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Review

Analytical methods to determine phosphonic and amino acid group-containing pesticides

Constantine D. Stalikas*, Constantina N. Konidari

University of Ioannina, Department of Chemistry, Laboratory of Analytical Chemistry, Ioannina 45110, Greece

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Abstract

A comprehensive view on the possibilities of the most recently developed chromatographic methods and emerging techniques in the analysis of pesticides glyphosate, glufosinate, bialaphos and their metabolites is presented. The state-of-the-art of the individual pre-treatment steps (extraction, pre-concentration, clean-up, separation, quantification) of the employed analytical methods for this group of chemicals is reviewed. The advantages and drawbacks of the described analytical methods are discussed and the present status and future trends are outlined.

Keywords: Reviews; Sample preparation; Water analysis; Fruits; Soil; Forensic analysis; Environmental analysis; Phosphonic acids; Amino acids; Pesticides

Contents

*Corresponding author. Tel.: +30-651-98719; fax: +30-651-44831.
E-mail address: cstalika@cc.uoi.gr (C.D. Stalikas).

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1. Introduction

The term pesticides includes insecticides, herbicides, fungicides and various other substances used to control pests. The chemical classification is based on functional groups in their molecular structure or their specific biological activity on plagues. The presence of pesticide residues in the environment and the public concern about their possible toxic effects has forced official international institutions to establish maximum allowable concentration levels of these chemicals. In the European Union as well as in the USA through the National Pesticide Survey lists are established of compounds based on the human health and environmental risk [1–3]. The European Union has strict legislation on the occurrence of pesticides in water intended for human consumption, the maximum concentration of a pesticide should not exceed 0.1 μg/l while the sum of all pesticides must be below 0.5 μg/l [4,5]. Important areas in which monitoring takes place are the commodities on which the pesticides are applied, mainly fruits and vegetables, but due to the veterinary use or proliferation of pesticides in the food chain the pesticides are also monitored in products of animal origin. In the environment water and soil are the main areas of interest. The occurrence of pesticides in ground water largely depends on the physical and chemical properties [solubility, persistence (DT_{50}), leachability (K_{oc})] of the compound involved. For soil, persistent compounds that are not too mobile may be found. The above-stated risks and concerns about safety of drinking water and contamination of food have fuelled strong efforts by analytical chemists to develop methods able to detect residues at sub-μg levels in routine analysis.

Phosphonic and amino acid group-containing chemicals constitute an important category of pesticides which includes glyphosate [*N*-(phosphonomethyl)glycine] (GLYP), glufosinate [DL-homoalanin-4-yl-(methyl)phosphonic acid] (GLUF), bialaphos 4-[hydroxy(methyl)phosphinyl]-L-homoalanyl-L-alanyl-L-alanine (BIAL) and ampropylfos [(*RS*)-1-aminopropylphosphonic acid]. Mainly, GLYP, GLUF and BIAL are predominantly employed as non-selective, post-emergence contact herbicides and therefore the present review will focus on these.

GLYP is a widely used broad-spectrum, foliar-applied herbicide for vegetation control, introduced in the early 1970s by Monsanto. It is absorbed into the leaves and translocated through the plant to the roots and rhizomes via the phloem. On contact with soil, GLYP binds tightly with soil particles and so it is made unavailable for uptake by plants and no longer phytotoxic. It acts by interfering with the enzyme that catalyzes the sixth step in the shikimate pathway, 5-enol-pyruvylshikimate-3-phosphate synthetase (EPSP), has little chronic and neurotoxic effects and no obvious carcinogenic and mutagenic activity. Although GLYP mainly targets EPSP synthetase, photosynthesis and respiration are also affected. The physical, chemical and toxicological properties of GLYP have been well documented [6–8]. It is rapidly and completely degraded by soil microorganisms to water, carbon dioxide and phosphate [9]. The first step in the degradation pathway is essentially the cleavage to glyoxylate and aminomethylphosphonic acid (AMPA), that is also biologically degradable although slower than the parent compound [10,11]. It has been reported that GLYP is resistant to volatilisation and sunlight-mediated degradation [12]. Having pK_a values of 2.0, 2.6, 5.6, and 10.6, GLYP is a very polar and amphoteric compound [13]. Because of the advances in scientific knowledge, the US Environmental Protection Agency (EPA) requires that GLYP as well as a multitude of other pesticides, which were first registered years ago, be re-registered to ensure that they meet today's more stringent standards.

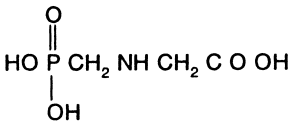
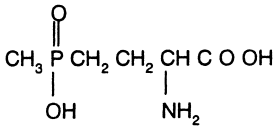
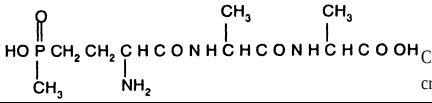
The ammonium salt of GLUF is currently being evaluated as a site preparation herbicide known by

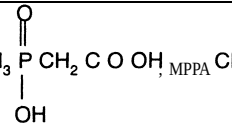
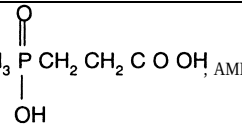
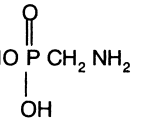
the trade names Ignite and Basta. GLUF is a very polar compound and its structural formula is similar to that of the older and widely used GLYP. It is a synthetic herbicide, related to the natural product BIAL produced by *Streptomyces viridochromogenes* L., both compounds containing phosphinothricin as the active ingredient [14]. Phosphinothricin inhibits the action of the enzyme glutamine synthetase, thus leading to the accumulation of toxic levels of ammonia by destructing ammonia metabolism and finally to plant death. It is anticipated that the worldwide usage of GLUF will increase greatly. Laboratory and field studies have shown that GLUF is readily biotransformed in soil. The parent compound half-life is ca. 3–11 days. For GLUF, the microbial degradation is the most important dissipation pathway with the degradation rate being dependent on soil characteristics and environmental conditions. Studies have shown the formation of two main metabolites during the degradation process. More specifically, the main metabolite is 3-methylphosphinicopropionic acid (MPPA) produced by the oxidative deamination of GLUF that is further degraded to 2-methylphosphinicoacetic acid [15]. The biotransformation potential of groundwater microorganisms with respect to the compound is unknown. Biotransformation of GLUF is not expected to be as rapid in groundwater as it is in soil because of the typically lower nutrient availabilities, relatively sparse microbial population and often colder temperatures of the groundwater environment. The relatively short half-life in soil suggests that there is a low potential for GLUF transport to groundwater if applied as recommended [16]. The potential effects of temperature, light, soil moisture and other factors on the adsorption translocation or metabolism of GLYP and GLUF have been investigated [17,18].

BIAL is a tripeptide-type antibiotic produced by *Streptomyces hygroscopicus* and is used as a herbicide. It is a commercially available natural phytotoxin, which consists of phosphinothricin and two L-alanine residues [19]. Compared to the other two herbicides previously presented, less information is available in the literature about BIAL.

Table 1 summarises data concerning the studied group of herbicides and affords the chemical structures of them and their principle metabolites.

Table 1
Structural formulae, physical characteristics and toxicology data of the pesticides

Pesticide	Formula	Form	Solubility in water (mg/l at 22°C)	Metabolites[a]	Acute oral LD_{50} for male rats (mg/kg)	Tradenames
Glyphosate (GLYP)		Odourless white powder	$>1.1\cdot10^{4}$	Aminomethylphosphonic acid (AMPA)	5600	Roundup, Rodeo, Sting
Glufosinate (GLUF)		Crystalline solid with pungent odour	$>1.4\cdot10^{6}$	3-Methylphosphinicopropionic acid (MPPA), 2-methylphosphinicoacetic acid	2000	Basta, Liberty Ignite
Bialaphos (BIAL)		Colourless crystals	$>1\cdot10^{6}$	L-Glufosinate	268	Meiji Herbiace

[a] 2-Methylphosphinicoacetic acid  , MPPA  , AMPA 

The difficulties in establishing simple methods for the extraction and determination of these compounds at residue levels are mainly due to their properties: relatively high solubility in the water, insolubility in organic solvents and favoured complexing behaviour. For reasons of late marketing (since the early 1980s) and its less widespread application, information on analytical methods for GLUF and its metabolite MPPA is poor in comparison to GLYP and its metabolite AMPA. Original methods for the detection of GLYP involved analysis by thin-layer chromatography [20,21] whereas later methods involved the employment of gas and liquid chromatographic techniques which require derivatization of the analytes, necessary for the chromatographic separation in gas chromatography (GC) and for improving detectability in liquid chromatography (LC) with fluorescence or UV detection.

It is known that the analytical methods for both research and regulatory purposes are multiresidue and single residue. The former are preferable because they provide the capability of determining different pesticide residues in a single analysis. Because GLYP, GLUF and BIAL are compounds with similar chemical structures, it is conceivable that the use of a common screening method which could also involve AMPA, MPPA and L-GLUF, the main metabolites of the parents GLYP, GLUF and BIAL would be possible. However, no or few attempts have been made up to now to present analytical methods spanning all the phosphorous-containing amino acid-type herbicides and some or all of their metabolites. These efforts for single and multiresidue analysis will be presented in this overview. Chromatographic methods have strict requirements for sample introduction while the achievement of high sensitivity necessitates the use of pre-concentration techniques. Therefore, a major part of this overview is devoted to the sample pre-treatment and clean-up of a diversity of sample matrices before the detection of the herbicides and their metabolites.

2. Chromatographic methods

2.1. Gas chromatography

2.1.1. Derivatization methods

The choice of a robust, simple and efficient derivatization reaction is the first and most critical step for a successful and reproducibly applicable GC method. Targeted analysis for phosphonic and amino acid herbicides requires prior derivatization in order to conveniently render them less polar and sufficiently volatile derivatives to be subsequently chromatographed.

