RESIDUES AND TRACE ELEMENTS

Rapid Determination of Fosetyl-Aluminum Residues in Lettuce by Liquid Chromatography/Electrospray Tandem Mass Spectrometry

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This paper describes a new method for the sensitive and selective determination of fosetyl-aluminum (Al) residues in vegetable samples. The method involves extraction with water by using a high-speed blender and subsequent injection of the 5-fold diluted extract into the liquid chromatograph. Fosetyl-Al is determined by liquid chromatography with electrospray tandem mass spectrometry after the addition of tetrabutylammonium acetate as the ion-pairing reagent. The method has been used to assay lettuce samples spiked at 2 and 0.2 mg/kg. Recoveries were satisfactory, with mean values of 98 and 106%, respectively, and relative standard deviations were <10%. The limit of quantitation was 0.2 mg/kg, and the limit of detection was as low as 0.05 mg/kg. Matrix-matched calibration was used for quantitation, and the addition of an internal standard improved repeatability. The developed method allows the accurate and rapid determination of low levels of fosetyl-Al residues in lettuce with very little sample handling and good sensitivity; it was shown to be robust by the analysis of almost 100 samples.

Fosetyl-Al, aluminium tris(*O*-ethyl phosphonate), is a fungicide used worldwide to control diseases caused by members of the *Peronosporales*, especially root and crown rots caused by various *Phytophtora* species and foliar diseases caused by some downy mildews (1). Although the mode of action of fosetyl-Al still remains controversial, it is probable that its breakdown product, the phosphonate anion, produces a direct antifungal effect on disease control, inhibiting growth and sporulation of a pathogen such as *Phytophthora* (2).

Because of its chemical characteristics (low molecular mass, ionic structure, and lack of UV absorption or fluorescence), fosetyl-Al is rather difficult to determine by conventional (reversed-phase) liquid chromatography (LC); therefore, it has been traditionally separated by high-performance ion chromatography (1–4), gas chromatography after derivatization (5), or microcolumn LC (6) and measured by using conductivity detection (1–4) or flame photometric detection (FPD; 5, 6).

When plant materials are analyzed, the complexity of the matrix necessitates a cleanup step, which is usually based on solid-phase extraction (1–3) or a derivatization step (5).

In addition, the low sensitivity and selectivity of conductivity detectors do not favor the determination of fosetyl-Al at residue levels in complex matrixes. In spite of the inherent higher selectivity of FPD, the determination of fosetyl-Al by coupling a flame photometric detector to a gas chromatograph or a liquid chromatograph requires a derivatization step (5) or microcolumn LC (6), respectively. Detection limits between 0.5 and 8 mg/kg have been proposed for the above-mentioned conventional techniques.

In recent years, the use of LC/mass spectrometry (LC/MS) for pesticide residue analysis of foods and vegetables has experienced significant growth due to its inherent selectivity and sensitivity. Moreover, when LC with tandem mass spectrometry (LC/MS/MS) is used, the initially formed precursor ion undergoes selective fragmentation achieved by collision-induced dissociation, which results in improved selectivity and sensitivity that make this technique quite adequate for analysis at residue levels (7). This trace-level detection is important in order to reach limits of quantitation that satisfy the regulatory requirements. In the case of fosetyl-Al, the European Union has established a maximum residue level (MRL) of 2 mg/kg in lettuce samples. Ideally, analytical methods with quantitation limits of ≥10 times lower than the MRL would be advisable to evaluate samples for compliance with the MRL.

Several recently published papers have described the use of MS/MS in target analyses for different pesticides by direct injection of a vegetable extract (8–10). Also, a few LC/MS/MS methods have been used to determine organic anions chemically similar to fosetyl, such as phosphonates (11), dibutyl phosphate (12), and alkyl phosphates (13), in complex matrixes. The high polarity of the alkyl phosphates required the use of an ion-pairing reagent to obtain sufficient retention time and allow the separation of highly polar interferences (salts) and analytes. Thus, a volatile ion-pairing reagent, tetrabutylammonium (TBA) acetate, was selected to prevent interface blockage (13).

