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Short communication

Rapid determination of glyphosate in cereal samples by means of pre-column derivatisation with 9-fluorenylmethyl chloroformate and coupled-column liquid chromatography with fluorescence detection

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Abstract

A rapid procedure for the determination of glyphosate in cereals has been developed. Convenient sample pretreatment is carried out by (i) a overnight standing extraction of 1.0 g homogenized sample with 20 ml of water, (ii) centrifugation of the samples, (iii) a passing of 2.5 ml of the clear layer through a 100 mg C_{18} solid-phase extraction cartridge and (iv) collection of the last 1.5 ml of the eluent into a calibrated tube. For the instrumental analysis, the efficient approach developed earlier for environmental water samples [J.V. Sancho, F. Hernández, F.J. LÛpez, E.A. Hogendoorn, E. Dijkman, P. van Zoonen, J. Chromatogr. A, 737 (1996) 75] was successfully adopted for the determination of glyphosate in the obtained cereal extracts. The procedure includes a 15 min derivatisation step of the analyte with 9-fluorenylmethyl chloroformate and a 16 times dilution step prior to instrumental analysis employing coupled-column LC with fluorescence detection. The developed procedure has a sample throughput of more than 25 samples per day and a limit of quantification of 0.5 mg/kg. The method was validated by analyzing freshly spiked cereal samples and samples with aged residues at levels between 1.0 and 10 mg/kg. The overall recovery of the freshly spiked samples was 86% ($n=10$) with a repeatability of 6.5% and a reproducibility of 9.5%. For samples with aged residues recoveries performed at different time intervals (range 80–150 days) did not differ significantly; the overall recovery ($n=10$) was 74% with a repeatability and reproducibility of 14 and 20%, respectively.

Keywords: Food analysis; Column-switching; Glyphosate; Pesticides

1. Introduction

Glyphosate [*N*-(phosphonomethyl)glycine] is a broad spectrum, non-selective post-emergence herbicide introduced in the early 1970s which has found a widespread agricultural and domestic use. The Dutch regulation [1] in harmonization with the European Union (EU) requires the annual monitoring of glyphosate in several types of foodstuffs [2] making the availability of an efficient screening method mandatory. Moreover, soybeans genetically modified for their resistance to the herbicide glyphosate have been recently introduced into the market.

Having pK_a values of 0.78, 2.29, 5.96 and 10.98 [3], glyphosate is a very polar and amphoteric compound. This feature and the poor detectability of glyphosate makes that existing analytical methodology always includes a derivatisation step prior to instrumental analysis. Both gas chromatography

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(GC) [4,5] and liquid chromatography (LC) [6–16] are used as a sensitive and selective technique in the residue analysis of glyphosate in agricultural and environmental samples. The availability of derivatisation techniques compatible with an aqueous extract or sample and the chromatographic separation makes LC an attractive technique. Three different procedures are generally used for the determination of glyphosate with LC: (i) post-column ninhydrin derivatisation and UV detection [6,7], (ii) post-column fluorogenic labeling with *o*-phthalaldehyde and fluorescence detection (FLD) [8–11] and pre-column derivatization using 9-fluorenylmethyl chloroformate (FMOC-Cl) with FLD [11–14]. The latter technique in combination with large volume injection (LVI) and coupled-column LC (LC–LC) has been successfully applied by us for the determination of glyphosate in environmental water [15] and soil [16] samples. This approach, based on the determination of glufosinate in water [17] allowed the direct processing of water samples or aqueous soil extracts after FMOC derivatisation and a dilution step enhancing significantly the sample throughput in comparison to existing methodology.

The aim of this study was to investigate the applicability of pre-column derivatisation with FMOC-Cl and LC–LC for the efficient determination of glyphosate in foodstuffs. An overview of matrices and the corresponding maximum residue limits (MRLs) for glyphosate according to the Dutch regulation [1] is given in Table 1. Based on this information grain was selected as a model commodity for these group of matrices for the development of a method for the determination of glyphosate down to a level of at least 1 mg/kg.

