ELISA calibration plot of alachlor in the different matrices was not significantly affected. The problem with the ELISA kit antibodies used for alachlor, however, was that they were not significantly cross-reactive with alachlor metabolites, therefore, the kit could not be used in regulatory analyses, despite the good results obtained for the parent compound (22).

In order to implement ELISA in regulatory screening applications, evaluation of a statistically relevant number of analyses of incurred and fortified samples taken from different

locations and different times of year is needed before this approach could be used in regulatory applications. ELISA results should be compared with results from traditional analysis as well. Based on the information gained from this detailed type of study, a significant target response could be set that would indicate the possibility of a violation. There have been a few instances in which this type of study has been performed, and ELISA results have been mixed. Regulatory agencies have not yet accepted ELISA as a valid general screening approach to pesticide analysis of foods and more work is needed if ELISA is to be used in this capacity. Alternate immunochemical or biochemical formats to ELISA, which are potentially faster, more quantitative, easier to perform, less expensive, and suitable for a wider range of pesticides are being investigated. Despite the advantages of ELISA versus many traditional approaches, the cost, time, labor, and format are still not ideal for analytical screening applications. Ideally, nondestructive means of analysis should be used in the field to monitor pesticide residues in a high percentage of products with minimal time, cost, and effort.

CONCLUSIONS

In the discussion, I tried to give an overview of recent technologies and to concisely assess the applicability of these technologies in pesticide residue analysis. Whether these assessments come true will depend on individual applications, instrumental capabilities, and skill of the analysts. I hope that the information presented will lead others to gather more detailed information on the topics of interest and make informed choices about whether or not to use these technologies in the development and implementation of more efficient ways to perform residue analyses.

In the future, it appears that miniaturization, computerization, and automation will become even more prominent in new techniques and technologies. The trend toward reduced need for clean-up will also likely continue. Someday, we will likely be able to probe a sample directly, with no other steps, and quickly quantify and confirm a wide range of pesticides. This is possible for some applications now, in which the technology, sample nature, and economics allow such an approach. Due to the sample complexity and low concentrations encountered, the ultimate approach to analysis, remote sensing, will probably never be used in pesticide residue analysis, at least not until the "tricorder" of Star Trek is invented.

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