Many reaction schemes have been discovered throughout the years in an attempt to derivatize the studied compounds. A derivatization procedure developed by Monsanto, the main producer of Roundup (GLYP formulation) and suggested by the EPA [22], has been adapted and used by many laboratories since 1977. In principle, it encompasses separate acetylation with trifluoroacetic anhydride, alkylation with diazomethane and detection by GC. Irreproducible results and low recoveries have been reported with this tedious methodology, especially with samples (crops, soils), which present formidable clean-up difficulties [23]. Furthermore, the method suffers from the drawback of employing diazomethane, a highly toxic, carcinogenic and explosive reagent.

Bearing this in mind, Moye and Deyrup focused their efforts on the utilisation of a relatively innocuous reagent, *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), a silylating reagent which had been used for the derivatization of sulfate, phosphate and other oxyanions and produces *tert*.-butyldimethylsilyl (tBDMS) groups at sites having active hydrogens [24]. It was attained to derivatize GLYP and AMPA using a single-step reaction, which is rapid, clean and accomplished with easily obtainable commercial reagent. The derivatives are stable upon storage and easily gas chromatographed under moderate conditions without the need for the removal or destruction of the reagent MTBSTFA. Briefly, the procedure involves evaporation of water at 100°C under a stream of nitrogen, addition of 4-(4-methyl-1-piperidinyl)pyridine as a catalyst and MTBSTFA to produce tBDMS groups after incubation at the foregoing temperature. Due to difficulties experienced with the lack of reproducibility and to prevent or reduce apparent adsorption of GLYP and AMPA on glass, special efforts were made to assess the most appropriate derivatization tubes. It was found that best results were obtained when experimentation was conducted in phosphoric acid-treated borosilicate glass tubes. Coating the tube

with phosphoric acid markedly increased yields, but low yields were still observed at low analyte levels. An alternative to this method which made use of a MTBSTFA–dimethylformamide (1:1) mixture led to stable and easily chromatographed derivatives without making any mention of the above reproducibility problems [25].

When problems with high detection limits and bad reproducibility remained unsolved, researchers turned their attention to the production of other derivatives. Deyrup et al. found that a mixture of fluorinated alcohol such as trifluoroethanol (TFE) with perfluorinated anhydride such as trifluoroacetic anhydride (TFAA) was a successful way to derivatize GLYP and AMPA [26]. Typically, the derivatization involves reaction under anhydrous conditions using TFE–TFAA at a ratio of 1:2 and heating at 90–100°C for 30 to 60 min. Compared to the MTBSTFA, this derivatization approach was found to be superior to the point that no special coating of the glass tube was needed and much better recoveries were obtained at low levels. Investigations which followed this publication were based on this derivatization concept for the determination of some of the studied herbicides in different environmental matrices [27–32].

Alferness and Iwata interestingly exploited the possibility of selecting certain alcohols to vary chromatographic retention and improve detectability eliminating potential interferences [33]. These authors presented a derivatization method that involves the direct addition of an aqueous extract or water sample to a mixture of TFAA and 2,2,3,3,4,4,4-heptafluoro-1-butanol to derivatize the analytes. The resulting derivatives of GLYP and AMPA despite their high molecular masses were thermally stable and exhibited rapid chromatographic elution that is typical of the fluorinated compounds. While the procedures for derivatizations with perfluorinated anhydrides require complete absence of water from the reaction mixture, the proposed method manages to get the analytes reacting with a premixed chilled reagent, the former being in the bulk aqueous matrix. This alleviates the difficulties mentioned, which arise from the irreversible adsorption of pesticides and their metabolites onto glass by virtue of the evaporation of sample before derivatization.

Trifluoroacetylation by trifluoroacetic anhydride followed by methylation with diazomethane has also been reported [34]. This method apart from the use of the undesirable diazomethane also requires many time-consuming steps and therefore is not judged workable.

Guinivan et al. first reported the alkylation with boron trichloride–2-chloroethanol and then acylation with heptafluorobutyric anhydride to produce 2-chloroethyl–*N*-heptafluorobutyryl derivatives [35]. The derivatized molecules were extracted from an aqueous environment with hexane. Heating of the reaction mixture at 110°C was required between the successive additions of the reagents.

The determination of GLYP by Curie point pyrolysis GC appearing in the Japanese literature does not offer a conventional and facile manner for such analysis [36]. In addition to above, the method cannot be an attractive prospect because of the needed sample workup and the low selectivity and sensitivity in the pyrogram.

Two convenient methods for screening GLYP, GLUF and AMPA were presented where herbicides and the metabolite were determined as their *N*-isopropoxycarbonyl- (*N*-*i*POC) and *N*-isobutoxycarbonyl (*N*-*i*BOC) methyl ester derivatives in water, food and soil samples [37,38]. The method requires neither vigorous derivatization conditions nor any clean-up step prior to detection; however the use of diazomethane with the already mentioned drawbacks seriously limits their applicability.

The notion of determining GLUF in soil by means of acetic acid and trimethyl orthoacetate (TMOA) was introduced by Smith [39], adopted by Tsuji and Akiyama for the simultaneous determination of GLUF, MPPA and GLYP in crops [40]. The reaction was satisfactorily applied by Ohtani et al. to develop a multiresidue method for the determination of GLUF, GLYP and five phenoxyalkanoic acid herbicides in river water with remarkably low detection limits and satisfactory recoveries [41]. The method is rather simpler than those previously published since it requires heating for a lesser period of time in the presence of all the reactants at the comparatively lower temperature of 80–90°C and ensures the simultaneous esterification of hydroxyl and carboxylic groups and the acetylation of amino groups of pesticides. Again, some precautions should be taken with a view to minimise adsorption of the analytes onto the glass walls of the reaction tubes. Sonication of the tubes for 5–10 min before incubation had an

enhancing effect on the reaction rate and yield of derivatization. Stalikas and Pilidis stressed that differences in the reaction rates and yields which were observed during derivatization are undoubtedly due to the presence or absence of certain functional groups in the molecules of the herbicides and their metabolites [42]. A chemometric optimisation developed for the derivatization of all the candidate molecules took into account the main and most limiting variables to this stage of method development: the concentrations of the reactants, the temperature and the reaction time This method performed a detailed investigation of the influential parameters for the simultaneous derivatization of the targeted molecules.

2.1.2. Chromatographic conditions and separation

Table 2 highlights the main characteristics of the most common GC methods for the herbicides and their metabolites so far published. As can be seen, the only way to obtain acceptable separation using GC is through chemical derivatization. The benefits from the use of the non-polar to moderately polar capillary chromatographic columns in GC can be found in the gain in sensitivity and detection limits as illustrated in the table.

BIAL is difficult to detect as an intact molecule by GC analysis. This fact, in addition to certain difficulties related to poor analytical reproducibility and sensitivity, have prevented many researchers from dealing systematically with it. Three publications have reported the analysis of BIAL along with other molecules of similar structure [25,32,42]. All of them present BIAL as emerging in the chromatogram as an intact molecule while one of them detects GLUF and BIAL as double peaks due to the occurrence of enantiomeric forms. A representative gas chromatogram including as many of the derivatized analytes as possible is depicted in Fig. 1.

2.1.3. Detection

Flame photometric detection (FPD), nitrogen–phosphorous detection (NPD) or the extreme sensitivity of electron-capture detection (ECD) seemed to be the preferable choices for detection after proper derivatization even though mass-selective detection (MS) has been adopted in this field of analysis.

FPD is based on the element-specific luminescence produced when sulfur or phosphorus compounds are burnt in a hydrogen-rich flame. The emission band for phosphorus species is detected at 526 nm. NPD was discovered by the observation that an alkali salt in the flame of a flame ionisation detection system enhanced the ionization of N and P compounds. Both detection methods obviously were good prospects and as such they have gained much popularity in the analysis of the analytes of interest since except for MPPA they contain both N and P atoms.

ECD can sensitively and selectively detect compounds with halogen atoms in their molecules. These features rendered GC–ECD a significant tool in the analysis of the structurally similar herbicides and their metabolites.

In recent years, ion trap detectors and benchtop quadrupole instruments were improved in their detector design and operation and acquisition software, leading to the widespread use of benchtop mass spectrometers in routine laboratories. The employment of MS detection for the analyte quantification after proper interpretation of mass spectra was proven to be a safe way to analyse all the studied compounds. Relevant application areas for the analysis of the concerned herbicide residues are foodstuffs, soil and water. The combination of the molecular ion confirmation and reasonable fragmentation pattern was a powerful means for the unambiguous identification and quantitation at residue levels in such matrices. Chemical ionization, electron impact ionisation quadrupole, ion-trap MS and ion-trap tandem MS coupled with packed or capillary GC were used [25,32,33,36,39,42–44].