Received December 3, 2002. Accepted by JS March 5, 2003.
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In this work, we investigated the direct injection of vegetable extracts by using LC with electrospray interface (ESI)–MS/MS with very little sample pretreatment for the rapid determination of fosetyl-Al residues in lettuce at the low mg/kg levels. After the method was validated, its applicability and robustness were checked by analysis of a large number of real samples.

Experimental

Reagents and Chemicals

(**a**) *Fosetyl-Al reference standard (83%).*—Dr. Ehrenstorfer (Augsburg, Germany).

(**b**) *Diethyl phosphate (DEP).*—Used as the internal standard (IS; Suppelco, Bellefonte, PA).

(**c**) *TBA acetate.*—98%; Sigma (St. Louis, MO).

(**d**) *Acetonitrile.*—LC grade; Scharlab (Barcelona, Spain).

(**e**) *Water.*—LC grade; obtained by purifying demineralized water in a Nanopure II system (Barnstead, Newton, MA).

(**f**) *Standard stock solution of fosetyl-Al.*—Prepared by dissolving 59.9 mg reference standard powder, accurately weighed, in 100 mL water to obtain a final fosetyl-Al concentration of 497 μg/mL.

(**g**) *Standard working solutions for the LC–MS/MS analysis and for fortification of samples.*—Prepared by diluting the stock solution with water.

(**h**) *Ion-pairing reagent.*—Prepared by dissolving 1.55 g TBA acetate powder in 10 mL water to obtain a final TBA acetate concentration of 500mM.

(**i**) *Standard stock solution of DEP.*—Prepared by dissolving 25 mg IS powder, accurately weighed, in 50 mL acetonitrile to obtain a final DEP concentration of 500 μg/mL.

(**j**) *Standard solutions containing DEP at 100 and 5 μg/mL.*—Prepared by diluting the stock solution with water.

Instrumentation

(**a**) *LC system.*—A Waters Alliance 2690 LC system (Waters, Milford, MA) was interfaced to a Quattro LC triple-quadrupole mass spectrometer (Micromass, Manchester, UK).

(**b**) *Column.*—Supelco Discovery C_{18} (5 μm, 2.1 × 50 mm); used for the LC separation at a flow rate of 300 μL/min. The mobile phase was a water–acetonitrile gradient in which the percentage of acetonitrile was changed linearly as follows: 0 min, 5%; 4.6 min, 15%; 4.7 min, 60%; 7 min, 60%; 7.1 min, 5%; and 11 min, 5%.

(**c**) *Mass spectrometer.*—Quattro LC (quadrupole-hexapole-quadrupole) mass spectrometer with an orthogonal Z-spray- electrospray interface (Micromass). For operation in the MS/MS mode, the collision gas was argon, 99.995% (Carburos Metalicos, Valencia, Spain) with a pressure of 2×10^{-3} mbar in the collision cell. Capillary voltages of 3.0 kV were used in the negative ionization mode. The interface temperature was set to 350°C, and the source temperature, to 120°C. Dwell times of 0.2 s/scan were chosen. A solvent delay of 2.25 min was selected to give an additional cleanup using the built-in divert valve controlled by Masslynx NT v.3.5 software. This software was also used to process the quantitative data obtained from the calibration standards and from lettuce samples.

(**d**) *Nitrogen generator.*—NG-7 (Aquilo, Etten-Leur, The Netherlands). Drying gas, as well as nebulizing gas, was nitrogen generated from pressurized air in a nitrogen generator. The nebulizer gas flow was set to ca 80 L/h, and the desolvation gas flow to 900–1000 L/h.

(**e**) *Single syringe pump.*—Directly connected to the interface; Model 11 (Harvard Apparatus, Holliston, MA); used to perform infusion experiments.