Table 1
Glyphosate MRLs of the Dutch Regulation [1]

Matrix	MRL (mg/kg)
Wheat and rye	5
Barley and oats	20
Mushrooms	50
Linseed	10
Rape seed	10
Soybean	20

2. Experimental

2.1. Chemicals

Glyphosate (content >99%) was obtained from Dr. S. Ehrenstorfer (Promochem, Wesel Germany). Acetonitrile, HPLC-grade, was from J.T. Baker (Deventer, Netherlands). Analytical grade potassium dihydrogenphosphate, disodium tetraborate decahydrate, orthophosphoric acid (analytical-reagent grade, 89% pure), hydrochloric acid (analytical-reagent grade, 37%), potassium hydroxide and FMOC-Cl were bought from Merck (Darmstadt, Germany).

HPLC-grade water was obtained by purifying demineralised water in a Milli-Q system (Millipore, Bedford, MA, USA).

A stock standard solution (approx. 500 μg/ml) of glyphosate and dilutions were prepared in HPLC-grade water. A 0.025 *M* and a 0.125 *M* disodium tetraborate buffer solution (pH 9) was prepared in HPLC-grade water. A 2 mg/ml FMOC-Cl solution was prepared in acetonitrile.

Acetonitrile–0.05 *M* phosphate, pH 5.5 in water (35:65, v/v) was used as the first (M-1) and second (M-2) mobile phase for the first column (C-1) and second column (C-2), respectively. The pH of the mobile phase was adjusted with 1 *M* potassium hydroxide solution. A rinsing mobile phase (MR) consisting of acetonitrile–water (65:35, v/v) was applied for the clean-up of C-1 between analysis.

Disposable 1-ml solid-phase extraction (SPE) cartridges containing 100 mg of C_{18} bonded phase (40 μm) were obtained from J.T. Baker. The cartridges were preconditioned with 3 ml of acetonitrile, 3 ml of water and 1 ml of sample or standard obtained after derivatisation with FMOC-Cl.

2.2. Equipment

A Baker-10 system of J.T. Baker was used to perform SPE. The HPLC modular system consisted of a Model 232 ASPEC autosampler from Gilson (Villiers-le Bel, France) equipped with two programmable high-pressure valves (type 7010, Rheodyne, Cotati, CA, USA), two Model 305 and one Model 306 isocratic LC pumps from Gilson, and a Model FP-920 fluorescence detector of Jasco

(Tokyo, Japan) set at 263 nm (excitation) and 317 nm (emission).

In the coupled column LC analysis a 30×4.6 mm I.D. column packed with 5 μm Hypersil ODS and a 250×4.6 mm I.D. column (C-2) packed with 5 μm Adsorbosphere NH_2 both from Alltech (Carnforth, UK) were used as C-1 and C-2, respectively. C-2 was kept at 30°C with a laboratory-made column oven connected to a Model 1441 circulating water system from Braun (Melsungen, Germany).

Recording of chromatograms and quantitative measurements of peak heights were performed with the PC-1000 integration software and a Model 800 DP integrator of Spectra-Physics (San Jose, CA, USA).

A pH meter Model 691 and Pipetmans (200 and 1000 μl) were from Metrohm (Herisau, Switserland) and Gilson, respectively. A Model 2-15 centrifuge was from Sigma (Osterode am Harz, Germany).

A Moulinette chopper machine of Moulinex (Paris, France) was used for grinding grain samples into flour.

2.3. Sample fortification

Freshly spiked samples were prepared by weighing 1.0 g of flour into a glass bottle followed by the addition of an appropriate volume (=2.0 ml) of spiking solution; the samples were allowed to stand overnight before extraction. Fortifications were made at levels of 10, 5.0 and 1.0 mg/kg, respectively.

Samples with aged residues were prepared by spiking 1 g flour samples to the levels of 1.0 and 10 mg/kg. After air-drying overnight, they were stored in the refrigerator at about 4°C; sample analysis was performed after 80, 140 and 150 days, respectively.

2.4. Sample pretreatment (cf. Fig. 1)

One g of grain flour was weighed into a 50-ml erlenmeyer flask with stopper. Next, 20 ml of HPLC-grade water were added and the erlemeyer was shaken vigorously for 1 min. After an overnight standing extraction the content was transferred into a 30-ml centrifuge tube and centrifuged for 10 min at 4500 rpm (3034 *g*). One ml of clear supernatant was brought onto a preconditioned 100 mg C_{18}-bonded silica cartridge and send to waste. The cartridge was removed from the SPE system and placed on top of a calibrated tube. 1.5 ml of the clear supernatant sample was transferred to the cartridge and by means of over pressure, i.e., with a syringe placed on top of the cartridge, passed through the cartridge and collected into the tube. After adding 200 μl of 0.125 *M* borate buffer and 1.0 ml of FMOC solution (2 mg/ml in acetonitrile) the tube was swirled and left at room temperature for 15 min. After reaction, 1.0 ml of the solution was pipetted into a 20-ml vial and 15 ml of borate buffer (0.025 *M*) was added and the vial was swirled again to perform a proper mixing.

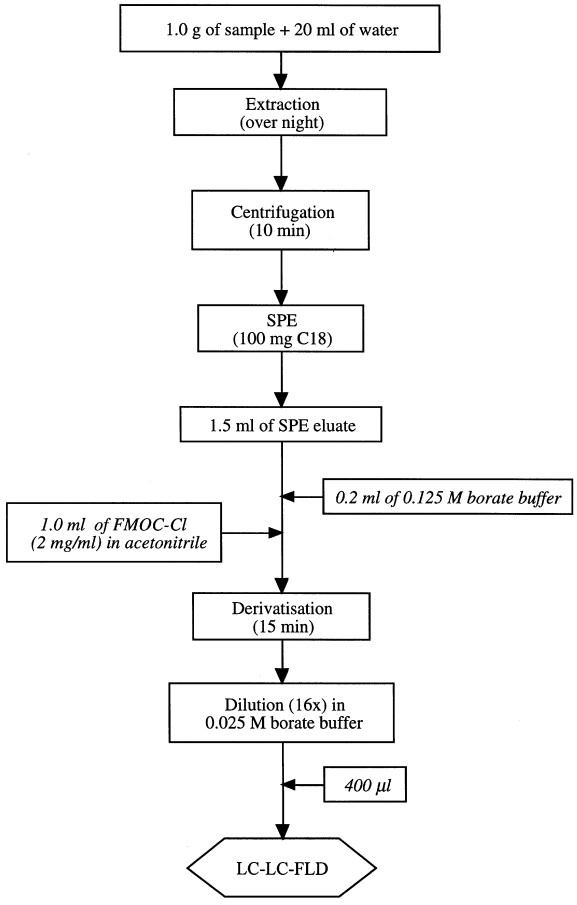

Fig. 1. Scheme of analysis.

2.5. LC analysis

The mobile phases were set at a flow of 1 ml/min. A volume of 400 μl of the solution obtained after derivatisation and dilution was injected onto C-1. After clean-up with 0.40 ml of M-1, C-1 was switched on-line with C-2 during 18 s to transfer the fraction (300 μl) containing the glyphosate derivative to C-2. Next, by means of the second high-pressure valve C-1 was cleaned with 5 ml of rinsing mobile phase and reconditioned with M-1 prior to the next injection.

Quantitation of glyphosate was done by external calibration with standard solutions of glyphosate in water obtained after derivatisation and dilution.

3. Results and discussion

3.1. General aspects

The aim of this study is to investigate whether the efficient approach developed for the determination of glyphosate and glufosinate in environmental samples [15,16] can be applied to foodstuffs. In order to comply with the products of the Dutch Regulation (see Table 1) grain was selected to represent the matrices.

The developed approach is based on the FMOC-derivatisation procedure [13] of the analyte followed by the on-line processing of the (diluted) solution with LC–LC using a C_{18} column (C-1) coupled to an ion-exchange amino column (C-2) as firstly applied for the determination of glufosinate in water [17].

FMOC-Cl is a very reactive reagent and the fact that in aqueous solution the reagent rapidly converts into FMOC-OH, the derivatisation procedure always provides a large excess of fluorogenic FMOC interferences. Fortunately, in case of amphoteric analytes like glyphosate and amino acids the excess of the rather non-polar FMOC-OH can be effectively removed. This is usually done by ionizing the FMOC analytes (anions) by means of pH adjustment of the aqueous phase followed by an extraction with a powerful organic solvent such as ethyl acetate.

In the LC–LC approach using a mobile phase of acetonitrile–phosphate buffer, pH 5.5 (35:65) glyphosate-FMOC will be ionized which will result in little C_{18} retention on C-1 and adequate retention on the amino column (C-2). The efficient clean-up is obtained by transferring an almost unretained small analyte-containing fraction to C-2. All interferences with more C_{18} retention, e.g., FMOC-OH are retained on C-1 and sent to waste by the rinsing mobile phase during the separation of the analyte on C-2. It appeared that sensitivity could be improved by large volume injection (up to 2.0 ml) after diluting the solution obtained after FMOC derivatisation with borate buffer. A sufficient decrease of the percentage organic modifier (from 37 to 2.3%, see Fig. 1) of the sample provided an adequate peak compression of the analyte during injection of volumes up to 2.0 ml.

3.2. Extraction

Compared to environmental samples the nature of grain is quite different requiring adjustment of the sample pretreatment, e.g., extraction, clean-up. Glyphosate is a systemic herbicide which requires a grinding of the grain into flour in order to extract adequately the analyte from the matrix.

Because of the amphoteric character of glyphosate extraction should preferably be done with an aqueous solution. However, such a polar solvent will proliferate a large amount of polar matrix interferences requiring a large effort in laborious sample pretreatment, e.g., liquid–liquid extraction [8,12,14] and off-line chromatographic techniques [5,9].

To be compatible with the solvent used for derivatisation and dilution a 0.02 *M* borate buffer solution (pH 9) was firstly selected as the extraction solvent in combination with a one-night over standing extraction. As a good compromise between the volume of the matrix and the solvent 1 g of flour was extracted with 20 ml of borate buffer. However, this solvent remains turbid after high-speed centrifugation and/or filtration over a 0.20-μm filter. Therefore, LC-grade water providing a clear solution after extraction was selected for further experimental work.

3.3. Clean-up

Firstly, experiments were performed without an additional clean-up step. Aiming at a limit of quanti-

fication of about 0.5 mg/kg a 16-fold dilution step after derivatization was applied and a sample injection volume of 400 μl was selected. However, under these conditions it was not possible to determine glyphosate-FMOC at the required sensitivity due to the high amount of interferences.

Assuming that a large part of the contamination is caused mainly by FMOC reaction products less polar than glyphosate-FMOC, an SPE clean-up as applied for the determination of ethylenethiourea in apple juice [18] was investigated. The clean-up approach (see Section 2) on the 100 mg C_{18} SPE cartridge is similar to that of the C-1 column in LC–LC analysis. A small volume of sample extract is passed through the cartridge and collected. Glyphosate is transferred quantitatively, while the less polar interferences will be retained effectively from the aqueous solution.

Glyphosate-FMOC is also very polar allowing the use of the SPE clean-up before or after derivatisation with FMOC-Cl. In both cases the fast SPE clean-up decreased significantly the amount of interferences and provided a good baseline separation of the analyte. SPE before derivatisation was selected on the basis of the slightly improved baseline.

The performance of the procedure is demonstrated in Fig. 2A showing the LC–LC–FLD analysis of an extract of grain sample spiked at the level of 5 mg/kg.

3.4. Results

The final procedure including aqueous extraction of the sample, simple SPE treatment and instrumental analysis with LC–LC–FLD is schematically presented in Fig. 1.

The LC–LC–FLD analysis of an extract of a flour sample spiked at the level of 1 mg/kg shown in Fig. 2B illustrates that a limit of detection (LOD, $S/N=3$) of 0.5 mg/kg is feasible.

When comparing the chromatograms of Fig. 2A Fig. 2B one can observe the (unexpected) appearance of a large and broad interference peak (I) in Fig. 2B which is not present in Fig. 2A. It appeared that the interference peak originates from a previous injection

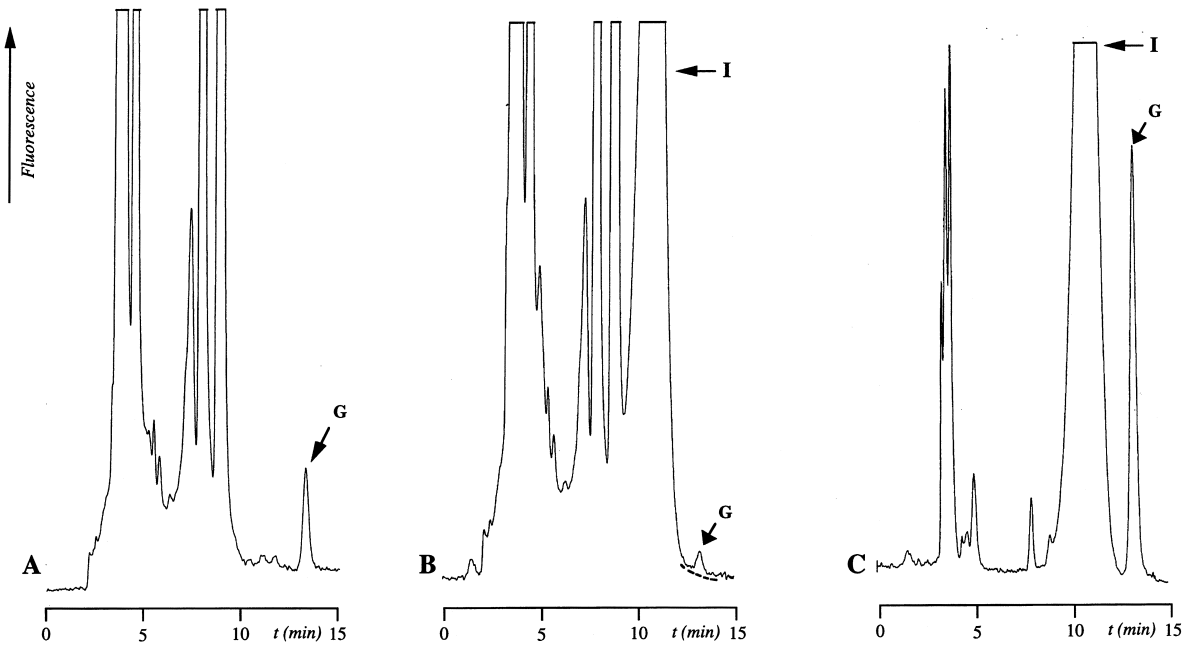

Fig. 2. LC–LC–FLD of extracts of grain flour samples freshly spiked with glyphosate at a level of 5 mg/kg (A) and 1 mg/kg (B) and of a glyphosate standard (C) in water (1 μg/ml). Cereal extracts obtained after sample pretreatment procedure given in Fig. 1 and standard after derivatization and dilution; G, glyphosate-FMOC; I, interference.

Table 2
Overall results of freshly spiked cereal samples ($n=10$)

Series	Spiked level (mg/kg)	No. of experiments	Mean recovery (%)	RSD (%)
1	10	3	88	5.3
2	5 and 10	2	99	7.1
3	1, 5 and 10	5	80	7.0
		Overall recovery (%)	86.3	
		Repeatability (%)	6.5	
		Reproducibility (%)	9.5	

and can be eliminated by increasing the run time with about 5 min. In order to visualize the origin of background interferences the LC–LC analysis of a blank cereal is indicated by the dotted line in Fig. 2B and of a glyphosate standard in water after FMOC derivatisation and dilution displayed in Fig. 2C.

The method was validated by the analysis of freshly spiked homogenized grain (flour) samples and homogenized grain samples with aged residues (see Section 2) on different days and levels. The overall results of the freshly spiked samples are summarised in Table 2. A mean recovery of 86%, a repeatability of 6.5% and a reproducibility of 9.5% are satisfactory results.

The validation data of the aged residues samples are given in Table 3. A mean recovery of 74%, a repeatability of 14% and a reproducibility of 20% emphasizes the usefulness of this approach concerning both extraction and instrumental analysis. An explanation for the relative high value of the mean recovery at day 140 cannot (yet) be given. However, the contribution of repeatability and the inter-period variation is almost equal, indicating the acceptability of the obtained data in this field of analysis.

4. Conclusions

The approach of pre-column derivatization with FMOC-Cl and LC–LC–FLD [15–17] has been extended successfully for the residue analysis of glyphosate in grain.

The high selectivity of the column-switching procedure combined with a preceding simple SPE sample pretreatment makes it possible to determine glyphosate down to a level of 0.5 mg/kg in grain.

The analytical procedure provides a throughput of at least 25 samples per day and has been effective for grain flour samples with aged residues stored over a period of 150 days.

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Table 3
Overall results of cereal samples with aged residues ($n=10$)

Aging (days)	Spiked level (mg/kg)	No. of experiments	Mean recovery (%)	RSD (%)
80	1 and 10	3	63.3	13.1
140	10	4	92.5	17
150	1 and 10	3	61.3	7.0
		Overall recovery (%)	74.4	
		Repeatability (%)	13.8	
		Reproducibility (%)	19.9	

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