2.2. High-performance liquid chromatography

2.2.1. Derivatization methods

As an alternative to the GC analysis of phosphonic and amino acid-type herbicides, a number of high-performance liquid chromatography (HPLC) methods have been published. The compatibility of the water samples with the reversed-phase chromatographic separation systems and the possibility of performing derivatization in aqueous solution made LC the preferred technique. The lack of chromophore or fluorophore and the current requirement for favourable detection limits necessitated derivatiza-

Table 2
GC analysis of the phosphonic and amino acid group containing pesticide residues in crops, foods and environmental matrices

Compounds	Detection	GC conditions[a]	Derivatization reagent	Retention time (min)	Limits of detection	Ref.
AMPA, GLYP (fruits)	ECD, MS(EI), MS(CI)	3.27 m×4 mm I.D., 10% DC-200 on Gas-Chroma Q T_c: 220°C	Boron trichloride–2-chloroethanol–HFBA	2.6, 14.3		[35,43]
AMPA, GLYP	FPD	1.8 m×2 mm I.D., Ultra-Bond SE-20 T_c: 200°C for GLYP T_c: 170°C for AMPA	MTBSTFA	3.1, 4.0		[24]
AMPA, GLYP	FPD, ECD	1.8 m×2 mm I.D., Ultra-Bond SE-20 T_c: 150°C for GLYP T_c: 140°C for AMPA	TFAA–TFE, TFE–HFBA	1.8, 2.5		[23]
AMPA, GLYP (soils)	NPD	1.8 m×2 mm I.D., Ultra-Bond SE-20 T_c: 150°C	TFAA–TFE	8, 11	0.01 mg/kg, 0.05 mg/kg	[27]
GLUF, MPPA (soils)	NPD	15 m×0.53 mm I.D. (1.5 μm), OV-17 T_c: 205°C for GLUF T_c: 140°C for MPPA	Acetic acid–TMOA	11.2, 11.5		[39]
AMPA, GLYP (plants)	NPD	1.8 m×2 mm I.D., Ultra-Bond SE-20 T_c: 200°C for GLYP T_c: 170°C for AMPA	TFAA–TFE	6.3, 10	0.01 mg/kg, 0.03 mg/kg	[28]
AMPA, GLYP (soils)	ECD	2.2 m×4.4 mm I.D., 1.5% OV-17+1.95% QF1 Chromosorb WHP. T_c: 160°C	TFAA–TFE	2.8, 4.6		[29]
MPPA, AMPA, GLYP, GLUF, BIAL (water)	MS (ion trap)	30 m×0.24 mm I.D. (0.25 μm), DB-1 T_c: 100°C–8°C/min–300°C (5 min)	MTBSTFA	15.6, 16.3, 20, 21, 21.6		[25]
AMPA, GLYP (soils, plants, animal matrices, waters)	MS(EI), MS(CI)	30 m×0.25 mm I.D. (0.25 μm), Durabond 5.625 T_c: 90°C (2 min)–30°C/min–290°C (3 min)	TFAA–HFB	5.3, 6.2	0.01 mg/kg, 0.01 mg/kg	[33]
AMPA, GLYP, GLUF (water, soil, crops)	FPD	15 m×0.53 mm I.D. (1.0 μm), DB-17 T_c: 170°C–10°C/min–270°C	Isopropylchloroformate–diazomethane	3.5, 5.8, 8.1	0.8 mg/kg, 1.2 mg/kg, 2.0 mg/kg	[38]
MPPA, GLYP, GLUF (crops)	FPD, MS(EI)	30 m×0.25 mm I.D. (0.25 μm), HP-5 T_c: 50°C (1 min)–30°C/min–170°C (1 min)–10°C/min–250°C (10 min)	Acetic acid–TMOA	7, 12, 14	0.02 mg/kg, 0.02 mg/kg, 0.02 mg/kg	[40]
AMPA, GLYP (water)	MS–MS (ion trap)	30 m×0.25 mm I.D. (0.25 μm), VA-5 MS T_c: 80°C (1.5 min)–30°C/min–260°C (1 min)–30°C/min–300°C	TFAA–HFB	4.0, 4.7	0.05 μg/l, 0.05 μg/l	[44]
AMPA, MPPA, GLYP, GLUF, BIAL (water)	ECD, MS(EI)	30 m×0.25 mm I.D. (0.25 μm), OV-5 T_c: 50°C (2 min)–5°C/min–280°C (5 min)	TFAA–TFE	8.8, 14, 15.4, 24.6 (double), 46 (double)	0.09 μg/l, 0.25 μg/l, 0.36 μg/l, 0.85 μg/l, 17 μg/l	[32]
MPPA, AMPA, GLUF, GLYP, BIAL (water)	MS(EI)	30 m×0.25 mm I.D. (0.25 μm), OV-5 T_c: 60°C (2 min)–5°C/min–180°C–15°C/min–280°C (5 min)	Acetic acid–TMOA	14.4, 16.0, 23.5, 26.8, 41.6	0.05 μg/l, 0.29 μg/l, 0.32 μg/l, 0.65 μg/l, 14 μg/l	[42]
AMPA, GLYP (water, soil)	MS(EI)	30 m×0.32 mm I.D. (0.25 μm), HP-5 MS T_c: 70°C (2 min)–30°C/min–170°C–120°C/min–270°C	TFAA–TFE	4.2, 5.09	0.05 μg/l, 0.003 μg/g	[31]

[a] T_c=Column temperature.
EI=Electron impact ionization; CI=chemical ionization.

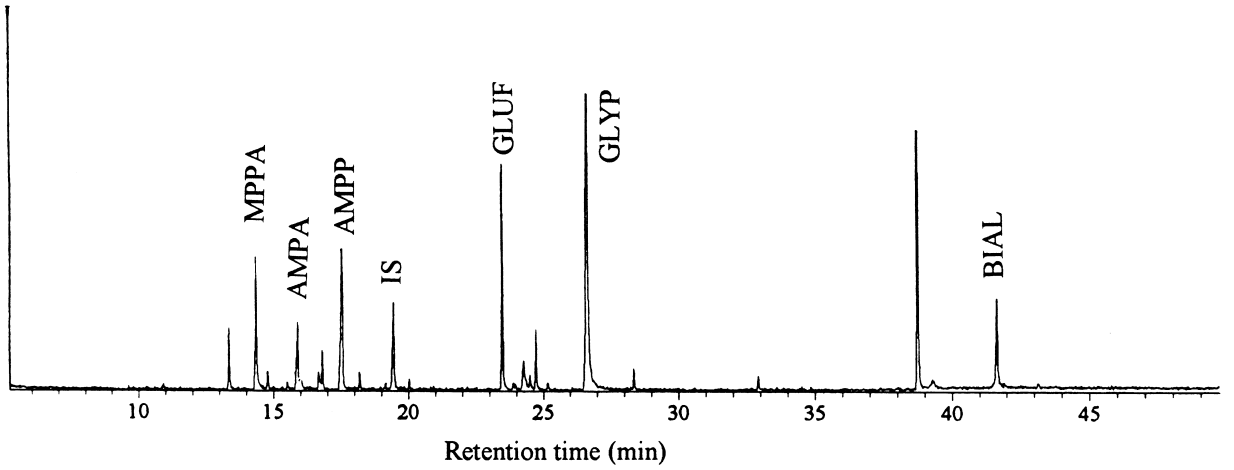


Fig. 1. Full scan gas chromatogram of a lakewater fortified with the analytes studied. MPPA: 3-methylphosphinico propionic acid, AMPA: aminomethylphosphonic acid (20 ng/ml), AMPP: ampropylfos (125 ng/ml), GLUF: glufosinate (368 ng/ml), GLYP: glyphosate (181 ng/ml), BIAL: bialaphos (1.11 μg/ml). The derivatization was performed with acetic acid and TMOA (from Ref. [42], with permission).

tion techniques for the determination of GLYP and GLUF residues and their metabolites in LC. The required selectivity and/or sensitivity in the LC analysis were reached via derivatization using either the pre- or the post-column mode. These methods are more straightforward than GC but in some instances require expensive or elaborate analytical equipment.

The method proposed by the EPA for the determination of GLYP is focused on a post-column derivatization approach as follows: water samples are filtered and directly injected into a cation-exchange column. The analytes of interest are separated isocratically and after elution from the analytical column at 65°C, they are reacted with *o*-phthalaldehyde–2-mercaptoethanol (OPA–ME) complex to give a fluorophore, which is detected by a fluorometer with excitation at 340 nm and detection of emission measured at >455 nm [45]. This method was adopted by Cowell and co-workers who paid most attention to organising an interlaboratory study toward the evolution of a method for GLYP and AMPA through testing a variety of matrices [46,47]. A similar, albeit more simplified clean-up procedure, which gives improved recovery, detection limit, reproducibility and low reagent consumption, was reported later [48]. All OPA–ME modifications employ an oxidizing reagent for converting GLYP to glycine which is an aqueous solution of $Ca(OCl)_2$ or NaClO forwarded to a post-column reactor maintained at 48°C [12,49–54].

A reagent, which has been shown to be highly reactive toward amines, possesses a non-polar highly fluorescent moiety and is commercially available in a pure form, is 9-fluorenylmethyl chloroformate (FMOC-Cl). The GLYP–FMOC derivative is readily formed under alkaline conditions. The procedure involves a pre-column derivatization step yielding the highly fluorescent derivatives of the analytes, which can then be determined at a fluorescence maximum of 315 nm [55–57]. The precipitation, which may be formed during the derivatization is washed out with diethyl ether and the process is repeated with a new portion of the reagent solution.

The traditionally used ninhydrin method was also coupled post-column with ion-exchange column chromatography. The absorbance detection was at 570 nm for the quantification of GLYP and AMPA in soils, sediments and foliage substrates. The use of a valve-switching technique allowed quantification of both analytes in a single chromatographic run [58]. An important paper published in 1997 by Sundaram and Curry compares the two post-column derivatization reactions of OPA–ME and ninhydrin with respect to recovery, reproducibility and minimum quantifiable limits [59]. The authors concluded that the fluorescence detection with OPA–ME exhibits

slightly superior characteristics although marked differences were observed between different commercial detectors. Another interesting feature of the above-mentioned paper is the application of the methods for the analysis of GLYP residues in glass fibre filter disks used as deposit collectors in aerial application.