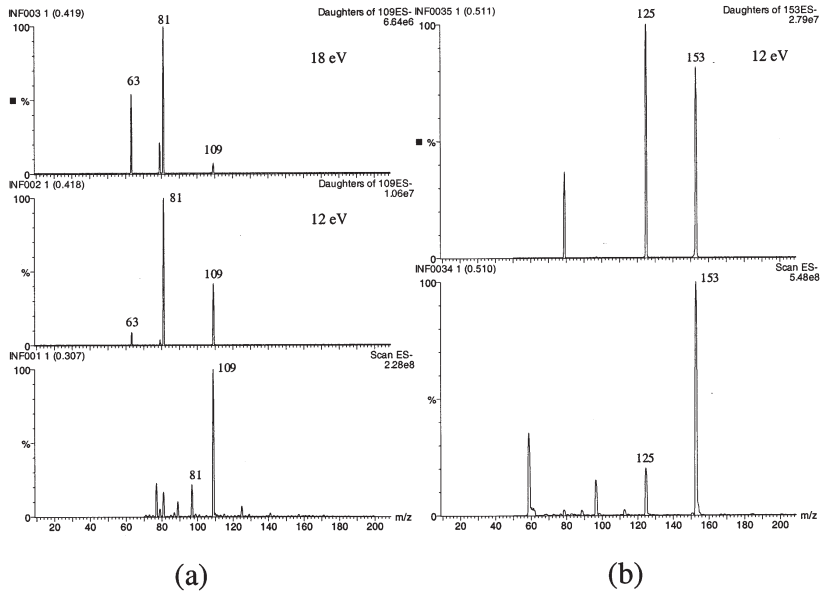

Figure 1. The negative-ion electrospray full-scan mass spectrum (bottom) and product-ion spectra (middle and top) of (a) fosetyl and (b) DEP, acquired by infusion of a 5 μg/mL standard solution.

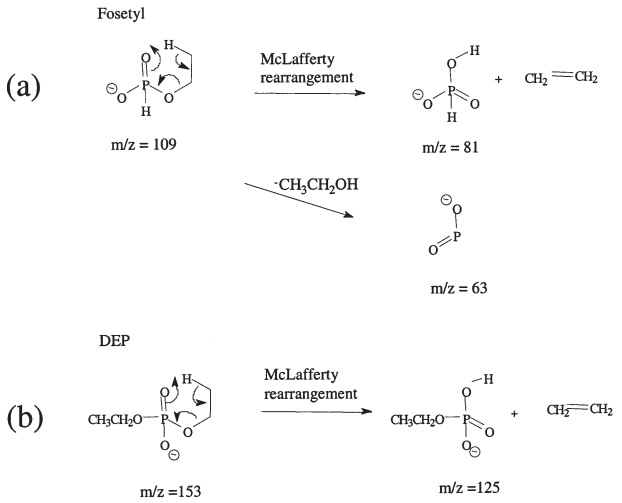

Figure 2. Fragmentation pathways of (a) fosetyl and (b) DEP.

Procedure

A 25 g portion of homogenized lettuce was accurately weighed (precision of 0.1 mg) and mixed with 60 mL LC grade water after the addition of 0.5 mL DEP solution at 100 μg/mL as the IS. The mixture was extracted for 2 min with a high-speed Ultra-Turrax T25 blender (Janke & Kunkel GmbH & Co., Staufen, Germany) at 8000 rpm and filtered by using a vacuum pump; the solid material was washed, and the filtrate was diluted with water to a final volume of 100 mL. If necessary, samples were centrifuged for 15 min to remove possible solid particulates, and finally they were diluted 5-fold, i.e., a 5 mL aliquot was diluted to 25 mL with LC grade water. Before analysis, 75 μL 0.5M TBA acetate solution was added to a 2 mL vial containing 1.5 mL diluted extract.

Matrix-matched standards were used for calibration. A blank lettuce sample was extracted with water, and the extract was diluted to 100 mL. A 5 mL aliquot was transferred to a 25 mL volumetric flask, and 5 mL standard solution (containing fosetyl-Al at 25–5000 μg/L) and 0.5 mL DEP solution at 5 μg/mL were added. Finally, the volume was diluted to 25 mL with water. As described for the samples, 75 μL 0.5M TBA acetate solution was added to a 2 mL vial containing 1.5 mL matrix-matched standard. Fosetyl-Al was determined by injecting a 20 μL aliquot into the LC/MS/MS system under the experimental conditions described in the *Instrumentation* section.

Samples for recovery experiments were fortified by adding 1 mL standard solution containing fosetyl-Al at 5 or 50 μg/mL to each 25 g portion of homogenized lettuce as described above to obtain a fortification level of 0.2 or 2 mg/kg, respectively. These fortified samples were equilibrated for 1 h before extraction.

Validation Study

The linearity of the method was evaluated by using the ratio of the areas obtained for the analyte standard and the IS for ≥8 standard solutions that were analyzed in duplicate.

Precision (repeatability), expressed as percent relative standard deviation (RSD), and recoveries were determined within-day by analyzing fortified lettuce samples in quintuplicate. This experiment was performed at 2 spiking levels, 0.2 and 2 mg/kg.

The limit of quantitation (LOQ) was established as the lowest concentration giving satisfactory recoveries (70–110%) and precision (<15%).

The limit of detection (LOD), defined as the lowest concentration producing a signal-to-noise ratio of 3, was estimated from chromatograms of fortified samples at the lowest concentration assayed.

Results and Discussion

MS Optimization

The negative-ion electrospray full-scan mass spectra and the MS/MS spectra of fosetyl and DEP are shown in Figure 1. All spectra were obtained from infusion, at a flow rate of 10 μL/min, of each compound at 5 μg/mL in acetonitrile–water (50 + 50, v/v). The negative-ion electrospray full-scan spectrum of fosetyl (Figure 1a, bottom) at a cone of 25 V shows a peak at *m/z* 109, corresponding to the molecular weight of the fosetyl anion. The MS/MS spectrum at 12 eV (Figure 1a, middle) shows an important fragment at *m/z* 81, the product of a McLafferty rearrangement. By increasing the collision energy to 18 eV (top), a minor fragment, at *m/z* 63, due to the loss of ethanol was obtained. In contrast, the negative-ion electrospray full-scan spectrum of DEP (Figure 1b), at a cone of 20 V, shows a peak at *m/z* 153, corresponding to the deprotonated molecule $[M\text{-}H]^-$. The MS/MS spectrum (collision energy of 12 eV) shows an important fragment at *m/z* 125 produced by a McLafferty rearrangement. The

Table 1. Optimized MS parameters for the determination of fosetyl-Al

Compound determined	Precursor ion, *m/z*	Cone, V	Collision energy, eV	Product ion, *m/z*
Fosetyl-Al[a]	109	25	12	81
DEP[b]	153	20	12	125

[a] Transition used for quantification.
[b] Internal standard.

Table 2. Results of the dilution study for the determination of fosetyl-Al in 10 individual lettuce samples spiked at 0.2 mg/kg

Extraction	Recovery, %[a]	RSD, %[b]	RSD (IS), %[c]	Absolute signal
Without dilution	21	57	38	2350
2-fold dilution	29	32	27	2100
5-fold dilution	64	12	6	1800
10-fold dilution	84	7	6	1350

[a] Calculated by external calibration with aqueous standards.
[b] RSD = Relative standard deviation.
[c] IS = Internal standard.

fragmentation pathways of these molecules are depicted in Figure 2. The selected reaction monitoring (SRM) transitions chosen for the determination of fosetyl-Al are shown in Table 1.

