Analysis of Multiple Herbicides in Soybeans Using Pressurized Liquid Extraction and Capillary Electrophoresis

Satoru Nemoto[†] and Steven J. Lehotay*[,‡]

Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 10300 Baltimore Avenue, Beltsville, Maryland 20705, and National Institute of Health Sciences, Division of Foods, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan

Several herbicides commonly used on soybeans are often difficult to extract, isolate, and quantify from the complex soybean matrix at low concentrations. Typical analytical methods for herbicide residues in soybeans are single analyte procedures using HPLC or GC after chemical derivatization. In this study, method development for the analysis of six polar herbicides in soybeans was performed using pressurized liquid extraction (PLE), which is also known by the trade name, Accelerated Solvent Extraction, and capillary electrophoresis (CE). In CE, a 50 mM ammonium acetate running buffer, pH 4.75, was able to separate imazaquin (Scepter), chlorimuron-ethyl (Classic), thifensulfuron-methyl (Harmony), acifluorfen (Blazer), bentazon (Basagran), and 2,4-dichlorophenoxyacetic acid in a 75 μm i.d., 83 cm capillary (65 cm to detector) within 30 min at 17 kV applied voltage. Chlorsulfuron (Glean) was used as an internal standard in the analysis, and detection was by UV absorbance at 240 nm in a high-sensitivity optical cell. PLE extracts required extensive cleanup prior to CE/UV analysis. Several cleanup techniques were investigated and compared, including liquid−liquid partitioning, gel-permeation chromatography, semipreparative HPLC, and solid-phase extraction with a variety of stationary and mobile phase combinations. A combination of techniques that provided the most efficient cleanup was selected in the final method. Four of the six herbicides could be determined by the method in samples fortified at tolerance levels with average recoveries of 71% and relative standard deviation (RSD) of 11%. At a higher spiking level, all of the herbicide recoveries were >70% with %RSDs < 10%, except for acifluorfen which gave more variable recoveries.

Keywords: *Residue analysis; herbicides; soybeans; capillary electrophoresis; pressurized liquid extraction*

INTRODUCTION

Soybeans are an important commodity in U.S. agriculture. Soybeans rival wheat as the top U.S. agricultural export (*Agriculture Fact Book 1994*), and a considerable share of U.S. soybean production is imported by Japan, South Korea, and other Asian countries. Nearly all nations require that pesticide residues in food are below established regulatory tolerance levels or free of pesticides for which no tolerance level has been established. Appropriate analytical methods of analysis must be available to verify that food products meet these conditions.

The most efficient approach for analysis in routine monitoring and enforcement purposes is often a multiresidue method that determines many analytes in a single procedure. There are several multiresidue methods that are designed for detection of hundreds of pesticides in a variety of commodities (*Pesticide Analytical Manual*, 1994; *Analytical Methods for Pesticide Residues in Foodstuffs*, 1996). However, many of the herbicides commonly applied on soybeans, such as sulfonyl ureas, imidazolinones, and others, cannot be analyzed using existing multiresidue methods. Analysis of these type of pesticides in soybeans often poses problems due to the complicated matrix, delicate nature of the pesticides, and low detection limits required. In these situations, single analyte methods have been developed, typically by the pesticide registrant for the particular pesticide/commodity pair (*Pesticide Analytical Manual*, 1987). However, a registrant has no obligation to develop new multiresidue methods to ease monitoring or enforcement applications.

This study was designed to develop a multiresidue (and multiclass) method for herbicides often applied on soybeans. The herbicides selected for the study are listed in Table 1, which includes their chemical structure, selected chemical properties, and U.S. tolerance level in soybeans. Five of these six herbicides are also often called by their trade names Blazer (acifluorfen), Basagran (bentazon), Harmony (thifensulfuron-methyl), Classic (chlorimuron-ethyl), and Scepter (imazaquin), while 2,4-D is an abbreviation for 2,4-dichlorophenoxyacetic acid. The herbicides are registered for use on soybeans in the U.S. (*Code of Federal Regulations*, 1996) and represent the sulfonyl urea (thifensulfuron-methyl and chlorimuron-ethyl), imidazolinone (imazaquin), phenoxy acid (2,4-D), and other (bentazon and acifluorfen) classes. Another imidazolinone registered for use on soybeans, imazethapyr (Pursuit), was originally included in the study, but it gave poor peak shape at the conditions used for analysis of the other herbicides.

Two automated methods of extraction, supercritical fluid extraction (SFE) and pressurized liquid extraction

* Author to whom correspondence should be addressed [e-mail slehotay@asrr.arsusda.gov; fax (301) 504-5048].

[†] National Institute of Health Sciences.

[‡] U.S. Department of Agriculture.

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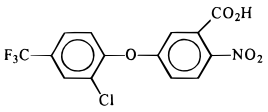
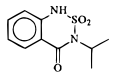
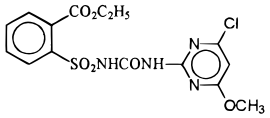
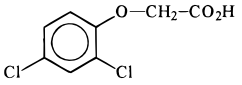
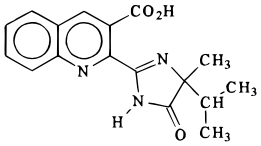
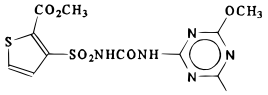
Table 1. Herbicides Included in the Study, Their Chemical Structures, Molecular Weights, Acid Dissociation Constants, and U.S. Tolerance Levels in Soybeans[a]

herbicide	structure	MW	pK_a	tolerance (ng/g)
acifluorfen		361.7	3.8	100
bentazon		240.3	3.3	50
chlorimuron-ethyl		414.8	4.2	50
2,4-D		221.0	2.8	200
imazaquin		266.3	3.8	50
thifensulfuron-methyl		387.4	4.0	50

[a] Most pK_a values are from the Pesticide Properties Database (http://www.arsusda.gov/rsml/ppdb3).

(PLE), were considered for investigations in this study. Due to the many potential benefits of SFE in terms of relatively high selectivity, reduced solvent use, and the savings in time, costs, and labor, SFE may be considered the first choice for evaluation in new method development (Lehotay, 1997). However, the extraction of amines, amides, imides, ureas, azoles, and similar nitrogen-containing chemicals, such as most of the target herbicides, are known to be problematic in SFE (Lehotay, 1997; Nemoto et al., 1997). Incomplete recoveries of the target herbicides with supercritical CO_2 in preliminary experiments to determine the effects of water, pH, salt, and trapping conditions ended further method development investigations of SFE in this application.

PLE combines aspects of SFE with traditional liquid-based extraction capabilities. The relatively new technique employs increased temperature and pressure to improve the speed of extraction and reduce the solvent consumption (Richter et al., 1996). PLE is similar to SFE in that it is easily automated, samples often require a dispersant, and extraction vessels are used for sequential extraction in a carousel. Lehotay and Lee (1997) compared PLE with SFE for the extraction of multiple pesticides from tomato and other matrices, and Obana et al. (1997) applied PLE for extraction of organophosphorus pesticides from foods. Conte et al. (1997) extracted the nicotinanilide herbicide, diflufenican, from soil using PLE, and Kreisselmeier and Dürbeck (1997) compared PLE with SFE and traditional methods for the extraction of surfactants from sediment.

For analysis, capillary electrophoresis (CE) using an extended path-length optical cell for absorbance detection was chosen on the basis of promising results for these types of herbicides demonstrated previously (Krynitsky and Swineford, 1995; Krynitsky, 1997; Garrison, 1994; Wigfield et al., 1993). CE is an aqueous-based method of low-volume analysis that can provide high separation power and low limits of detection (LODs), particularly for ionic molecules. CE has been studied extensively and applied in many applications (St. Claire, 1996), but the approach has not been frequently evaluated in pesticide residue analysis. HPLC has been the most common technique for these types of pesticides (Prince and Guinivan, 1988; Knutsson et al., 1992), but HPLC often has a reduced number of theoretical plates versus CE and typically generates a great deal of organic solvent waste. Chemical derivatization and gas chromatographic analysis is another potential option for these types of pesticides (Cessna, 1985; Sánchez-Brunete et al., 1994), but it is preferable to detect the analyte without chemical alteration. Also, these types of pesticides are good candidates for immunochemical analysis, but immunochemical methods are not typically multiresidue and the results are not necessarily quantitative (Kaufman and Clower, 1995; Lucas et al., 1995).

The large amount of matrix coextractives using PLE and the need for clean extracts in CE/UV analysis led to the need for extensive cleanup with these methods. Several cleanup techniques, such as liquid−liquid partitioning, solid-phase extraction (SPE), semipreparative HPLC, and gel-permeation chromatography (GPC), were evaluated to help eliminate matrix interferences. The use of UV absorbance for detection combined with the complexity of the matrix and diversity of the pesticides led to a challenging task in achieving the selectivity needed for removal of interferants while maintaining acceptable analyte recoveries.

MATERIALS AND METHODS

Chemicals. Pesticide standards were obtained from the U.S. Environmental Protection Agency. A solution of 20 μg/mL each of thifensulfuron-methyl, chlorimuron-ethyl, imazaquin, and bentazon, 40 μg/mL of acifluorfen, and 80 μg/mL of 2,4-D was prepared in acetonitrile which served as the spiking solution and working standard. The concentrations were chosen on the basis of the ratios of the tolerance levels for the herbicides in soybeans (Table 1). A solution of 106 μg/mL of chlorsulfuron (Glean) in acetonitrile was used as the internal standard for CE. Soybeans that were certified to be free of pesticides were provided by Montague Farms (Center Cross, VA). Acetonitrile (MeCN), acetone, methanol (MeOH), ethyl acetate (EtOAc), cyclohexane, dichloromethane (DCM), hexane, and other solvents used in the study were HPLC grade or higher quality, and water was obtained from a water purifier (Sybon-Barnstead, Dubuque, IA). Ammonium acetate, sodium acetate, acetic acid (HOAc), HCl, NaCl, Na_2SO_4, NaOH, $Na_2B_4O_7$, H_3BO_3, sodium dodecyl sulfate (SDS), $NaHCO_3$, and other chemicals were ACS grade or better from Fisher (Fairlawn, NJ), Sigma (St. Louis, MO), or Aldrich (Milwaukee, WI). The diatomaceous earth material, Hydromatrix (HMX), which was used as a sample dispersant in PLE, was obtained from Varian (Harbor City, CA). The PLE instrument used zero-grade N_2 (Potomac Airgas) for purging the vessels and instrument pneumatics.

Acetate running buffer solutions were prepared for CE by making individual solutions of HOAc and ammonium acetate at the desired final concentration of the buffer (typically 50 mM). Then equal portions of the solutions were mixed together to give the desired volume, and final adjustments in pH, as measured with a pH meter, were made by adding small amounts of the appropriate solution to the mixture. The buffer

was degassed and filtered through a 0.2 μm filter before use in CE. A fresh 4 mL inlet buffer solution was used after approximately six sample injections, and the 40 mL outlet buffer solution was changed generally every other sample set.

The following SPE cartridges were evaluated: octadecylsilane (Waters, Milford, MA), silica (Waters), alumina-neutral (J. T. Baker, Phillipsburg, NJ), anion exchange (International Sorbent Technology, Mid Glamorgan, UK), and aminopropyl (Supelco, Bellefonte, PA). Various solvent combinations and pH were tested to determine herbicide elution volumes, recoveries, and cleanup aspects. All cartridges were treated just before addition of the sample extract by rinsing with the elution solvent followed by the solvent comprising the extract.

Apparatus and Procedures. The CE instrument was a Crystal 300 (Thermo CE, Franklin, MA). Unless otherwise noted, the following conditions were used for analysis. A 75 μm i.d. capillary of 83 cm total length, 65 cm to the detector, fitted with a 3 mm path-length, high-sensitivity optical cell (LC Packings, San Francisco, CA) was used for separations, and a Model 785A programmable absorbance detector (Applied Biosystems, Foster City, CA) set at 240 nm was used for detection. The capillary oven temperature was set to 20 °C, and a 50 mM ammonium acetate buffer, pH = 4.75, was the running buffer for CE. The voltage applied was 17 kV, which typically gave 50 μA current. Injection was for 0.4 min at 40 mbar, which gave a volume of approximately 90 nL as calculated from the Poiseulle equation (calculation software provided by Thermo CE). After each run, 0.1 M NaOH was flushed through the capillary for 2 min at 2000 mbar followed by the buffer for 1 min. Data acquisition and peak integration were performed using the instrument software, and a spreadsheet program was used to calculate results.

PLE was performed with an ASE 200 instrument (Dionex, Sunnyvale, CA). The instrument permits vessel sizes of 11, 22, and 33 mL, and extracts are collected in 40 or 60 mL vials. In the final method, the extraction solvent was 3:7 0.05 M HCl:MeCN, pH = 2, with extraction conditions of 2000 psi, 50 °C, a 10 min static time, 100% solvent flush of the vessel (1 cycle), and a 60 s purge with nitrogen. For the 11 mL vessels, approximately 24 mL of solvent was used per extraction. The soybeans were ground using a centrifugal mill (Udy, Fort Collins, CO) to pass through a 60-mesh screen. Samples were mixed 2:1 soybean:HMX (w/w) prior to extraction in PLE. Whatman (Maidstone, U.K.) grade D28 (1.9 μm pore size) filter paper disks, precut to fit the extraction vessels, were used to help contain the samples in the PLE vessels.

Semipreparative HPLC was performed using a Supercosil SPLC-18 column, with 25 cm length, 1 cm i.d., and 5 μm particle size (Supelco). The column was connected to a Model 1050 HPLC pump (Hewlett-Packard, Little Falls, DE), Model 490 UV/vis detector (Waters) set at 240 nm, Model 3396 Series II integrator (Hewlett-Packard), and a six-port injection valve (0.5 mL or 1 mL injection volume). The conditions for the semipreparative HPLC cleanup step were a 3 mL/min flow rate with the following gradient elution program: 40:60 MeCN:0.15% HOAc from 0 to 5 min, then 80% MeCN by 7 min through 16 min, and a return to 40/60 after 16 min which was followed by a 10 min reequilibration time. Collection of the analyte fraction was from 8 to 14 min when the mobile phase was 80:20 MeCN:0.15% HOAc. For GPC experiments, the same pump and injector were used as in HPLC, and the column was a 1 cm i.d., 40 cm long glass column packed with 200−400 mesh S-X3 styrene divinylbenzene (Biorad, Hercules, CA) in 1:1 EtOAc:cyclohexane.

Evaporation of extracts in 40 mL vials was performed using a Speedvac SC200 (Savant, Farmingdale, NY) or with a laboratory nitrogen blowdown device. Because the method was designed to minimize solvent volumes and glassware needs, the extracts were generally kept in 40 mL vials with Teflon-lined twist caps, or in 15 or 50 mL graduated centrifuge tubes when accurate measurement of a final volume was needed. For convenience liquid−liquid partitioning was often conducted by removing the upper layer from a partitioned extract in a 40 mL vial using a Pasteur pipet. To check the pH of extracts at certain points in the final method, a small drop of the extract was placed on pH strips with a Pasteur pipet. For filtration of extracts prior to HPLC or CE, 0.2 μm pore size PTFE filters, designed for 1 mL volumes, were used.

Table 2. LODs (ng/mL and pg Injected for a 90 nL Volume) of the Herbicides in Water, Migration Times (t_m) ± Standard Deviation (SD), and Improvement in the Precision of Identifying Analyte Peaks by Using the Ratio of t_m versus the t_m of the Internal Standard, Chlorsulfuron

herbicide	LOD (ng/mL)	LOD (pg)	$t_m \pm$ SD[a] (min)	t_m/t_m i.s. ± SD[a]
chlorimuron-ethyl	5.0	0.38	16.8 ± 1.1	0.9217 ± 0.0035
thifensulfuron-methyl	3.4	0.26	17.2 ± 1.1	0.9453 ± 0.0032
imazaquin	2.4	0.18	17.8 ± 1.1	0.9760 ± 0.0012
chlorsulfuron (i.s.)			18.2 ± 1.2	1
acifluorfen	13	0.98	20.0 ± 1.3	1.0947 ± 0.0063
bentazon	7.3	0.55	24.7 ± 1.8	1.356 ± 0.024
2,4-D	14	1.0	25.4 ± 1.8	1.392 ± 0.028

[a] $n = 27$.

RESULTS AND DISCUSSION

Capillary Electrophoresis. The first step in the development of the analytical method was the determination of the optimal wavelengths for absorbance detection of the pesticides. With the use of the wavelength scanning function of the detector, 240 nm was found to be the most favorable wavelength for the range of pesticides studied. Absorbance maxima (5 nm detector bandwidth) occurred at approximately 235, 245, 240, and 225 nm for thifensulfuron-methyl, chlorimuron-ethyl, imazaquin, and bentazon, respectively. Longer wavelengths (e.g. 280 nm) were feasible for acifluorfen and 2,4-D to help avoid potential matrix interferants, but they also gave higher responses at 240 nm. Table 2 lists the (LODs of the herbicides in water at 240 nm using the final CE conditions. LODs are the concentrations at which S/N = 3 in the analysis of the herbicides. LODs were determined by taking one-fifth of the peak-to-peak noise, which provides the standard deviation of the noise (σ), multiplying by 3, and dividing by the slope of the calibration curve using peak heights as the signal (3σ/sensitivity).

To determine optimal CE separation conditions for analysis of the herbicides, several different buffers and parameters were evaluated. The final conditions were very similar to those of Krynitsky (1997), who separated 13 sulfonyl urea herbicides within 25 min by CE. In this study, experiments were conducted using borate, phosphate, carbonate, citrate, formate, and other buffers, such as 2-[*N*-morpholino]ethanesulfonic acid (MES), but none gave better separation for the six herbicides than 50 mM acetate buffer at pH 4.75. Figure 1 displays a typical electropherogram of a standard mixture of the herbicide analytes plus the internal standard, chlorsulfuron, using the final CE conditions.

The effects of pH, ionic strength, buffer additives (micelles and organic solvents), counterions, applied voltage, and temperature were also studied using the different buffers. The most dramatic differences in the separation were encountered by changing buffers, but, for a particular buffer, the effect of pH within the buffer's range was not as pronounced. For example, citrate buffer at pH 4.76 required much longer time than acetate buffer at pH 4.75 to perform the separation. For buffers at pH > 6, the addition of a micelle, such as SDS, seemed to be required to improve separation (the electroosmotic flow increases with increasing pH when positive voltage is applied to the inlet buffer solution).

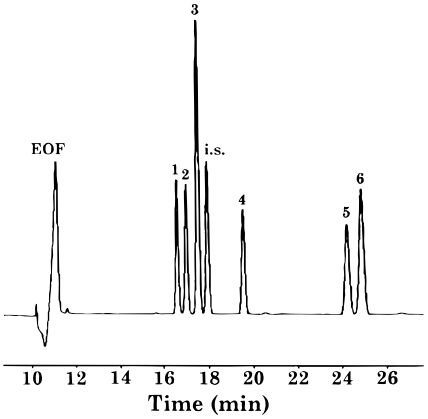


Figure 1. Electropherogram obtained with the final CE conditions of the herbicide analytes in water: (1) chlorimuron-ethyl, 0.2 μg/mL; (2) thifensulfuron-methyl, 0.2 μg/mL; (3) imazaquin, 0.2 μg/mL; (4) acifluorfen, 0.4 μg/mL: (5) bentazon, 0.2 μg/mL; and (6) 2,4-D, 0.8 μg/mL, i.s. = chlorsulfuron, 0.21 μg/mL, and EOF = electroosmotic front.

The use of more concentrated buffers helped maintain separation for real samples, but the higher ionic strength also extended analysis time and limited the voltage that could be applied before excessive heat was generated. Furthermore, background detector noise increased with increasing buffer concentration and when micelles were added to the buffer. The 50 mM acetate buffer concentration was sufficient to compensate for the effect of matrix components during injection and keep heating within acceptable limits. MeCN was also added to the samples and buffers to determine its effects at 4−12.5% concentrations (Krynitsky, 1997), but no significant improvement was observed in this case. Also, little difference was observed between sodium and ammonium as the counterion for the acetate buffer, but ammonium was better for facilitating the possible use of mass spectrometric detection in the future. The choices of voltage and temperature were made to maintain acceptable resolution between the peaks and to give the shortest analysis time while staying within the linear region of an Ohm's law plot of current versus voltage. Sodium borate buffer at pH 9−10 with the addition of SDS was also feasible for achieving separation of the herbicides within 40 min, but CE with ammonium acetate buffer containing no micelles gave a lower LOD and a shorter separation time.

A common problem in the application of CE/UV to analysis of real samples has been potential difficulties in the accurate identification of analytes due to fluctuations in their migration times. Migration times may fluctuate significantly from sample to sample due to slight differences in the matrix and the effect of changing pH and ionic strength of the running buffer during each run. An important aid in the assignment of peaks in this study was the use of an internal standard. The main purpose of the internal standard was to serve as a marker, and analyte peaks were assigned on the basis of the consistent migration time relative to the marker. Table 2 provides the fluctuations of the migration times and relative migration times of the herbicides using the CE method and shows the improvement gained in the use of the internal standard. Chlorsulfuron (Glean), a sulfonyl urea herbicide not registered for use on soybeans, was selected as the internal standard because it gave a good response at 240 nm and appeared near the middle of the electropherogram. Ideally, two internal standards would be used which appear toward the beginning and end of the run (as well as a neutral species to accurately mark the electroosmotic front), but limited attempts to find suitable compounds were unsuccessful. Furthermore, chlorsulfuron is not an ideal choice because it has higher potential to appear in a soybean or other agricultural sample than an unrelated compound. For this reason, chlorsulfuron should not be used for quantitative purposes in unknown samples. In these studies of fortified samples, results were very similar whether the internal standard was used in quantitation or not. Also, the use of standards in matrix blanks gave the same results as the use of standards in water.

Pressurized Liquid Extraction. Due to the polar nature of the herbicide analytes and high water solubilities (depending on pH), initial studies of PLE of the herbicides were performed using water as the extraction solvent. Water is the most desirable solvent to use in terms of low cost, nontoxicity, wide availability, and many other factors. The selectivity gained by being able to alter pH and ionic strength, combined with the capability of PLE to alter other extraction properties through the control of heat and pressure, made the use of water in PLE an exciting possibility. However, investigations of water-based extraction led to the conclusion that it was not possible to use 100% water solutions for PLE of soybeans due to low and variable extraction volumes. The high viscosity of water coupled with the high levels of carbohydrates and proteins in soybeans made PLE difficult, independent of pH, temperature, and pressure, unless an organic solvent was also added. Soybean extracts with water were basic, and due to the acidic nature of the analytes, an acidic solution was beneficial for extraction. The manufacturer did not recommend using a liquid with pH < 2, but an existing method for 2,4-D required highly acidic solution for extraction (Newsome and Collins, 1989). The solvent is the most important parameter in extraction using PLE, and increasing temperature and pressure does not necessarily compensate for solvent or pH effects.

For example, the effect of pH was determined by altering the HCl concentration in the aqueous fraction of a 70% MeCN extraction solution. As shown in Figure 2, chlorimuron-ethyl and thifensulfuron-methyl gave low recoveries from soybeans for 0.1 and 1 M HCl concentrations, while 0.05 and 0.01 M concentrations gave acceptable recoveries for all pesticides studied. Recovery of bentazon appeared to be affected by pH to a lesser extent than the sulfonyl ureas, but the other pesticide recoveries were not affected much by the acid concentration. In this study, therefore, a 7:3 solution of MeCN:50 mM HCl (pH = 2) was used as the extraction solvent.

Other experiments were conducted in PLE to determine the effects of temperature, pressure, static extraction time, volume, and number of extraction cycles. The results echoed findings from previous researchers who determined that pressure had minor effects, as did the other parameters except temperature and solvent (Richter et al., 1996). Increasing temperature in this case accomplished little except to increase the level of matrix coextractives. Based on these experiments, the PLE conditions for the chosen sample size (3 g of soybean + 1.5 g of HMX in an 11 mL vessel) were set to be a 10 min static time, a single extraction cycle, and a temperature of 50 °C.

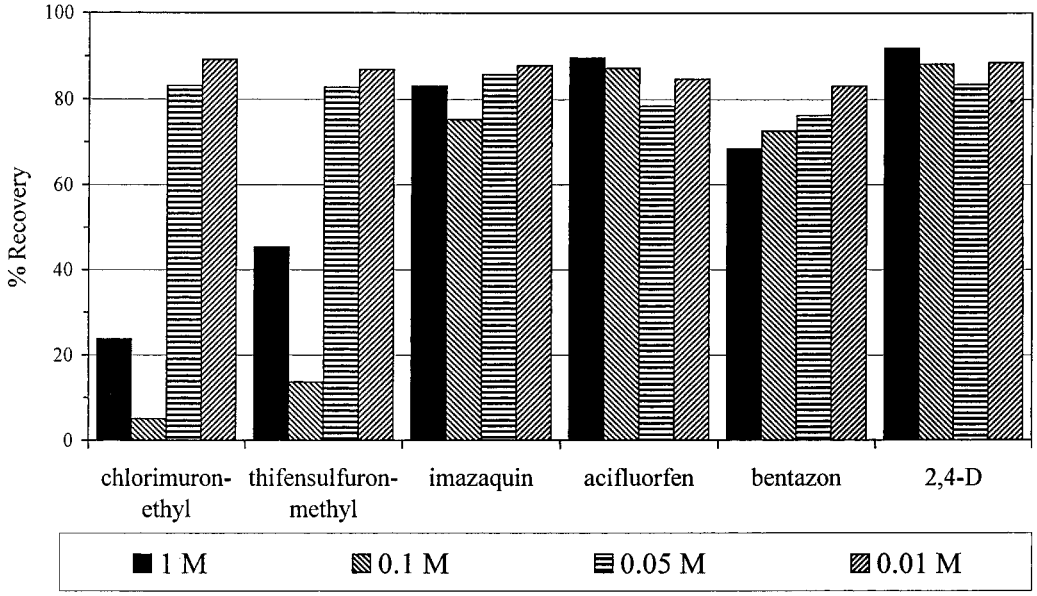


Figure 2. Effect of HCl concentration in extraction solvent (7:3 mixture of MeCN:HCl solution) on PLE of the herbicides from fortified soybean. PLE conditions were 2000 psi, 100 °C, a 5 min extraction time, 100% flush volume, one cycle, and a 1 min N_2 purge for the 11 mL vessel containing 3 g of soybean + 1.5 g of HMX.

Hexane Partitioning. After extraction of soybeans with PLE, extensive cleanup was required before analysis with CE/UV. Lipid coextractives were reduced by using the aqueous MeCN solution at pH of 2 for extraction; the use of a more nonpolar organic solvent or a higher pH increased lipid content in the extracts. Removal of lipids was achieved by partitioning the extract with hexane, and the herbicides remained 100% in the lower acidified, aqueous MeCN layer. Nakamura et al. (1996) demonstrated the utility of removing lipids from soybean extracts with this approach.

Gel-Permeation Chromatography. Nakamura et al. (1996) also demonstrated the effectiveness of GPC for separation of lipids from pyrethroid insecticides in soybeans, and this approach was also tested for the herbicides in this study. A 1 cm i.d., 40 cm long GPC column of S-X3 was used to separate lipids, and potentially other high-MW interferants, from the herbicides. Figure 3 shows the herbicide elution results from the GPC column for a 1 mL injection volume and 1 mL/min flow rate using different mobile phases. A mobile phase of 1% HOAc in EtOAc eliminated the peak tailing associated with 1:1 EtOAc:cyclohexane and EtOAc mobile phases. However, subsequent analysis of soybean extracts after GPC at these conditions showed that the technique had little effect on the interferants in CE/UV. The hexane partitioning step was the easier, faster, and more cost-effective approach for removing lipids for these herbicides.

Solid-Phase Extraction. Several types of SPE cartridges were evaluated for cleanup of soybean extracts: octadecylsilane (C-18), alumina-neutral (alumina-N), silica, aminopropyl ($-NH_2$), and anion exchange (SAX). Table 3 summarizes many of the experiments conducted using SPE, but the following text provides more details. A difficulty with all of the cleanup procedures for the multiresidue/multiclass mixture of herbicides was that the differences in the chemical properties of the analytes limited the selectivity that could be achieved in removing interferants. For example, C-18 retained four of the six herbicides when they were loaded onto the cartridge in water (pH 7), but low pH was required for bentazon and 2,4-D to be retained. Furthermore, HOAc solutions (up to 100 mM) did not provide as consistent or as high recoveries as stronger acids such as HCl. Extracts in 10−50 mM HCl gave consistent recoveries near 100% provided that the herbicides, particularly the sulfonyl ureas, were not stored long in the acid solutions. Once the herbicides were loaded on the column, more than 20 mL of water (for a 500 mg cartridge) could be used to rinse unretained components without causing the herbicides to elute. Acetone (7 mL) was used to elute the herbicides after the water rinse. The C-18 procedure was able to remove salts effectively, but due to the low pH required to retain all of the herbicides, it was not especially good for removing other matrix components.

Similar tradeoffs in selectivity were made with the other SPE cartridges tested in order to obtain high recoveries of all six herbicides. In the case of SAX, only 10 mL of MeOH was needed to elute chlorimuron-ethyl, thifensulfuron-methyl, and imazaquin from a 1 g cartridge, while 2,4-D needed 50 mL and acifluorfen and bentazon were completely retained. With MeCN as the elution solvent, 2,4-D was also retained and much larger volumes were needed to elute the other three herbicides. The SAX cartridge was very useful for cleanup for sulfonyl ureas (Krynitsky, 1997), but it could not be extended for use with the other herbicides except imazaquin.

In initial studies with soybean extracts, the final extracts were often turbid and filtration with 0.2 μm filters was ineffective in removing the causing factor. Despite the turbidity, the electropherograms were unaffected except by a large peak at the electroosmotic front, which indicated that a neutral substance was the source of the problem. Silica was found to be very effective for removing the component causing the turbidity (as were other techniques tested subsequently), but many of the other interferants remained. Acetone, MeOH, $MeCl_2$, MeCN, EtOAc, and water were tested as loading, rinse, and elution solvents in experiments with silica. As before, the need to obtain high recoveries for

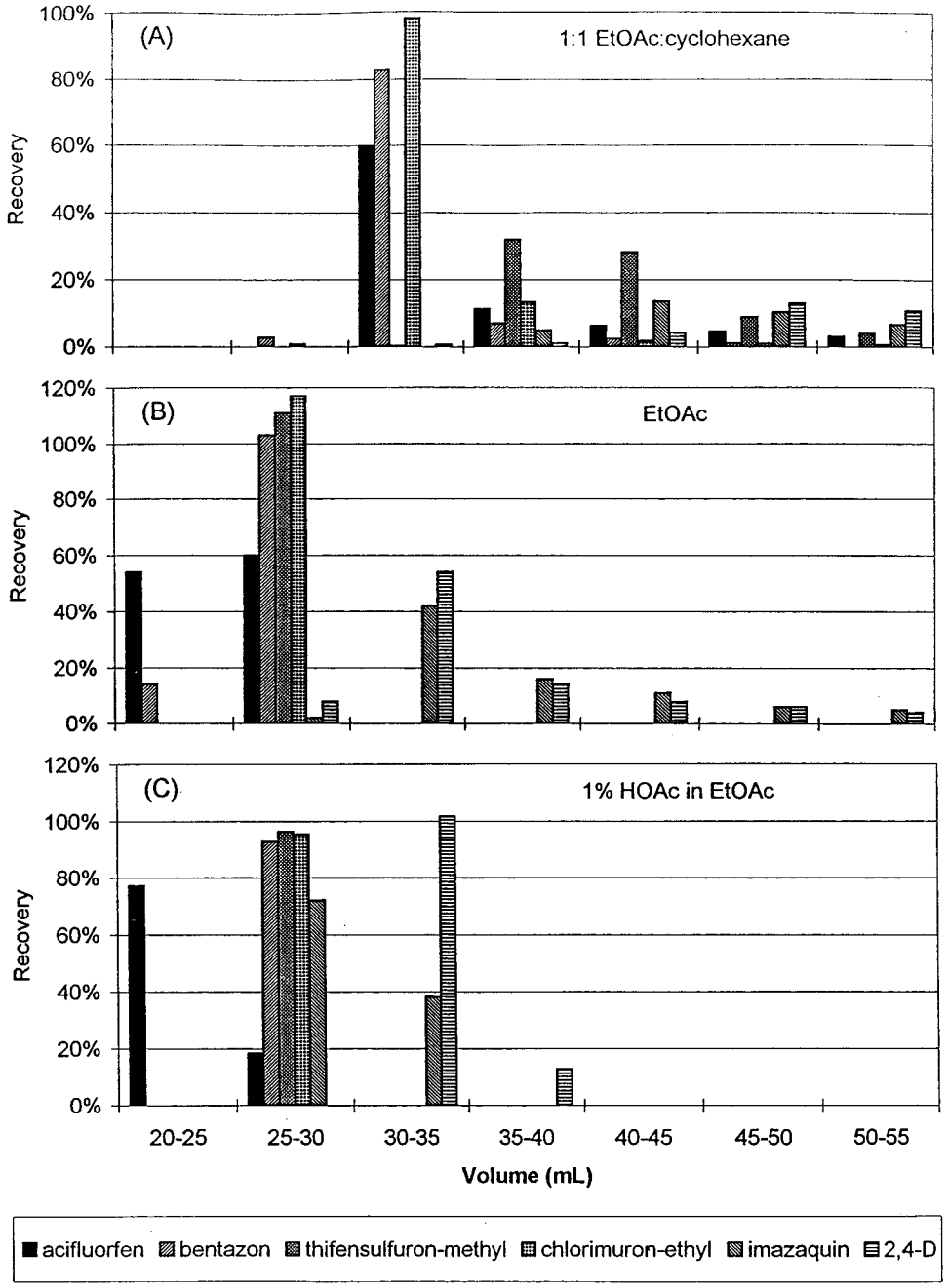


Figure 3. Elution of herbicides in 5 mL of collected fractions from the GPC system using mobile phases of (A) 1:1 EtOAc:cyclohexane, (B) EtOAc, and (C) 1% HOAc in EtOAc.

Table 3. Summary of Some of the SPE Procedures Evaluated for Cleanup of the Herbicides from Soybean Extracts

SPE cartridge	extract solution	washing solution	elution solution	result
C-18	10 mL of 50 mM HCl	10 mL of H_2O	7 mL of acetone	>90% recoveries; salts removed
silica	5 mL of 20:80 acetone:hexane	10 mL of 20:80 acetone:hexane	15 mL of 20:80 MeOH:acetone	>90% recoveries; turbidity removed
SAX	(A) 5 mL of MeCN		(A) 50 mL MeCN	(A) 0% bentazon, acifluorfen, and 2,4-D
	(B) 5 mL of MeCN		(B) 50 mL of MeOH	(B) 0% acifluorfen, bentazon
$-NH_2$	(A) 5 mL of MeCN		(A) 50 mL of MeOH	(A) no herbicides eluted
	(B) 5 mL of MeOH	(B) 10 mL of MeOH	(B) 10 mL of 0.1 M K_2HPO_4 (pH 6−8)	(B) >85% recoveries except chlorimuron-ethyl
	(C) 5 mL of MeOH	(C) 10 mL of MeOH	(C) 10 mL of 0.1 M $NaHCO_3$	(C) >85% recoveries; some interferants removed
alumina-N	(A) 5 mL of MeOH	(A) 10 mL of MeOH	(A) 10 mL of 2/98 HCl:acetone	(A) >80% recoveries; poor cleanup
	(B) 5 mL of MeOH	(B) 10 mL of MeOH	(B) 10 mL of 0.1 M K_2HPO_4 (pH 6−8)	(B) >85% recoveries; independent of pH 6−8
	(C) 5 mL of MeOH	(C) 10 mL of MeOH	(C) 8 mL of 0.1 M $NaHCO_3$	(C) >95% recoveries; some interferants removed

all components precluded obtaining high selectivity. All herbicides were retained on silica (690 mg) with DCM and EtOAc, whereas acetone was able to elute chlorimuron-ethyl, thifensulfuron-methyl, and bentazon. The most polar solvents, MeOH and water, were able to elute all of the herbicides in <10 mL. After further

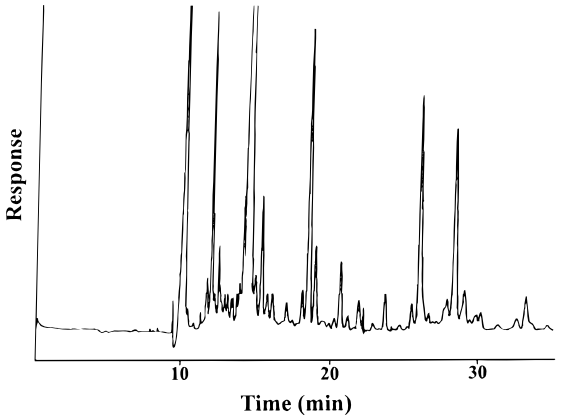


Figure 4. CE/UV electropherogram of a blank soybean extract using the final method except for the semipreparative HPLC cleanup step.

experimentation, a solution of 20:80 MeOH:acetone worked well to elute the pesticides completely within 15 mL, but electropherograms of soybean extracts were too complex to perform quantitation at low levels.

Alumina-N and $-NH_2$ were found to be effective at removing several interfering peaks in CE. In the cases of C-18, SAX, and silica, the retention properties and elution order of the herbicides was different among the different phases tested, but alumina-N and $-NH_2$ behaved rather similarly in this application. All of the herbicides were more strongly retained on these stationary phases than the others evaluated, and strongly acidic organic solvent solutions (e.g. 2−4% concentrated HCl in acetone) were required to elute the herbicides in their protonated form, especially for 2,4-D and acifluorfen. HOAc solutions in DCM were able to effectively elute the sulfonyl ureas and bentazon in 10 mL from alumina-N, but at pH 7, none of the herbicides came off of the 1 g cartridges using 10 mL MeCN, DCM, acetone, EtOAc, or MeOH (water gave partial recoveries). This was useful for washing the cartridge of unretained components in the extracts with the different solvents, but the use of strong acid for elution did not provide much selectivity. Instead, it was determined that the herbicides all were eluted from the cartridges within 10 mL using 0.1 M $NaHCO_3$ (pH = 8.3) which improved cleanup aspects. Solutions of 0.1 M sodium phosphate (pH 6−8) also gave complete elution from alumina-N or $-NH_2$ within 10 mL, but saturated NaCl and $NaSO_4$ solutions did not work as well. Elution with 0.1 M $NaHCO_3$ gave higher herbicide recoveries and was more effective for cleanup than 0.1 M phosphate buffer. The combination of alumina-N and 0.1 M $NaHCO_3$ was most effective for cleanup of soybean extracts versus the other SPE cartridges studied, but as Figure 4 shows, the extracts obtained using the final method, except for the semipreparative HPLC step, were not suitably clean for quantitation of the herbicides by CE/UV.

Semipreparative HPLC. HPLC and CE are often considered competitive approaches in the separation and analysis of thermally labile compounds. However, due to fundamental differences in the HPLC and CE separation processes, the techniques can be complementary in that HPLC can effectively cleanup extracts for CE (injection volumes in CE are generally too small for use in cleanup for HPLC). In this study, HPLC was initially evaluated as an alternative to analysis using CE, and separation of the six herbicides was accomplished

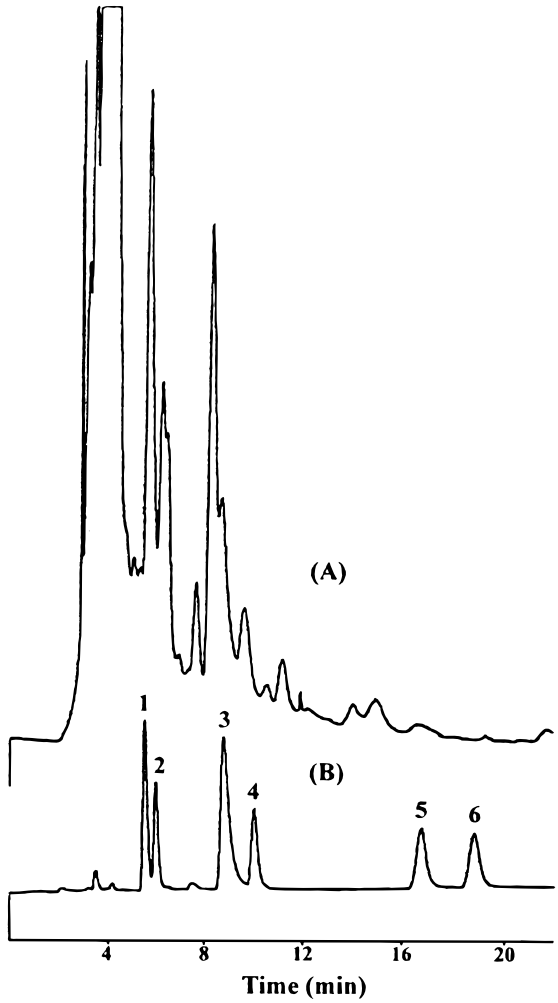


Figure 5. HPLC chromatograms of (A) soybean extract prepared by the final method (except for the HPLC step) and (B) standard solution of the pesticides containing (1) thifensulfuron-methyl, 4 μg/mL; (2) bentazon, 4 μg/mL; (3) imazaquin, 4 μg/mL; (4) 2,4-D, 16 μg/mL; (5) acifluorfen, 8 μg/mL; and (6) chlorimuron-ethyl, 4 μg/mL. The isocratic HPLC mobile phase was 60:40 1% HOAc:MeOH, a 500 μL injection volume, and a 4 mL/min flow rate using the 1 cm i.d., 25 cm long, 5 μm particle size C-18 column.

quickly by modifying the reversed phase HPLC conditions of Knutsson et al. (1992). However, HPLC/UV gave higher LODs than CE/UV for the systems studied, despite the nearly 10 000-fold greater injection volume used in HPLC, and matrix interferants were worse in HPLC/UV than in CE/UV. Figure 5 shows chromatograms for a standard mixture of the herbicides and for a soybean extract using the same cleanup procedure for CE analysis as that shown in Figure 4.

An interesting difference in the HPLC separation with respect CE was the different separation order of the pesticides. Bentazon and 2,4-D appeared much earlier in the HPLC separation, whereas chlorimuron-ethyl, which appeared first in the CE method, eluted last in the HPLC method. This effect also undoubtedly occurred with matrix interferants, and a peak that coeluted with a particular herbicide in CE was most likely to have been separated from that herbicide in HPLC. The collection of fractions at the expected retention times for the individual pesticides in HPLC confirmed this aspect, but again, the need for high recoveries of all six herbicides forced the collection of HPLC fractions that contained interferants in CE. Experiments were

Table 4. Summary of Liquid−Liquid Partitioning Experiments (Boldfacing Represents Phase into Which Herbicides Partition)[a]

solvents	procedure	result
hexane **MeCN**	2 × 10 mL of hexane (MeCN can be acidic)	lipids removed by hexane 100% recovery of herbicides in MeCN
MeCN **saltwater**	add NaCl to aqueous MeCN	partial recoveries in both phases at any pH
EtOAc 0.2 M HCl	2 × 10 mL EtOAc	>95% recovery of herbicides in EtOAc
EtOAc **base**	(A) 0.1 M NaOH (B) 2 × 10 mL 0.1 M $NaHCO_3$ (C) 4 × 10 mL 0.1 M $NaHCO_3$	(A) NaOH degraded imazaquin and thifensulfuron-methyl (B) >90% recoveries, except acifluorfen (75%) (C) >90% recoveries, including acifluorfen
base DCM	10 mL of DCM and 0.1 M $NaHCO_3$ or 0.1 M NaOH	>90% recoveries in aqueous layer except chlorimuron-ethyl which appears in DCM layer
0.2 M HCl **DCM**	2 × 10 mL DCM	100% in DCM

[a] Acid/base partitioning with organic solvents was effective in removing polar interferants from soybean extracts.

conducted using different mobile phases to minimize these interferants without sacrificing recoveries, and a mobile phase gradient of MeCN and 0.15% HOAc solution listed in Materials and Methods was found to be the most practical.

Liquid−Liquid Partitioning. Liquid−liquid partitioning between organic solvents and acid/base solutions was an effective cleanup procedure. Table 4 summarizes the results from the experiments using liquid−liquid partitioning. An inconvenience of the PLE method was that the extract was contained in a solvent mixture of MeCN and water. The separation of the water from the MeCN with complete recoveries in one of the phases could have saved an evaporation step in the overall procedure. The addition of salt to the mixture separated MeCN from the saturated salt solution, but attempts to easily partition all herbicides completely into one of the phases through the use of acidic, neutral, or basic conditions were not successful. Partial recoveries for some of the herbicides were obtained in each phase at pH 2 and 7, while at pH ≈ 8.5, all analytes partitioned into the aqueous phase except bentazon which gave 44% recovery in the MeCN phase.

In other experiments, aqueous acidic or basic solutions were partitioned with the organic solvents, EtOAc or DCM. HCl solution, 0.2 M, was used as the acid in each experiment and 0.1 M NaOH or 0.1 M $NaHCO_3$ constituted the base solutions. In the acid solutions, the herbicides partitioned readily into the DCM or EtOAc phases. Under basic conditions, the herbicides remained in the aqueous layer when partitioned with either EtOAc or DCM, but if the herbicides were dissolved in the organic layer to begin with, it took longer to drive the pesticides from the organic solvent into the aqueous phase. This was presumably because the herbicides did not deprotonate as easily in the organic solvents. Increasing the pH with the use of NaOH rather than $NaHCO_3$ helped speed the process, but the strong base degraded imazaquin and thifensulfuron-methyl, particularly in the experiments with EtOAc. With $NaHCO_3$, acifluorfen tended to give the most problems with incomplete recoveries. In the final method, liquid−liquid partitioning from the organic phase into a base was not performed due to the more inconsistent recoveries.

Final Method. After much experimentation, the extraction and analytical conditions using PLE and CE were established, and high recoveries of the herbicides were achieved through a variety of cleanup approaches. However, the choice of which cleanup techniques to use and the order in which to perform them in the final method was not entirely straightforward. Essentially, the most effective cleanup steps were chosen, and their order in the overall method was designed to provide the most convenience. Minimal experimentation was conducted to change the order of the cleanup steps or to remove a particular step to see the effect on the analysis. The most useful cleanup procedures were liquid−liquid partitioning with hexane, DCM, and EtOAc, semipreparative HPLC, and SPE with alumina-N and C-18. Unfortunately, solvent evaporation steps were needed after nearly every cleanup procedure, and water, MeCN, and EtOAc solutions were not conducive to rapid evaporation. Furthermore, it was important not to store the sulfonyl ureas in water or acidic solution for extended periods of time.

Figure 6 schematically outlines the final procedure used for analysis of fortified soybean samples. This overall method is given in more detail as follows: (1) mix 3 g of ground soybean with 1.5 g of HMX in a weigh boat, load mixture in an 11 mL extraction vessel, and perform PLE; (2) partition extract twice with 10 mL of hexane saturated with MeCN (discard upper layers); (3) evaporate extract until volume is ≈7 mL; (4) add 10 mL of H_2O and ≈0.4 mL of 1 M HCl to make pH ≈ 2 and partition extract twice with 10 mL of EtOAc; (5) add 1 mL of H_2O to combined EtOAc layers and evaporate to ≈1 mL; (6) add 10 mL of MeOH and perform SPE with alumina-neutral (add extract + 10 mL of MeOH rinse, air-dry cartridge for 10 s, and elute with 8 mL of 0.1 M $NaHCO_3$); (7) add 10 mL of H_2O to extract and ≈1.2 mL of 1 M HCl to make pH ≈ 2 and partition with 10 mL DCM twice; (8) evaporate combined DCM layers just to dryness using nitrogen blowdown; (9) add 1.5 mL of 40:60 MeCN:0.15% HOAc, filter through a 0.2 μm filter, and inject 1 mL in the semipreparative HPLC system; (10) collect the 8−14 min fraction and evaporate the extract to ≈4 mL; (11) add 10 mL of 10 mM HCl (pH ≈ 2), sonicate for 2 min, and desalt using the octadecylsilane SPE step (apply extract, rinse with 10 mL of 10 mM HCl and 10 mL of H_2O, and elute with 7 mL of acetone); (12) add 1 mL of H_2O and evaporate to ≈1 mL; (13) add H_2O to make final volume = 5 mL, add 10 μL of internal standard solution, filter through a 0.2 μm filter, and inject in CE.

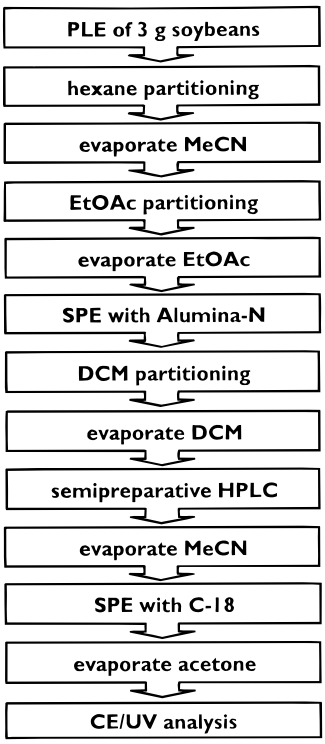


Figure 6. Flow diagram of the final method for the herbicides in soybeans.

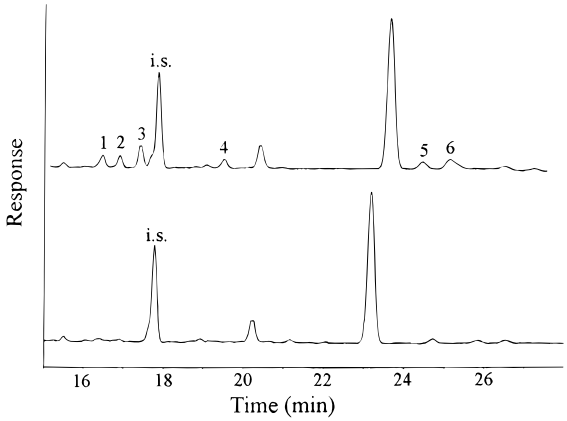


Figure 7. CE/UV electropherograms of soybean extracts: (top) soybean extract from final method fortified at tolerance levels with (1) chlorimuron-ethyl, (2) thifensulfuron-methyl, (3) imazaquin, (4) acifluorfen, (5) bentazon, and (6) 2,4-D and (bottom) blank soybean extract, i.s. = 270 ng/mL chlorsulfuron.

For an efficient worker, the extraction and cleanup steps for the method could be performed for a set of six samples in a single day which would enable analysis performed overnight, but there are stopping points available when the extracts are contained in EtOAc, MeOH, DCM, and acetone. The large amount of labor, time, and materials used in the final method was a disappointment, but this was necessary to meet the objectives of developing the multiresidue method using CE/UV analysis.

Analysis of Fortified Samples. Table 5 presents the results from analyses of fortified soybean samples using the final method. The method worked well over the course of several trials at high fortification levels with average recoveries >70% and relative standard deviation (RSD) < 10%, except for acifluorfen which gave an RSD of 23%. Figure 7 shows electropherograms of a soybean blank extract and an extract fortified at tolerance levels in the soybean; LODs were calculated from these fortified extracts, accounting for the recoveries. The LODs for the herbicides were 2−10 times below the regulatory tolerance levels (Tables 1 and 5), but only imazaquin gave an acceptably low LODs for regulatory analysis.

In samples fortified at the tolerance levels, losses of the sulfonyl ureas, chlorimuron-ethyl and thifensulfuron-methyl, increased, possibly due to increased degradation at lower concentrations. Chlorimuron-ethyl was not detected in the extracts, nor was bentazon due to an insufficiently low LOD in relation to the spiking level. Subsequent analysis of the extracts using HPLC/MS was able to confirm and quantify the presence of these herbicides below the LOD of the CE/UV analytical method (Krynitsky and Lehotay, 1998).

CONCLUSIONS

A multiresidue method of analysis using PLE and CE/UV for six herbicides from five different chemical classes in soybeans was accomplished, but only through the use of a lengthy and laborious cleanup procedure. Even then, the LODs with CE/UV detection were not quite low enough, except for imazaquin, to use the method for regulatory analysis. Perhaps fewer cleanup steps would be needed for water, soil, cereals, or other sample types to provide a more practical method. Generally, multiresidue approaches save time, labor, and expense versus single analyte methods, but for soybeans, it was more unclear if the use of several more selective methods would have been more efficient. The inherent tradeoff between recoveries and selectivity for a diverse set of analytes led to difficulties in minimizing the amount of cleanup that was necessary for the complex soybean extracts.

Another fundamental limitation in this multiresidue approach was the use of the relatively nonselective detection method, UV absorbance. The CE/UV system with the high-sensitivity optical cell provided a superior alternative to HPLC/UV for separation and analysis of the herbicides to HPLC in both clean and relatively complex matrixes. Until recently, there have been no

Table 5. Recoveries, Relative Standard Deviation (RSD), and Calculated LODs ($S/N = 3$) of the Herbicides Fortified in Soybeans Using the Final Procedure

herbicide	high spike recovery[a] (%)	RSD ($n = 14$) (%)	low spike recovery[c] (%)	RSD ($n = 3$) (%)	LOD (ng/g)
chlorimuron-ethyl	88.6	4.4	ND		11
thifensulfuron-methyl	86.5	9.9	53	12	11
imazaquin	81.3	7.2	84.1	4.0	5.2
acifluorfen	72	23	70	20	36
bentazon	88.6[b]	6.1[b]	ND		26
2,4-D	77.3	8.1	76.1	6.5	85

[a] Spiking level of 6.7 times tolerances. [b] $n = 11$. [c] Spiking levels at tolerances; ND = not detected.

exceptional alternatives to UV absorbance for HPLC and CE detection in multiresidue analysis at trace levels. With the use of electrospray ionization/mass spectrometry (ESI/MS), the universally selective detection and chemical confirmation properties of mass spectrometry becomes possible at low concentrations in complex matrices. Krynitsky and Lehotay (1998) evaluated ESI/MS for the same set of analytes in soybean samples and demonstrated the advantages of that approach.

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