In 1986 Lundgren proposed a new method for the pre-column derivatization of GLYP and its metabolite AMPA in soil [60]. The essential innovation was the use of 1-fluoro-2,4-dinitrobenzene which reacts smoothly with the amino group at room temperature in saturated sodium tetraborate solution. It was found that the formed derivatives in the aqueous phase decompose slowly in daylight. Hence the samples were stored in the dark whereas direct sunlight was avoided during derivatization.

In the pre-column mode, the formation of tosylated derivatives of GLYP and AMPA can also take place under mild conditions (50°C, for 30 min) [61–63]. The reaction proceeds readily in alkaline ambience with the use of *p*-toluenesulfonyl chloride.

Morin (3,5,7,2′,4′-pentahydroxylflavone) is known to be amenable to complexation with Al^{3+} and many other metal ions to form a highly fluorescent solution, which is the basis for the fluorimetric determination of the metal ions. In the presence of phosphate, the emitted fluorescence of the Al^{3+}-morin decreases and this drop can be correlated with the amount of the phosphate in the sample [64,65]. The use of Al^{3+}-morin as a post-column reagent is in indirect detection, which will be detailed in an ensuing section of this review.

Tris(2,2′-bipyridyl)ruthenium(II) has proven to be very attractive for the determination of compounds with secondary or tertiary aliphatic nitrogen, one compound with such a structural feature being GLYP [66]. The possibility of using tris(2,2′-bipyridyl)ruthenium(II) electrogenerated chemiluminescence (ECL) for the separation and detection of GLYP was scrutinised, but its applicability would seem restricted [67].

2.2.2. Chromatographic conditions and separation

Table 3 lists the main characteristics of the most common HPLC methods appearing in the literature. As GLYP, GLUF, BIAL and their metabolites are acidic, they can conveniently be separated by anion-exchange chromatography. When using FMOC-Cl, it is felt that anion-exchange chromatography would be a satisfactory separation mode since neither the phosphoric nor the carboxylic acid moieties are derivatized. The EPA recommends a cation-exchange column for the determination of GLYP before the post-column production of a fluorophor [45].

The versatility of HPLC allowed the quantification of GLYP and AMPA of both analytes in a single chromatographic run after combining an ion-exchange column with the valve-switching technique for eliminating late-eluting co-extracted interferences [58].

An interesting publication in 1994 demonstrated that the combination of direct large-volume injection and coupled-column reversed-phase LC is a suitable technique for the rapid, sensitive and selective determination of GLUF in environmental water samples after derivatization with FMOC-Cl [68]. The dimensions of the first C_{18} column made possible the injection of large sample volumes (sensitivity) and the performance of an efficient clean-up (selectivity) between the polar analyte and the large excess of UV-absorbing early interferences. The coupling of an amino column for the anion-exchange enabled the separation of the fluorescent GLUF derivative. The single residue method was later improved and a method for the simultaneous determination of GLUF, GLYP and AMPA with the same technique was reported [69]. The method proved suitable for screening purposes due to high sample throughput. The obtained chromatogram from a spiked water sample is illustrated in Fig. 2. Recently, the same authors adapted this technique to the analysis of GLYP in soil and foodstuffs [70,71]. The basic procedure involves injection of crude sample extract on the first chromatographic column where a pre-separation is performed with a certain volume of the first mobile phase, and switching with the second analytical column where the final separation of the analytes is performed using a second mobile phase.

Reversed-phase ion-pair HPLC using tetraethylammonium bromide as counterion reagent to control the retention has shown appreciable separation between GLYP and AMPA derivatives [60]. Ultimately, by using anion-exchange chromatography, it is possible to incorporate tris(2,2′-

Table 3
HPLC analysis of the phosphonic and amino acid group containing pesticide residues in crops, foods and environmental matrices

Compounds	Detection	HPLC conditions[a]	Derivatization reagent[a]	Retention time (min)	Limits of detection	Ref.
AMPA, GLYP (water, soil)	UV (240 nm or 280 nm)	250×4.6 mm I.D., Develosil ODS-5 MP: 0.2 *M* phosphate, pH 2.3–ACN (85:15, v/v) Flow-rate: 1 ml/min	*p*-Toluenesulfonyl chloride	10, 15	8 μg/l, 10 μg/l	[61,63]
AMPA, GLYP (fruits)	FL (254 nm, 313 nm)	250×4.6 mm I.D., Hypersil APS MP: 0.05 *M* NaH_2PO_4, pH 5.0–MeOH (40:60, v/v) Flow-rate: 0.5 ml/min	FMOC-Cl		0.1 mg/kg	[56]
GLYP, AMPA (soil)	UV (405 nm)	100×0.8 mm I.D., Nova-Pak C_{18} MP: 0.02 *M* tetraethylammonium bromide, 0.05 *M* NaH_2PO_4, pH 3.2–ACN (5:1) for 7 min–(1:5) in 8 min. Flow-rate: 1 ml/min	1-Fluoro-2,4-dinitrobenzene	5.8, 10.1	0.05 mg/kg, 0.1 mg/kg	[60]
GLYP (water, soil)	FL (254 nm, 313 nm)	150×4.6 mm I.D., LiChrosorb-NH_2 MP: 0.1 *M* KH_2PO_4, pH 5.4–ACN (15:85, v/v) Flow-rate: 1.5 ml/min	FMOC-Cl	4.0	10 μg/l, 5 mg/kg	[57]
GLYP, AMPA (water)	FL (285 nm, 310 nm)	30×3.9 mm I.D., Spherisorb-NH_2 MP: 1.5% KH_2PO_4, pH 5.8–ACN (85:15, v/v) Flow-rate: 1.3 ml/min	FMOC-Cl	20.6	0.02 μg/l, 0.02 μg/l	[53]
GLYP (fruits)	FL (230 nm, 418 nm)	250×4 mm I.D., Aminex A-27 MP:0.3% H_3PO_4+0.03% H_2SO_4, pH 2.2 Flow-rate: 0.6 ml/min	OPA–ME	17	0.05 mg/kg	[50]
GLYP	ECL	250×4.1 mm I.D., PRP-X100 anion exchange MP: 0.1 m*M* $Ru(bpy)_3^{2+}$ in ACN–0.01 *M* phosphate, pH 9.8 (1:9) Flow-rate: 1 ml/min	Tris(2,2′-bipyridyl)-ruthenium(II)	19.3	1.7 μg/l	[67]
GLYP (soil)	FL (270 nm, 315 nm)	250×4.0 mm I.D., Alltech NH_2 MP: 0.05 *M* KH_2PO_4, pH 6.0–ACN (75:25) Flow-rate: 1 ml/min	FMOC-Cl		0.5 mg/kg	[23,104]
GLYP (crops, vegetables)	FL (360 nm, 400 nm)	250×4.0 mm I.D., Ionosphere anion exchange MP: phosphate, pH 2.1 Flow-rate: 0.5 ml/min	OPA–ME		0.05 mg/kg	[51]
GLYP, AMPA (water)	FL (340 nm, 455 nm)	250×4.1 mm I.D., PRP-X400 cation exchange MP: 0.005 *M* KH_2PO_4 in 4% MeOH, pH 2.1 Flow-rate: 0.4 ml/min	OPA–ME	13, 17	2 μg/l, 2 μg/l	[52]
GLUF (water)	FL (263 nm, 317 nm)	30×4.6 mm I.D., Nucleosil C_{18} (C1) and 250×4.6 mm I.D./Adsorbosphere NH_2 (C2) MP: ACN–0.05 *M* phosphate, pH 5.5 (35:65) (C1), and ACN–0.1 *M* phosphate, pH 5.5 (35:65) (C2). Flow-rate: 1 ml/min	FMOC-Cl	8.0	0.25 μg/l	[68]
GLYP, GLUF, AMPA (wastewater)	ESI/MS	250×4.6 mm I.D., Inertsil ODS-2 MP: ammonium acetate 5 m*M*–ACN (90:10) (46:54) in 20 min Flow-rate: 1 ml/min	FMOC-Cl	13.2, 15.1, 18.0	0.03 μg/l, 0.1 μg/l, 0.03 μg/l	[75]
GLYP, AMPA (water, crops)	FL (340 nm, 455 nm)	300×4.6 mm I.D., Animex A-9 MP: 0.005 *M* KH_2PO_4 in 4% MeOH, pH 1.9 Flow-rate: 0.5 ml/min	OPA–ME		0.05 mg/kg, 0.05 mg/kg	[46]
AMPA, GLYP	FL (270 nm, 315 nm)	300×4.0 mm I.D., μCarbohydrate (pentylamine) MP: 0.1 *M* phosphate, pH 4.0–ACN (75:25) Flow-rate: 1 ml/min	FMOC-Cl	7, 18		[55]
GLYP (crops)	FL (263 nm, 317 nm)	30×4.6 mm I.D., Hypersil ODS (C1) and 250×4.6 mm I.D., Adsorbosphere NH_2 (C2) MP: ACN–0.05 *M* phosphate, pH 5.5 (35:65, v/v) for both C1 and C2 Flow-rate: 1 ml/min	FMOC-Cl	13	0.5 mg/kg	[71]

Table 3. Continued

Compounds	Detection	HPLC conditions[a]	Derivatization reagent[a]	Retention time (min)	Limits of detection	Ref.
GLYP, AMPA (water)	FL (330 nm, 465 nm)	150×4.0 mm I.D., cation exchange, K^+ form MP: 0.005 *M* KH_2PO_4, pH 2.0–2.5% KOH (100:0, v/v) for 15 min–(0:100) in 17 min Flow-rate: 0.7 ml/min	OPA–ME	6.8, 12.0	2 μg/l, 4 μg/l	[54]
GLYP, AMPA (formulation)	FL (400 nm, 480 nm)	150×4.1 mm I.D., PRP-X100 (C1) or 250×4.1 mm I.D., PRP-X400 (C2) MP: 25 m*M* $NaNO_3$, pH 9.5 (C1) 10 m*M* HNO_3, pH 2.0 (C2) Flow-rates: 1 ml/min (C1) 0.5 ml/min (C2)	Al^{3+}-morin	24.7, 7.0 (C1) 9.2, 18.5 (C2)	14 mg/l, 40 mg/l	[72]
AMPA, GLUF, GLYP (water)	FL (263 nm, 317 nm)	30×4.6 mm I.D., Spherisorb ODS-2 (C1) and 250×4.6 mm I.D., Adsorbosphere NH_2 (C2) MP: 0.05 *M* phosphate, pH 5.5–ACN (65:35, v/v) Flow-rate: 1 ml/min	FMOC-Cl	7.9, 14.0, 18.6	1 μg/l, 1 μg/l, 1 μg/l	[69]

[a] MP=Mobile phase, ACN=acetonitrile, FL=fluorescence, ECL=electrogenerated chemiluminescence.

bipyridyl)ruthenium(II) in the mobile phase, thus eliminating the need for any post-column reagent addition in the determination of GLUF using electrogenerated chemiluminescence detection.

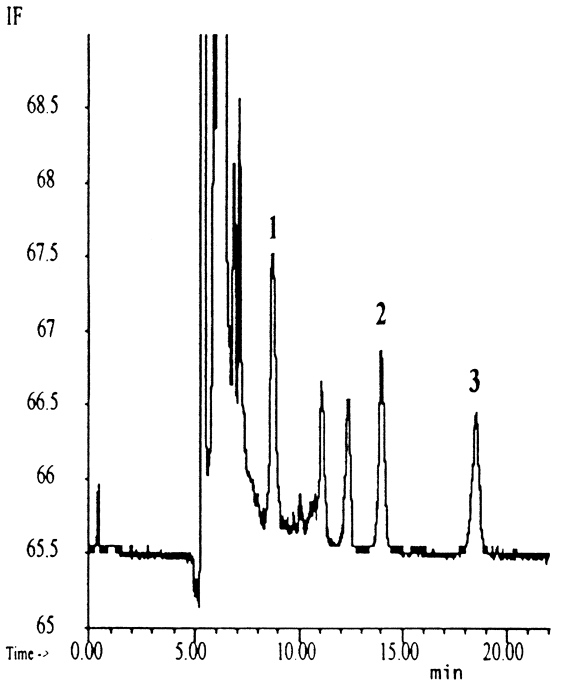


Fig. 2. LC–LC–fluorescence detection of a surface water sample spiked at a level of 4 μg/l. Peaks: 1=AMPA, 2=glufosinate, 3=glyphosate (from Ref. [69], with permission).

2.2.3. Detection

The conventional UV–Vis and fluorescence detectors are most frequently used in HPLC for the direct analysis of GLYP, GLUF and AMPA after appropriate derivatization. The use of Al^{3+}-morin as a post-column reagent helped to develop an indirect fluorescence detection of GLYP and AMPA, since the decrease in Al^{3+}-morin fluorescence in the analyte band due to the presence of the phosphoric acid relative to the fluorescent background is monitored [72].

Another aspect recently proposed by a French scientific group uses the classical amino acid analyser (Beckman 6300) with the program for biological fluids as a simple and rapid method for the diagnosis and monitoring of GLYP poisoning [73].

Tris(2,2′-bipyridyl)ruthenium(II) and electrogenerated chemiluminescence detection merits some consideration from the research point of view but this detection technique demands a specific and dedicated flow-through cell construction.

As HPLC itself is not accepted as a pure identification technique, hyphenated techniques were developed to get around it. Covering a wider range of polar pesticides with GLYP included, Schroeder examined different MS interfaces, like thermospray, electrospray and atmospheric pressure chemical ionisation with regard to their suitability for substance-specific detection by flow injection analysis even without preceding LC separation [74]. Fluorescence itself, is a non-specific detection technique and

frequently requires MS confirmation to ascertain the identity of analyte peaks in a chromatogram of a real-life sample. The progress in LC–MS coupling and the possibility of gaining additional structural information by means of the new MS techniques prompted its use in the analysis of the herbicides studied and their metabolites, as well. Vreeken et al. developed a selective and sensitive method for the determination of GLUP, GLYF and AMPA by means of solid-phase extraction–HPLC–electrospray ionization mass spectrometry after pre-column derivatization with FMOC-Cl [75]. Three ion signals resulted through a special scan routine, viz. the $[M-H]^-$, ion signal from the derivative during the LC–MS scan and two product-ion signals formed upon collision induced dissociation. The figures of merit of the method were good enough and although the method has been developed for drinking and surface waters, it can also be applied for wastewater screening.

2.3. Ion chromatography

Very few detection methods for GLYP without derivatization are reported in the literature. Ion chromatography (IC), since its introduction in the mid-1970s, has been a useful tool for detecting ionic substances quickly and conveniently [76]. GLYP has a strongly ionized phosphate group as a result of its pK_a values. This motivated Zhu et al. to explore the potential of determining GLYP by suppressed conductivity ion chromatography [77]. The main objective was to develop a simple and sensitive method for the determination of GLYP in aquatic samples placing emphasis on a simple clean-up procedure. The technique of conductivity suppression was found to reduce the background signal in the range of about two orders of magnitude leading to a significant increase in sensitivity and a detection limit of 42 μg/l. A mixture of sodium carbonate (9.0 mmol/l) and sodium hydroxide (4.0 mmol/l) was used as mobile phase which compensated for good peak shape and short elution time of common interfering anions. An AS4SC separating column (Dionex) and an anion self-regenerating suppressor (ASRS-I) with electrochemical methods were employed.

Electrospray coupled to LC–MS is used more and more for the determination of organic micropollutants, especially in aqueous matrices. The integration of a suppressor module into an IC–MS–MS system enabled the simultaneous determination of polar organic trace compounds in water, with GLYP and AMPA included [78]. The technique utilises a suppressor module, which is switched into the eluent flow between the separation column and the mass spectrometer. Suppressor increases sensitivity in IC due to the exchange of interfering cations with H^+ and in this way the minimum detectable amount is 1 μg/l.

3. Enzyme-linked immunosorbent assay

A substantially new approach now presented is enzyme-linked immunosorbent assay (ELISA). Since the time that ELISA came into its own, it has been recognised as a valuable tool in residue analysis and compliments or even surpasses conventional analytical methods providing rapid sample testing and accurate results [79–81]. ELISA has been used successfully for the quantitative analysis of numerous pesticides in water matrices with little or no matrix interferences [82,83]. The potential of the performance of a competitive indirect ELISA for the detection and quantification of GLYP in water was recently and uniquely conceived [84]. Contrary to the expensive and time-consuming HPLC and GC methods, ELISA provided a sensitive, cost-effective and efficient method for analysing environmental samples containing GLYP. The proposed assay is more rapid than the other conventional methods (HPLC, GC) since as many as 40 samples can be analysed simultaneously in a few hours. Correlation of HPLC and ELISA estimates exhibited good agreement between the two analytical methods for GLYP. Nevertheless, the limited solubility of GLYP in organic solvents needed to synthesize the immunogens and coating conjugates, the tedious GLYP polyclonal antisera production and the cross-reactivity to AMPA and glyphosphine, a structurally related herbicide, are still limiting factors to its widespread use. The high limit of detection is another barrier to the routine basis use of ELISA since it was determined to be 7.6 μg/ml even though a simple pre-concentration step allowed it to go down to 0.1 μg/ml.

4. Capillary electrophoresis

In recent years, capillary electrophoresis (CE) has become an all-purpose analytical technique. Yet, integrated methods for this category of compounds have scarcely been published. In the early 1990s an undertaking to determine GLUF and AMPA by CE proved successful. This method utilised *p*-toluenesulfonyl chloride for derivatization prior to separation with 0.1 *M* boric acid–sodium hydroxide buffer (pH 9.6) containing 10% methanol at an applied potential of 30 kV. Elution was completed within 15 min and the recoveries of GLYP and AMPA from spiked sera were in the range 78–89% [85]. Another method incorporated ribonucleotides into the background electrolyte at a concentration of 5 m*M* with 2 m*M* diethylenetriamine as electroosmotic flow modifier to provide the signal for indirect photometric detection [86]. GLUP formulation was analysed with positive and negative polarity CE and the results were in agreement with the claimed certified values. While the first method provided improved detection limits over the second, analyte derivatization should add to the total analysis time. A more recent method employed indirect detection using a phthalate background electrolyte containing tetradecyltrimethylammonium bromide as an electroosmotic flow modifier to provide mobility and charge matching with GLYP [87]. The same authors showed that field-amplified sample injection could give pre-concentration for improving sensitivity by a factor of up to 1000. In this way, the method can reach detection limits of 2 μg/l. Fig. 3 provides an electropherogram of a Roundup herbicide solution where the overall analysis is apparently completed within 10 min.

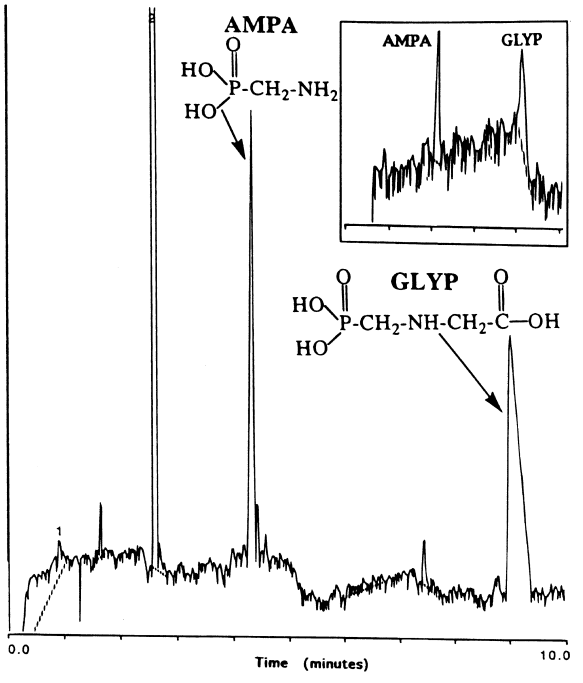


Fig. 3. Electropherogram of Roundup herbicide solution. The sample contained 1 ml of the commercial solution dissolved in few drops of methanol and diluted to 100 ml with triply deionized water. Vacuum injection for 1.5 s, +30 kV applied for separation, current: 6 μA, indirect photometric detection at 259 nm. Inset: peaks for AMPA and GLYP at their limit of detection (from Ref. [86], with permission).

5. Other analytical techniques

In the early days of pesticide residue analysis, colorimetric methods were used on a routine basis on the assumption that specific reactions allowed selective detection. That was the case for GLYP, the oldest of the studied herbicides. The organic phosphate in GLYP was oxidised with hydrogen peroxide to the orthophosphate, which was then measured colorimetrically as the phosphomolybdate heteropoly blue complex at 830 nm [88]. When samples were free of phosphorus-containing substances, the method offered such advantages as being simple, rapid and capable of being performed with common laboratory instrumentation. At best, microgram quantities could be detected while the method seriously lacks specificity and consequently did not find applicability in the determination of GLYP in field samples.

In a later attempt to determine GLYP spectrophotometrically in wastewater a method was developed based on its reaction with excess Br^- and the subsequent colour fading of rhodamine B by the excess Br^- in acetate buffer solution. Possible interferences were removed by application of cation-exchange resin but the limit of detection was as high as 0.4 mg/l [89].

Other reported analytical methods refer to the detection and determination of GLYP and its metabolite by differential pulse polarography, thin-layer

chromatography (TLC) and nuclear magnetic resonance (NMR).

GLYP was nitrosated by treatment with H_2SO_4–KBr–$NaNO_2$ after it had been concentrated on an anion-exchange column. The destruction of the excess of HNO_2 followed the determination of GLYP by differential pulse polarography using the −0.78 V wave [90].

TLC does not belong to the methods of choice for pesticide trace analysis. It would seem that there are a very limited number of articles on the application of TLC to the analysis of the reviewed herbicides. TLC detection of GLYP and AMPA was based on the separation on cellulose plates using either a methanol–water solvent system containing a small amount of NaOH, or ethanol–water–ammonia–trichloroacetic acid–acetic acid. Copper nitrate and Rhodamine B were over-sprayed on the previously developed ninhydrin spots. The methods were implemented to residues in treated field bindweed and spiked distilled water samples [20,21,91].

Proton and ^{31}P-NMR for characterising GLYP and AMPA by measuring the ^{31}P shift was an isolated case of NMR usage, which was not followed up [92].

Tsunoda in a comparative study reports on the analyses of the herbicides of interest and their metabolites in formulations, physiological fluids and food by TLC, GC–MS, fast atom bombardment (FAB) MS, HPLC, LC–MS, IR and NMR [93]. The tBDMS derivatives of the three herbicides and their metabolites and 19 amino acids were simultaneously chromatographed and well separated by GC–ion trap MS on a DB-1 fused-silica capillary column. LC coupled with atmospheric pressure chemical ionization MS enabled the discrimination of L-2-amino-4-[(hydroxy)(methyl)phosphinyl]butyric acid (the major metabolite of BIAL and enantiomer of DL-GLUF) from GLUF.

6. Sample pre-treatment – applications

6.1. General considerations

The development of an analytical method involves several steps; from the sampling and treatment to the detection of the concerned analytes. It has been estimated that two-thirds of the total time required for the analysis is spent in sample preparation and pre-treatment steps before the final determination, these being the main sources of errors in an analytical procedure [94]. Pre-concentration of analytes is required when their concentration in the sample tested is low, or when the sample volume that can be introduced in the assay is small. Clean-up of the sample is dictated by the need to remove compounds interfering with the detection of the analytes of interest (e.g., co-eluting and detected at the same wavelength in a UV detector) or to eliminate compounds that influence the performance of the instrumentation (e.g., ionisation of the analyte in the interface of a mass spectrometric detector). Furthermore, the analytes of interest should be present in a solvent compatible with the analytical system. This rationally signifies the presence of volatile solvent for GC and miscible with the mobile phase for HPLC.

Water samples depending, however, on their origin, require little pre-treatment and clean-up, if needed. As far as food and soil samples are concerned the procedures followed are more laborious which in some instances inevitably result in low recoveries and high detection limits. The challenge for the analyst involves overcoming such problems and ensuring the accuracy of the analytical method with emphasis on keeping the method as simple as possible. Methods to cope with these difficulties are subsequently addressed for the specific matrices.

In principle, clean-up methods for the analytes are based on ion-exchange chromatography due to their ionic form. The use of an ion-exchange column in the pre-concentration or clean-up step appeared to be very effective and that is the reason that almost all the reported investigations extensively use anionic and cationic resins to isolate analytes from matrix interferences.

Based on the above considerations, some general pre-treatment procedures will be given with respect to the different sample matrices.

6.2. Water samples

The selection of the sampling procedure is the basis of the environmental studies for consistent and reliable analytical results and should be considered as an integral part of the whole analytical protocol.

The specific subject of sampling plans and strategies for different types of water is treated in books and reviews [95,96]. The use of borosilicate glass with PTFE-lined tops, which is recommended for water samples containing pesticides, is not the way to go with the analytes which this review deals with. That is, because of their high tendency to adsorb to the inner walls of the glass recipient, which surely produces erroneous results. This fact warrants collecting samples in full polypropylene bottles wrapped in aluminium foil to avoid photochemical degradation whereas care should be taken so that samples do not come into contact with the glass prior to derivatization. The samples are subjected to a filtration step by passing them through a 0.45-μm filter. Pre-concentration is possible through evaporation of a certain volume of water sample and redissolving in the minimum volume to achieve high pre-concentration factor. Usually, this procedure takes place in a rotary evaporator (rotary film, vortex-type) with a water bath at around 50°C.

Classical liquid–liquid extraction using organic solvent(s) (e.g., hexane, dichloromethane), which is followed by evaporation to near dryness, when needed, and subsequent GC analysis, is not applicable here due to the high polarity and water solubility of GLYP, GLUF, BIAL and their metabolites. Owing to this behaviour researchers turned their attention to the study of extraction materials some of which exhibited relative success to the isolation of the analytes. Disposable cartridges packed with LiChrolut EN were used for pre-concentration allowing sufficiently high percentage of extraction of GLYP and AMPA. If coupled with a strong anion-exchange column (Amberlite IRA 410-OH^- form) this polymeric resin can overcome problems related to the presence of organic and inorganic interferences while at the same time isolate quantitatively GLYP and AMPA [54]. Anion-exchange resin in the hydroxide form was also the choice of other researchers for clean-up because it has the lowest selectivity and therefore would be easily exchanged by another ion. Taking into account the acid dissociation constants, water samples are importantly treated using an eluent with high relative selectivity. Sodium citrate buffer showed such improved characteristics and was therefore preferred at the appropriate pH value [48,52].

Recently, the utilisation of supported liquid membrane technique for the extraction of GLYP was examined [97]. To this end, a cationic carrier (quaternary ammonium salt) was incorporated into the membrane phase and the method was applied in standard samples. Its usefulness to the analysis of real samples in conjunction with analytical methods or detection systems remains to be explored.

In terms of speed, efficiency and sample throughput, the techniques described in Refs. [68–71] are the most convenient combining on-line sample pre-treatment and detection.

Minimal pre-treatment is required for samples analysed by CE method. The water sample is extracted with dichloromethane to remove organic compounds and the aqueous phase is concentrated to very small volume by rotary evaporation.

6.3. Fruits and crops

Certain features characterise analytical methods for the pesticide residue analysis in a complex matrix such as fruits, vegetables, feeds or food. Most commonly, the chromatographic methods used for the final determination require extraction of the residues from the matrix and subsequent clean-up procedure before they become suitable for analysis. Searching the literature there seems to be that in two cases the proposed methods do not call for sample pre-treatment taking advantage of the specificity of FPD [37,38].

Worldwide the first step of a conventional solvent extraction for pesticide residues in food is homogenisation of a mixture of wet sample and a water soluble solvent such as acetone, acetonitrile, or ethyl acetate in the presence of sodium sulfate [98,99]. Also, aqueous acetonitrile, water–chloroform and water alone have been preferable because they allow good penetration in the aqueous part of crop where the polar analytes accumulate.

The similarity of most of the analytes to the naturally occurring amino acids and small amino sugars contributes to the difficulty in determining residues of these compounds in crops and animal products. This requires the use of lengthy clean-up procedures that sometimes involve both anion- and cation-exchange columns for the extraction and isolation of the target analytes. More specifically, the

elimination of sugars and pigments from fruit samples, is feasible by means of passage of water extracts through a gel permeation column in a low pH eluent where the sugars are removed by permeation and the pigments by strong adsorption [35]. Additional sugar and unwanted co-extractives soluble in the water has been reported to be eliminated with a small cation-exchange column as an extra step before derivatization and subsequent gas chromatographic detection of GLYP and AMPA [33]. Samples with high oil content, such as nutmeats, soybean oil, etc., or even more products of animal origin wherein fat often holds large (triglycerides) cannot be injected into the GC system. This leads to additional requirements in sample preparation, which can be fulfilled by chloroform extraction. However, the need for the chloroform partition to remove fats and oils should be evaluated for each matrix requiring analysis.

Exhaustive clean-up including solvent partitioning, charcoal elimination of pigments, large volume anion-exchange and separation of the GLYP and its metabolite by cation exchange has been proposed by the EPA [22]. Later, a modified method suggested blending and macerating of the plant materials with water–chloroform. The aqueous fraction is partitioned with hexane and ethyl acetate and then is subjected to charcoal treatment for the removal of pigments followed by column chromatography using a cation-exchange resin for removal of sugar [28].

Another pre-treatment approach adopts the following steps: percolation of crop aqueous extracts sequentially through anion-exchange (HCO_3^- form) and gel permeation (Bio-Gel) and clean-up of the trifluoroacetyl-trimethyl derivative of GLYP using silica gel adsorption HPLC in order to decrease GC interferences. It is noteworthy that omission of either the anion-exchange or gel permeation clean-up step or application of them in reverse order gave unacceptable levels of GC interferences with all the relevant detectors.

The clean-up procedures proposed in the literature for the GC determination encompass isolation and pre-concentration of the analytes before the step of derivatization. However, one method applies additional clean-up of the derivatization products through florisil column chromatography to get rid of derivatized substances, such as free fatty acids, amino acids and organophosphoric acids which may interfere with the gas chromatograms [40]. Even if such a post-derivatization clean-up step is integrated in the procedure, the removal of pigments, starch and proteins should necessarily precede the derivatization using the known reaction schemes [33].

The sample pre-treatment for HPLC analysis is in general less demanding, yet indispensable in any case. A methodology applied for different plant matrices employed a biphasic aqueous–organic extraction of the matrix followed by clean-up of the supernatant aqueous extract utilising initially iron-loaded Chelex 100 resin (ligand-exchange) and then AG1-X8 (anion-exchange) resin columns [46]. Although validated through an interlaboratory study, it was not followed up by other researchers. A more convenient and less complex and tedious procedure involves the following steps: An amount of the crop to be analysed is suspended in high purity water and the suspension stands overnight. The sample is centrifuged and an aliquot is brought onto a preconditioned C_{18} cartridge before is being derivatized [71].

Crop (wheat) intended to be analysed by CE was blended with water for 2 min, centrifuged and the supernatant was passed through a 0.2-μm filter before analysed.

6.4. Soil

The interaction between the soil matrix and the analytes is stronger than in food so that bound residues exhibited different extraction behaviour than the non-bound fraction. Many investigators have experienced low and irreproducible recoveries from many soils, which are believed to be associated with the sorption of GLYP to soil clays and organic matter [100]. Furthermore, there is evidence that GLYP binds to soil minerals in a manner similar to the inorganic phosphate [101]. Pertinent experiments showed that for most sorbent–solvent systems studied, the amount of GLYP extracted was increased at elevated pH values, suggesting that sorption occurs through an ion exchange and hydrogen bonding [23]. Problems related to the extraction method for the analysis of GLYP, GLUF and their metabolites in soils prompted some investigations and gave rise to controversy. The developed ex-

traction schemes aimed at conforming to the different forms of the herbicides of interest as well as their metabolites in the soil and exhibited different extraction capabilities. Finally, it was made clear that the development of a unified procedure was severely hampered by the fact that soil composition varies from sand with a low organic matter content, to heavy loamy soils with high organic matter contents.

For the elimination of relevant interferences, clean-up procedures for soils are more or less pertinent to those used for food samples. Similar to the method of the EPA as applied for fruits and crops is that proposed by Konar and Roy [28] who adapted it to the analysis of GLYP and AMPA in soil. The extraction of the analytes from the soil was attained using phosphoric acid. While the results obtained were reported to be reproducible, the recoveries of both analytes from fortified soils were low. This was suggested to be able to extract the soluble and the weakly adsorbed GLYP although the extraction of strongly sorbed GLYP may be achieved by using longer shaking periods or a larger number of extractions.

The ion-exchange clean-up included passage from both anion- and cation-exchange resin, the latter being regenerated prior to use following a standard procedure [29]. Simple batchwise anion-exchange workup is also proposed using resin in the HCO_3^- or Cl^- form [57,60].

6.5. Forensic

Further to the classical environmental and food matrices there is some interest in the need to involve these compounds in forensic examination and emergency toxicology. Despite the low mammalian toxicity, human fatalities have been reported after suicidal ingestion of GLYP. Moreover, many Japanese cases of accidental and suicidal poisoning have been reported with herbicides containing GLUF-ammonium [102]. Also, the suicidal ingestion of the herbicide Harbie (BIAL-sodium 18%) caused hypotension and cardiovascular effects, the latter is suspected to be due to L-GLUF, the active metabolite of BIAL [103].

In a generalised procedure, blood samples which are going to be tested for the phosphonic and amino acid-type herbicides are treated by passing over an ion-exchange resin (carbonate form) and eluting with HCl–methanol. Necessary tests are required to establish the optimum conditions of elution of the analytes before being derivatized with one of the methods described in the relevant section [37].

Tissue samples (muscle, kidney, liver) are extracted with 0.1 *M* HCl and 25 ml chloroform for fat removal. Then, they are macerated and the aqueous extract is filtered for subsequent process. Cation-exchange clean-up and evaporation of sample to dryness is required before derivatization for GC analysis [33].

7. Quality assurance measures

In evaluating the analytical results it is necessary to consider the basic quality assurance parameters. Accuracy is regarded as one of them and the easiest way to achieve it is the use of certified reference materials (CRMs). The production of a lyophilised reference material might remedy the current lack of CRM in the analysis of this category of herbicides and their metabolites, although this seems to present insurmountable difficulties. The lack of stability in aqueous matrices and hydrolysis and photolysis as the main mechanism of degradation has discouraged researchers from including them in such preparations.

In view of the absence of CRM blanks, standards, and spiked control samples should be included in each sample series for quality assurance purposes. Ultimately, strict measures to judge the ruggedness of a developed analytical methods should be the comparison of the actual recovery and the variation in recovery as a function of the: compounds, analyst, matrix and fortification level.

8. Conclusions

Summing up, there have been a great variety of methods for the determination of these pesticides over the last 20 years. A lot of them reach the detection limits dictated by the current stringent regulations for food and environmental matrices. The solving of the dilemma for the technique of choice remains difficult and depends on several parameters.

The chromatographic methods continue to be the most popular for residue analysis. Nowadays, almost all the laboratories are familiar with or have the facilities required for pre- or post-column labelling techniques. HPLC can more readily automated coupling on-line solid-phase extraction and incorporating either pre- or post-column derivatization. LC–MS supported by the new atmospheric pressure ionization interfaces is becoming a powerful tool for these analyses for reasons already mentioned.

Although the absence of derivatization reduces the analytical errors, low detection limits with IC and conductivity suppression at levels of 0.1 μg/l are hardly achieved.

GC–MS methods provide sensitive and selective detection of the herbicides and their metabolites with no need for lengthy clean-up. Since, in the last few years, GC–MS has not been found to be a cost-effective alternative there seems to be little reason to use the less specific GC-only methods.

CE has not yet been accepted by the laboratories for routine analysis. What is more, for the compounds studied CE exhibits relatively high detection limits.

Finally, it is envisaged that in the near future the development of immunoassays, immunosensors, which is a particularly active area, will encompass these herbicides in the research plans.

References

[1] Directive 76/464/EEC, Pollution Caused by Certain Dangerous Substances Discharged into the Aquatic Environment of the Community (Black List), Off. J. Eur. Commun., L129/7 (1976).
[2] US Environmental Protection Agency, National Pesticide Survey. Phase I Report PB-91-125765, National Technical Information Service, Springfield, VA, 1990.
[3] US Environmental Protection Agency, National Survey of Pesticides in Drinking Water Wells. Phase II Report EPA 570/9-91-020, National Technical Information Service, Springfield, VA, 1992.
[4] Council Directive 80/778/EEC, European Union, Brussels, 1980.
[5] Council Directive 91/414/EEC, European Union, Brussels, 1991.
[6] S.D. Duke, in: P.C. Kearney, D.D. Kaufman (Eds.), Herbicides – Chemistry, Degradation and Mode of Action, Marcel Dekker, New York, 1988, p. 1.
[7] J. Malik, G. Barry, G. Kishore, BioFactors 2 (1989) 17.
[8] Environmental Health Criteria: Glyphosate, World Health Organization, Geneva, 1994.
[9] C. Bolognesi, S. Bonatti, P. Degan, E. Gallerani, M. Peluso, R. Rabboni, P. Roggieri, A. Abbondandolo, J. Agric. Food Chem. 45 (1997) 1957.
[10] M.L. Rueppel, B.B. Brightwell, J. Achaefer, J.T. Marvel, J. Agric. Food Chem. 25 (1977) 517.
[11] L. Torstensson, in: E. Grossbard, D. Atkinson (Eds.), The Herbicide Glyphosate, Butterworths, London, 1985, p. 137.
[12] J.W. Leung, J. Environ. Sci. Health, Part B 29 (1994) 341.
[13] P. Sprankle, W.F. Meggitt, D. Penner, Weed Sci. 23 (1975) 229.
[14] S. Duke, J. Lydon, Weed Technol. 1 (1987) 122.
[15] H. Behrendt, M. Matthies, H. Gildemeister, G. Gorlitz, Environ. Toxicol. Chem. 9 (1990) 541.
[16] M.J. Faber, G.R. Stephenson, D.G. Thompson, J. Agric. Food Chem. 45 (1997) 3672.
[17] D.M. Anderson, C.J. Swanton, J.C. Hall, B.G. Mersey, Weed Res. 33 (1993) 139.
[18] D.M. Anderson, C.J. Swanton, J.C. Hall, B.G. Mersey, Weed Res. 33 (1993) 149.
[19] T. Murakami, H. Anzai, S. Imai, A. Satoh, K. Nagaoka, C.J. Thompson, Mol. Gen. Genet. 205 (1986) 42.
[20] P. Sprangle, C.L. Sandberg, W.F. Meggit, D. Penner, Weed Sci 26 (1978) 673.
[21] J.C. Young, S.U. Khan, P.B. Marriage, J. Agric. Food Chem. 25 (1977) 918.
[22] Pesticide Analytical Manual, Vol. II, US Environmental Protection Agency, Washington, DC, 1977, Transmittal No. 77-3.
[23] C.J. Miles, H.A. Moye, J. Agric. Food Chem. 36 (1988) 486.
[24] H.A. Moye, C.L. Deyrup, J. Agric. Food Chem. 32 (1984) 192.
[25] N. Tsunoda, J. Chromatogr. 637 (1993) 167.
[26] C.L. Deyrup, S.-M. Chang, R.A. Weintraub, H.A. Moye, J. Agric. Food Chem. 33 (1985) 944.
[27] D.N. Roy, S.K. Konar, J. Agric. Food Chem. 37 (1989) 441.
[28] S.K. Konar, D.N. Roy, Anal. Chim. Acta 229 (1990) 277.
[29] P.L. Eberbach, L.A. Douglas, J. Agric. Food Chem. 39 (1991) 1776.
[30] P.S. Mogadati, J.B. Louis, J.D. Rosen, J. AOAC Int. 79 (1996) 157.
[31] E. Boerjesson, L. Torstensson, J. Chromatogr. A 886 (2000) 207.
[32] C.D. Stalikas, G.A. Pilidis, M.I. Karayannis, Chromatographia 51 (2000) 741.
[33] P.L. Alferness, Y. Iwata, J. Agric. Food Chem. 42 (1994) 2759.
[34] J.N. Seiber, M.M. McChesney, R. Kon, R.A. Leavitt, J. Agric. Food Chem. 32 (1984) 678.
[35] R.A. Guinivan, N.P. Thompson, W.B. Wheeler, J. Assoc. Off. Anal. Chem. 65 (1982) 35.
[36] M. Hida, T. Mitsui, Y. Fujimura, Bunseki Kagaku 38 (1989) 87.
[37] H. Kataoka, K. Horii, M. Makita, Agric. Biol. Chem. 55 (1991) 195.
[38] H. Kataoka, S. Ryu, N. Sakiyama, M. Makita, J. Chromatogr. A 726 (1996) 253.

[39] A.E. Smith, J. Agric. Food Chem. 37 (1989) 267.
[40] M. Tsuji, Y. Akiyama, M. Yano, Anal. Sci. 13 (1997) 283.
[41] Y. Ohtani, H. Hayashi, Y. Higuchi, Mizu Kankyo Gakkaishi 20 (1997) 108.
[42] C.D. Stalikas, G.A. Pilidis, J. Chromatogr. A 872 (2000) 215.
[43] R.A. Guinivan, N.P. Thompson, W.B. Wheeler, J. Agric. Food Chem. 30 (1982) 977.
[44] A. Royer, S. Beguin, J.C. Tabet, S.S. Hulot, M.A. Reding, P.Y. Communal, Anal. Chem. 72 (2000) 3826.
[45] EPA Method 547, Determination of Glyphosate in Drinking Water by Direct-Aqueous-Injection HPLC, Post-Column Derivatization and Fluorescence Detection, Office of Research and Development, US Environmental Protection Agency, Cincinnati, OH, 1990.
[46] J.E. Cowell, J.L. Kunstman, P.J. Nord, J.R. Steinmetz, G.R. Wilson, J. Agric. Food Chem. 34 (1986) 955.
[47] M.E. Oppenhuizen, J.E. Cowell, J. Assoc. Off. Anal. Chem. 74 (1991) 317.
[48] Y.Y. Wigfield, M. Lanouette, Anal. Chim. Acta 233 (1990) 311.
[49] H. Moye, C. Miles, S. Scherer, J. Agric. Food Chem. 31 (1983) 69.
[50] T.E. Archer, J.D. Stokes, J. Agric. Food Chem. 32 (1984) 586.
[51] L.G.M.Th. Tuinstra, P.G.M. Kienhuis, Chromatographia 24 (1987) 696.
[52] M.P. Abdullah, J. Daud, K.S. Hong, C.H. Yew, J. Chromatogr. A 697 (1995) 363.
[53] R. Gauch, U. Leuenberger, U. Muller, Z. Lebensm. Unters. Forsch. 188 (1989) 36.
[54] E. Mallat, D. Barcelo, J. Chromatogr. A 823 (1998) 129.
[55] H.A. Moye, A.J. Boning Jr., Anal. Lett. 12 (1979) 25.
[56] R. Roseboom, C.J. Berkhoff, Anal. Chim. Acta 153 (1982) 373.
[57] R.L. Glass, J. Agric. Food Chem. 31 (1983) 280.
[58] D.G. Thompson, J.E. Cowell, R.J. Daniels, B. Staznik, L.M. McDonald, J. Assoc. Off. Anal. Chem. 72 (1989) 355.
[59] K.M.S. Sundaram, J. Curry, J. Liq. Chromatogr. Rel. Technol. 20 (1997) 511.
[60] L.N. Lundgren, J. Agric. Food Chem. 34 (1986) 535.
[61] S. Kawai, B. Uno, M. Tomita, J. Chromatogr. 540 (1991) 411.
[62] M. Tomita, T. Okuyama, S. Watanabe, B. Uno, S. Kawai, J. Chromatogr. 566 (1991) 239.
[63] G. Forlania, A. Mangiagallia, E. Nielsena, C.M. Suardib, Soil Biol. Biochem. 31 (1999) 991.
[64] D. Lands, S. Edmonds, Mikrochim. Acta 6 (1966) 1013.
[65] S.E. Meek, D.J. Pietrzyk, Anal. Chem. 60 (1988) 1397.
[66] J.B. Noffsinger, N.D. Danielson, J. Chromatogr. 387 (1987) 520.
[67] J.S. Ridlen, G.J. Klopf, T.A. Nieman, Anal. Chim. Acta 341 (1997) 195.
[68] J.V. Sancho, F.J. Lopez, F. Hernandez, E.A. Hogendoorn, P. van Zoonen, J. Chromatogr. A 678 (1994) 59.
[69] J.V. Sancho, F. Hernandez, F.J. Lopez, E.A. Hogendoorn, E. Dijkman, P. van Zoonen, J. Chromatogr. A 737 (1996) 75.
[70] J.V. Sancho, C. Hidalgo, F. Hernandez, F.J. Lopez, E.A. Hogendoorn, E. Dijkman, Int. J. Environ. Anal. Chem. 62 (1996) 53.
[71] E.A. Hogendoorn, F.M. Ossendrijver, E. Dijkman, R.A. Baumann, J. Chromatogr. 833 (1999) 67.
[72] M.J. Lovdahl, D.J. Pietrzyk, J. Chromatogr. 602 (1992) 197.
[73] F. Parrot, R. Bedry, J.-C. Favarel-Garrigues, J. Toxicol.-Clin. Toxicol. 33 (1995) 695.
[74] H.F. Schroeder, Environ. Monit. Assess. 44 (1997) 503.
[75] R.J. Vreeken, P. Speksnijder, I. Bobeldijk-Pastorova, Th.H.M. Noij, J. Chromatogr. A 794 (1998) 187.
[76] P.E. Jackson, C.A. Pohl, Trends Anal. Chem. 16 (1997) 393.
[77] Y. Zhu, F. Zhang, C. Tong, W. Liu, J. Chromatogr. A 850 (1999) 297.
[78] K.-H. Bauer, T.P. Knepper, A. Maes, V. Schatz, M. Voihsel, J. Chromatogr. 837 (1999) 117.
[79] B.D. Johnson, J.C. Hall, J. Agric. Food Chem. 44 (1996) 488.
[80] J.S. Parnell, J.C. Hall, J. Agric. Food Chem. 46 (1998) 152.
[81] J.C. Hall, R.J.A. Deschamps, M.R. McDermott, Weed Technol. 4 (1990) 226.
[82] F.M. Rubio, J.A. Itak, A.M. Scutellaro, M.Y. Selisker, D.P. Herzog, Food Agric. Immunol. 3 (1991) 113.
[83] T.S. Lawruk, C.S. Hottenstein, J.R. Fleeker, J.C. Hall, D.P. Herzog, F.M. Rubio, Bull. Environ. Contamin. Toxicol. 52 (1994) 538.
[84] B.S. Clegg, G.R. Stephenson, J.C. Hall, J. Agric. Food Chem. 47 (1999) 5031.
[85] M. Tomita, T. Okuyama, Y. Nigo, B. Uno, S. Kawai, J. Chromatogr. 571 (1991) 324.
[86] S.A. Shamsi, N.D. Danielson, Anal. Chem. 67 (1995) 1845.
[87] M.G. Cikalo, D.M. Goodall, W. Matthews, J. Chromatogr. A 745 (1996) 189.
[88] R.L. Glass, Anal. Chem. 53 (1981) 921.
[89] W. Dong, X. Chen, Z. Lang, S. He, J. Ying, Fenxi Huaxue 25 (1997) 1210.
[90] J.O. Broenstad, H.O. Friestad, Analyst 101 (1976) 820.
[91] M.T.H. Ragab, Chemosphere 7 (1978) 143.
[92] M.L. Rueppel, J.T. Marvel, Org. Magn. Reson. 8 (1976) 19.
[93] N. Tsunoda, Hochudoku 12 (1994) 104.
[94] R.E. Majors, LC–GC Int. 4 (1991) 10.
[95] L.H. Keith, