A rapid method was developed for the trace-level determination of glyphosate in olives. After extraction of the glyphosate with water–dichloromethane and simultaneous removal of the olive oil, an aliquot of the aqueous extract is derivatized with 9-fluorenylethoxycarbonyl chloride; FMOC-Cl) to produce a highly fluorescent derivative. A 2 mL aliquot of this extract is injected directly into a coupled-column liquid chromatography system with fluorimetric detection (LC/LC–FD). The procedure was validated by recovery experiments at 3 spiking levels; recoveries ranged from 80 to 97% with relative standard deviations of 3–6%. The limits of detection and quantitation were estimated to be 0.01 and 0.05 mg/kg, respectively. The method was also applied to other plant materials, i.e., tomato plants, strawberry plants, and pear trees (branches, leaves, and fruits) suspected to be contaminated by glyphosate. In all these cases, the extraction was performed in aqueous media. The derivatization reaction was modified by increasing the FMOC-Cl concentration, to ensure a quantitative reaction between analyte and reagent in the presence of high levels of coextractives, which also react with FMOC-Cl. The final determination was by LC/LC–FD, yielding a rapid, selective, and sensitive method for the determination of glyphosate residues in these samples. The method was tested with real-world samples after application of glyphosate to the surrounding area of crops.

Glyphosate ([N-phosphonomethyl]glycine) and glyphosate-trimethylsulfonium (trimethylsulfonium N-[phosphonomethyl]glycine) are, respectively, the active ingredients in the commercial herbicides Roundup, marketed by Monsanto, and Touchdown, marketed by Zeneca Ag Products. They are nonselective, postemergence herbicides used for control of many grasses and broadleaf weeds and are registered for a number of preplant and postharvest uses and other noncrop uses. One of the important uses of glyphosate is weed control in the canopy of olive trees before harvest, which allows a reduction in recollection costs; however, because of the lack of information about glyphosate residues in olives, the collection of fallen olives from the ground could make herbicide applications a possible residue concern.

Recently (summer 1998), several analyses of tomato plants for glyphosate residues were required in our laboratory because it was suspected that they were contaminated with this widely used herbicide. To conduct the analyses, we first developed a method to determine residues of glyphosate at low levels in tomato plants.

Chemical properties of glyphosate that contribute to its effectiveness as a herbicide also make its determination difficult, especially at residue levels in the variety of environmental matrices where it might be found (1). Its polar nature and high water solubility make solvent extraction difficult and limit the options for using gas chromatography (GC) (2–7). The similarity of glyphosate to naturally occurring amino acids and amino sugars further contributes to the difficulty in determining residues in crops and animal products. This situation has usually required the use of lengthy cleanup procedures, involving both anion- and cation-exchange columns (6, 7). The lack of a chromophore or fluorophore also makes it necessary to use derivatization techniques for the determination of glyphosate by liquid chromatography (LC) (8–19), which also typically involves several cleanup steps.

Previous works have demonstrated that coupled-column liquid chromatography (LC/LC) combined with large-volume injection is an adequate technique for the rapid, sensitive, and selective determination of polar pollutants in environmental samples (20–22). In the case of glufosinate, glyphosate, and the main metabolite of glyphosate, amino-methylphosphonic acid (AMPA), different procedures for water (23, 24) and soil (25) have been developed, offering low limits of detection without preconcentration and sample cleanup. This approach yields a rapid and robust procedure for monitoring programs. A schematic presentation of an LC/LC system is shown in Figure 1. One of the most relevant aspects of this technique is the separation power of the first
column, C-1, which combines automated sample cleanup (enhancement of selectivity) with simultaneous enhancement of the overall sensitivity of the method as a result of the relatively large volume injected.

The aim of this work was the development of an LC/LC method for the rapid trace-level determination of glyphosate in different types of plant material. The developed method was validated for olives and subsequently tested for other types of plant materials, i.e., tomato plants, strawberry plants, and pear-tree leaves suspected to be contaminated with glyphosate. The method first involves a derivatization step with 9-fluorenylmethyl chloroformate (9-fluorenylmethoxycarbonyl chloride; FMOC-Cl) followed by the use of a short C_{18} separation column, for large-volume injection and efficient separation between analyte and excess reagent, coupled to a second amino separation column for anion-exchange separation of the derivatives.