LC Optimization

Fosetyl-Al is a highly polar compound; thus, it is difficult to obtain enough retention in a C_{18} column to remove salts and polar matrix components, which could interfere by decreasing the response of the MS analyte (13). Several solutions to this problem have been described, such as applying a specific column that allows the use of 100% aqueous solutions (14) or using ion-pair chromatography (15, 16). However, the use of nonvolatile substances is not recommended because nonvolatile ion-pairing reagents destabilize the electrospray process and contaminate the interface (14). In the present work, the selected option was the formation of an ion pair by using TBA acetate, which is the most common volatile ion-pairing reagent for LC/MS (13, 17). In some cases, the use of 1mM TBA acetate in the mobile phase has been a good solution as, for example, in the determination of alkyl phosphates in urine samples (13). However, this option was found to be unsatisfactory for the determination of fosetyl-Al in lettuce extracts because of the loss of sensitivity, which prevented us from achieving the expected LOD values.

Thus, the effect on both the response of the analyte and the retention time was studied by adding different concentrations of TBA acetate directly to the vial containing the solution to be injected into the LC/MS/MS system. The optimum concentration that yielded the best signal with enough retention and good peak shape was 25mM. This was obtained by the addition of 75 μL 500mM TBA acetate solution to 1.5 mL sample extract.

We checked that the TBA-fosetyl formed eluted as an ion pair through the column without any TBA acetate in the mobile phase because no chromatographic differences (retention time and peak shape) were obtained with respect to the data when TBA acetate was added to the mobile phase. The main reason for the removal of TBA acetate from the mobile phase was to improve sensitivity.

Method Optimization

With regard to sample analysis, after direct injection of an aliquot of the 100 mL lettuce extract and external calibration with aqueous standards, a recovery of only 21% was obtained. This low recovery was the result of ionization suppression from the coextracted components of the matrix. Nevertheless, the sensitivity obtained was still sufficient to achieve the required MRL; therefore, matrix-matched external standard calibration was a feasible approach to obtain acceptable recoveries. This approach assumes homogeneity between the blank matrix and samples containing unknown levels of analyte. To confirm this homogeneity, 10 lettuce samples of different origins and varieties were fortified at the 0.2 mg/kg level and analyzed. The response variation, expressed as RSD, was as high as 57%, making the procedure unfeasible.

To reduce the differences between matrixes, the dilution of lettuce extracts with LC grade water was tested as a fast and simple way to improve homogeneity and minimize matrix interferences. The results in Table 2 show that the higher the dilution, the lower the RSD that was obtained, indicating gradual matrix homogenization with dilution improves recovery. According to the RSD results, both 10- and 5-fold dilution would be adequate for accurate quantitation by matrix-matched calibration (RSD < 15%).

Table 2 also shows the relationship between dilution and absolute response obtained in the determination of fosetyl-Al. Unexpectedly, the absolute response did not decrease significantly after dilution. A possible cause of this effect could be

Table 3. Validation results for the developed LC/MS/MS procedure for the determination of fosetyl-Al residues in lettuce

Fortification level, mg/kg	Recovery, %[a]	RSD, %[b]
0.2	106	10
2	98	2

[a] $n = 5$.
[b] RSD = Relative standard deviation.

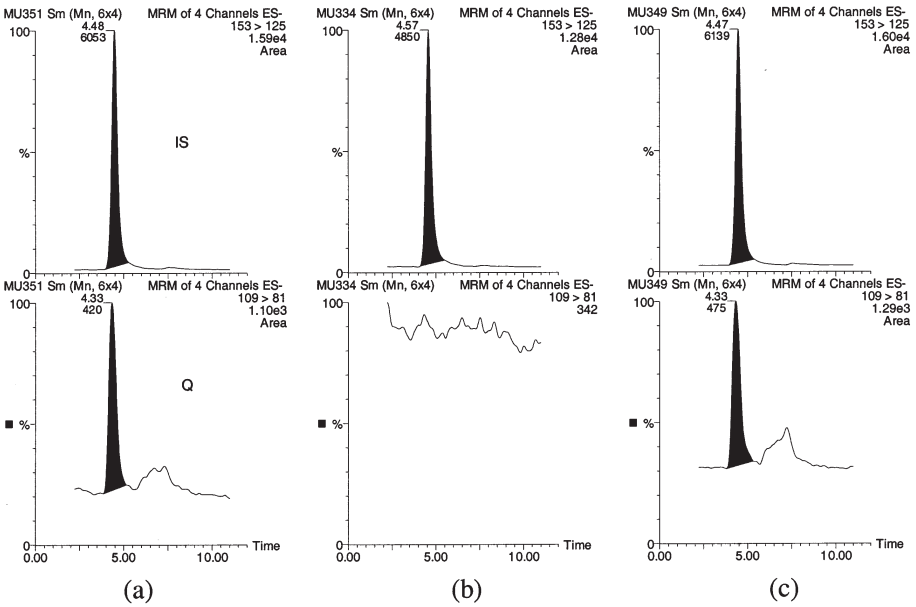

Figure 3. SRM chromatograms of (a) a matrix-matched standard of fosetyl-Al (50 ng/mL), (b) a blank, and (c) a lettuce extract spiked at 0.2 mg/kg. IS = DEP; Q = quantitative transition of fosetyl.

matrix ion suppression caused by compounds coextracted with the analyte. These interferences would be minimized by sample dilution, and analyte ionization would be improved; therefore, a higher-than-expected response would be obtained, depending on the dilution factor. Matrix-matched calibration with a 5-fold diluted matrix was selected as a compromise between repeatability and sensitivity. On the other hand, recovery values obtained by external calibration with aqueous standards gradually increased with matrix dilution, and quantitative values (84%) were obtained with a 10-fold dilution. In this way, external calibration with aqueous standards could be used, but only after the lettuce matrix is diluted by a factor of 10, and with a significant loss of sensitivity.

To achieve accurate quantitation without a decrease in sensitivity, the use of an IS was also checked. Because of the unavailability of a labeled IS, an analogue compound, DEP, was assayed. In this case, the coelution of the IS and analyte would be advisable (18); this was achieved by applying an LC gradient. As Table 2 shows, the rather high matrix content still present in the nondiluted and 2-fold diluted lettuce extracts could enhance the ionization differences between the analyte and the IS, as well as the chromatographic differences. Under these conditions, the use of the analogue IS was not adequate for the accurate quantitation because matrix effects were not compensated for.

However, a 5-fold dilution produced an adequate RSD. Consequently, a 5-fold dilution for both samples and matrix-matched standards was chosen, with the addition of DEP as the IS. Because DEP is one of the metabolic products of organophosphorus pesticides, samples were not treated with any pesticide that could be metabolized as DEP before this method was applied.

Method Validation

The optimized method described above was validated before it was applied to real-world samples. Linear calibration curves were obtained for both aqueous and matrix-matched standards in the range 5–1000 μg/L, with a correlation coefficient r = 0.9993. Precision and accuracy (expressed as recovery) are reported in Table 3. The method was found to be precise (RSD < 10%) and accurate, with satisfactory recoveries (98–106%), an LOQ of 0.2 mg/kg, and an estimated LOD of 0.05 mg/kg.

Typical chromatograms for standard solutions and lettuce samples (blank and spiked at the lowest level assayed) are shown in Figure 3. The chromatograms were obtained after direct injection of the 5-fold diluted solutions/extracts. The total chromatographic run time was about 11 min.

Application to Field-Treated Samples

The optimized procedure was applied to the analysis of about 80 samples from field residue trials. With each batch of 6–10 samples, a calibration curve was prepared for analyte concentrations between 5 and 1000 μg/L by injections before and after those of the sample extracts. In addition, 2 quality control (QC) samples were injected in every batch of samples. The QC samples were blank lettuce samples fortified at the LOQ level and at 10 times the LOQ level. In all cases, the individual QC recoveries were satisfactory (between 70 and 110%), showing the robustness of the procedure. These results allowed us to estimate the reproducibility of the method by using the QC recovery values obtained in the analysis of samples over a period of approximately 4 months. The results obtained were satisfactory both at the LOQ level (98% average recovery and 13% RSD) and at 10 times the LOQ level (99% average recovery and 8% RSD). All samples and standards were

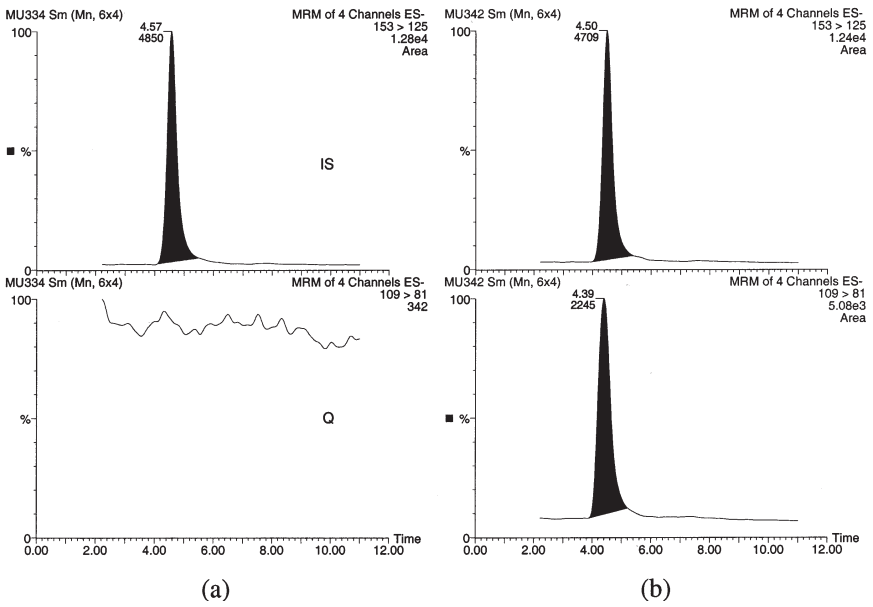

Figure 4. SRM chromatograms of field lettuce samples (a) untreated and (b) treated with fosetyl-Al and containing fosetyl-Al at 2.22 mg/kg. IS = DEP; Q = quantitative transition of fosetyl.

injected in duplicate. Levels of the fosetyl-Al residues were consistent with the elapsed time between application and collection of samples.

Figure 4 shows typical chromatograms for one untreated sample and one sample treated with fosetyl-Al and collected 7 days after the application.

Conclusions

This work has demonstrated that LC/ESI–MS/MS is a sensitive and selective technique for the determination of fosetyl-Al residues in vegetable samples at sub-ppm levels. Because fosetyl is found as an anionic species in aqueous solutions, the addition of an ion-pairing reagent such as TBA acetate is required for its sufficient retention on a reversed-phase LC column. The developed method described in this paper allows a short chromatographic run time (about 11 min) and fast and simple sample pretreatment, compared with previous methods reported in the literature.

When applied to lettuce samples, matrix calibration with raw extracts was shown to be an insufficiently robust approach to obtain satisfactory results when lettuce samples from different origins and varieties were analyzed. However, divergences were corrected by simply diluting the samples with water. In addition, dilution of samples did not lead to an important loss of absolute response, allowing the analysis of 5-fold diluted extracts without significant loss of sensitivity. The use of an analogue IS, such as DEP, although not compulsory, allowed the analytical characteristics of the method, mainly repeatability, to improve. The developed method was satisfactorily applied to field-treated samples, and its suitability and robustness were demonstrated.

Acknowledgments

We are very grateful to Probelte SA (Murcia, Spain) for financial support and for providing the lettuce samples, and to Serveis Centrals d'Instrumentació Científica (SCIC) of University Jaume I for the use of the Quattro LC triple-quadrupole mass spectrometer.

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