**Experimental**

**Chemicals**

Glyphosate (content >99%) was obtained from Riedel-de Haën (Seelze, Germany). Acetonitrile, ethyl acetate, and dichloromethane, all LC grade, were purchased from Scharlau Science (Barcelona, Spain). Analytical reagent-grade potassium dihydrogen phosphate, disodium tetraborate decahydrate, orthophosphoric acid (50% purity), hydrochloric acid (37%), potassium hydroxide, and FMOC-Cl were purchased from Merck (Darmstadt, Germany). LC-grade water was obtained by purifying demineralized water in a Nanopure II system (Barnstead, Newton, MA).

Stock standard solutions (ca 400 µg/mL) of glyphosate as well as diluted standards were prepared with LC-grade water. Solutions of 0.125M borate buffer (pH 9) were prepared in LC-grade water, and solutions of FMOC-Cl at 1000 and 5000 µg/mL in acetonitrile were used for derivatization. A 0.025M borate buffer solution was used to dilute extracts before LC/LC analysis.

The mobile phases (both M-1 and M-2) in the LC/LC method were acetonitrile–0.05M phosphate buffer, pH 5.5 (35 + 65, v/v). The pH of the aqueous buffer was adjusted to 5.5 with 2M KOH and 1M HCl.

**Equipment**

The modular LC system (Figure 1) consisted of a Model 233XL sampler (Gilson, Villiers-le-Bel, France); a high-pressure valve, equipped with a 2.0 mL loop and used to perform large-volume injections; a second high-pressure valve, used to perform column switching and time controlled by the sampler; a Model 9013 ternary gradient LC pump (Varian, Walnut Creek, Walnut Creek, CA); a Model 2150 isocratic LC pump (LKB, Bromma, Sweden); a Model 1046A fluorescence detector (Hewlett-Packard) set at 263 nm (excitation) and 317 nm (emission); a 30 × 4.6 mm id first-separation column (C-1) packed with 10 µm Spherisorb ODS-2 (Scharlau Science); and a 250 × 4.6 mm id second-separation column (C-2) packed with 5 µm Spherisorb NH₂ (Scharlau Science).

Chromatograms were recorded and peak areas were measured quantitatively with a Hewlett-Packard LC Chem Station (software version G1034A). A MicropH 2001 pH meter and Pipetmans (200, 1000, and 5000 µL) were obtained from Crison Instruments (Barcelona, Spain) and Gilson, respectively.

**Procedures**

**Olives.**—The olives were washed twice with 200 mL water and were subsequently chopped and homogenized. A 10 g sample portion was mixed with 20 mL dichloromethane and 20 mL water, and the mixture was mechanically shaken for 60 min and then centrifuged (2500 rpm for 15 min).

A 1 mL aliquot of supernatant water extract was pipetted into a 20 mL glass tube together with 0.5 mL 0.125M borate buffer and 1.0 mL FMOC-Cl reagent at 1000 µg/mL. The tube was shaken for 5 s and left at room temperature for 30 min. After the reaction, 17.5 mL 0.025M borate buffer was added, and the tube was swirled for thorough mixing.

![Figure 1. LC setup for column switching. AS = sample injector with 2 mL loop; HV = 6-port high-pressure valve; P-1 = gradient LC pump; P-2 = isocratic LC pump; C-1 = first separation column; C-2 = second separation column; M-1 and M-2 = mobile phases on C-1 and C-2, respectively; FD = fluorescence detector; I = integrator system; and W = waste.](image-url)
Other plant materials.—First, the plants were chopped and homogenized. Then 5 g sample was mixed with 10 mL water, and the mixture was mechanically shaken for 60 min and centrifuged (3000 rpm for 15 min).

A 0.5 mL volume of supernatant water extract was pipetted into a 20 mL glass tube together with 1 mL 0.125 M borate buffer and 1.0 mL FMOC-Cl reagent at 5000 µg/mL. The tube was shaken for 5 s and left at room temperature for 30 min. After the reaction, 17.5 mL 0.025 M borate buffer was added, and the tube was swirled for thorough mixing.

LC/LC analysis.—The mobile phases were set at a flow rate of 1 mL/min. A 2.00 mL volume of the diluted solution obtained after derivatization was injected onto C-1. After cleanup with 2.15 mL M-1 (injection volume included), C-1 was switched online with C-2 for 0.25 min to transfer the fraction containing the glyphosate derivative to C-2. After the transfer, C-1 was rinsed and conditioned with M-1 while the derivative was separated on C-2 with M-2. The analyte was quantitated by external calibration with standard solutions in water, or in blank extract in the case of olives, which were processed after the precolumn derivatization procedure.

Results and Discussion

In our previous work (23–25) on the determination of glyphosate in aqueous and soil samples, adequate LC/LC conditions were established, enabling cleanup and large-volume injection on the first short C18 column, and efficient separation of the analyte on the second amino separation column. From our experience in analysis for glyphosate, glufosinate, and AMPA, a mobile phase of acetonitrile-0.05 M aqueous phosphate buffer, pH 5.5 (35 + 65, v/v) was selected for both columns to provide satisfactory results. We found that sensitivity could be enhanced considerably by large-volume injection after dilution of the derivatized sample with a volume of borate buffer. Our aim was to decrease the acetonitrile content for better peak compression of the analyte on the first column during the 2 mL injection step. In the present study we investigated the potential of this approach for the determination of glyphosate in plant material.

Olives

First, the olives were washed to remove possible glyphosate deposited on them, so that only the amount of compound taken up by the olives, which could contaminate the crop and the processed olive oil, would be determined. Then the extraction step was optimized by studying different solvents, i.e., mixtures of organic phase (dichloromethane or ethyl acetate) and aqueous phase (acid, neutral, or basic). A water-immiscible organic solvent, dichloromethane, was used to remove the olive fat and avoid an emulsion. The pH of the
aqueous phase was also checked because glyphosate had been extracted from acidic soils and plant material using both acid and alkaline media (10, 26). The best results were obtained with dichloromethane–water, pH 7 (50 + 50), which provided a clear and accessible aqueous extract free of as many coextractives as possible. Three layers appeared after the extraction and centrifugation steps. The bottom layer contained the olive fat dissolved in dichloromethane, the middle layer contained the olive cake, and the top layer was a clear aqueous extract containing the analyte. A 1 mL aliquot of this aqueous extract was pipetted directly into the derivatizing tube, and the procedure was followed as stated in the Experimental section. It is noteworthy that a more concentrated borate buffer was needed, compared with that used in previous work (23–25), to properly maintain the pH of the derivatization reaction.

An 8-fold dilution was used after precolumn derivatization to decrease the acetonitrile content to 5% to obtain good peak compression (24). Olive sample extracts were processed with LC/LC by using accurate, adjusted cleanup and transfer times for glyphosate (cleanup, 2.15 min; transfer, 0.25 min). Figure 2 shows the chromatograms obtained with this procedure for a glyphosate standard at 100 µg/L and a blank untreated olive sample fortified with glyphosate at 100 µg/L. A small decrease in peak height observed for the olive sample was due to the presence of matrix components during the derivatization step; therefore, all standards were prepared in blank olive extract to solve this problem. Extracts contain-

<table>
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<th>Av. recovery, %</th>
<th>RSD, %</th>
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<td>6</td>
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<td>0.05</td>
<td>84</td>
<td>4</td>
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* n = 5.

Table 1. Recovery of glyphosate from olives by the LC/LC method

Figure 4. Chromatogram, obtained by LC/LC, for a field-treated olive sample, showing a glyphosate content of 0.11 mg/kg.

Figure 5. Chromatograms, obtained by LC/LC, for (bottom) a blank untreated tomato plant sample extract fortified with glyphosate at 400 µg/L and prederivatized with 5000 ppm FMOC-Cl, (middle) a glyphosate standard of 400 µg/L, and (top) a blank untreated tomato plant sample extract fortified with glyphosate at 400 µg/L and prederivatized with 500 ppm FMOC-Cl.
ments with blank olive samples fortified with glyphosate at 3 levels. The recoveries and relative standard deviations (RSDs) are listed in Table 1, which shows acceptable values (>80%) and RSDs of 3–6% (n = 5). Figure 3 shows the chromatograms obtained for an olive sample fortified with glyphosate at 0.05 mg/kg and for the blank olive sample, from which a limit of detection of 0.01 mg/kg was estimated for the developed method. Therefore, the limit of quantitation was found to be 0.05 mg/kg, and the statistical limit of detection was 0.006 mg/kg (3 times the standard deviation calculated for the 0.05 mg/kg level). However, from a practical point of view, it seems that a limit of detection of 0.01 mg/kg is more realistic. The values appear to be adequate for monitoring glyphosate residues in these types of samples. Differences in the retention time of glyphosate shown in Figures 2 and 3 are due to a small degradation of the stationary phase of the second column. This difference does not affect the robustness of the coupling procedure because the cleanup and

![Chromatograms](image)

Figure 6. Chromatograms, obtained by LC/LC, for (A) healthy and unhealthy tomato plants, showing a high level of glyphosate in the latter, saturating the fluorescence detector, and (B) an unhealthy tomato plant extract diluted by a factor of 1000, showing a high level of glyphosate, 554 mg/kg.
transfer times depend only on the short C$_{18}$ column. The shift of the glyphosate retention time to smaller values was checked periodically by injection of standard solutions.

The LC/LC procedure was applied to the analysis of olives treated with glyphosate in the canopy of olive trees and collected 1 or 7 days after treatment. The glyphosate concentrations found in olive samples reached maximum values of 0.11 mg/kg (1 day after treatment) and 0.08 mg/kg (7 days after treatment). Figure 4 shows the chromatogram obtained for the olive sample extract with the highest level of glyphosate residue. Moreover, glyphosate was also determined in the first water washings to obtain the total glyphosate residue on/in the olive sample and to calculate the percentage of glyphosate that can be taken up by the olives. The values obtained refer to the olive sample (200 mL water washings/200 g olives) and ranged between 0.07 and 0.23 mg/kg.

**Other Plant Materials**

Tomato plants were also studied for the determination of glyphosate residues in our laboratory in a case of possible glyphosate contamination from an unknown source. In this case, we analyzed the plant, not the tomato fruit. We used only water for the extraction of this kind of nonfatty plant material, based on the results obtained for the olive samples. We also omitted the dichloromethane partitioning step because there was no need to remove coextracted fat, and we used only a neutral aqueous phase. The sample size was decreased to 5 g to maintain the sample/solvent ratio of 1/2, and the centrifugation speed was increased slightly to obtain a clear water extract.

To check the effect of the matrix on the derivatization reaction yield, we compared the chromatograms obtained for a glyphosate standard at 400 µg/L and a blank tomato plant extract fortified with glyphosate at 400 µg/L. As shown in Figure 5, no glyphosate peak appears in the chromatogram for the blank tomato plant extract. This can be explained by the presence of large amounts of endogenous compounds that also react with the FMOC-Cl reagent. As indicated earlier, this effect was also observed for olives to a much lower extent. For tomato plant extracts, the derivatization step was slightly modified by decreasing the extract volume from 1 to 0.5 mL, and increasing both the borate buffer volume to 1 mL and the FMOC-Cl concentration to 5000 µg/mL, which was a 10-fold increase in the FMOC-Cl/sample ratio relative to the ratio in our previous work on water, soil, and olives. Under these conditions, the glyphosate peak height obtained was comparable to that for the standard prepared in LC water, as shown in Figure 5; consequently, all the standards used for calibration and quantitation were prepared in pure water.

With these new derivatization conditions, healthy and unhealthy tomato plants were analyzed by the procedure described for other plant materials in the Experimental section, and the chromatograms shown in Figure 6A were obtained. These chromatograms indicate the presence of an enormous amount of glyphosate in the unhealthy tomato plants.

To quantify the amount of glyphosate in the sample, the aqueous extract was diluted by a factor of 1000 before the derivatization step; alternatively, the sample size was decreased to 1 g and then diluted by a factor of 200. In both cases, the residue levels found were similar, reaching concentrations as high as 554 mg/kg with RSDs of 10% ($n = 6$). The absence of glyphosate residues in the healthy tomato plants provided further evidence that the apparent source of illness in the unhealthy plants was the high level of this herbicide. Figure 6B shows the chromatogram obtained for the unhealthy tomato plant after dilution by a factor of 1000.

The method applied to tomato plants was also used to determine the presence of glyphosate in strawberry plants and pear trees. The herbicide was detected in all the unhealthy samples, and no residues were detected in the control samples.

**Conclusions**

A rapid, sensitive, and selective method with high sample throughput was developed for the determination of glyphosate residues in different plant materials. The method was validated for the determination of glyphosate residues in olives with recoveries of >80% and a limit of quantitation of 0.05 mg/kg. The developed method was also applied to other types of plant materials with satisfactory results. It was shown to be a very robust method for analysis of real samples to confirm the presence of glyphosate residues in possible cases of contamination. The use of 2 columns with different retention mechanisms (reversed phase and weak anion exchange), the short selective transfer of the analyte fraction to the second column, and the selective fluorescence detection provide a very powerful approach for confirmation of glyphosate residues.

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**References**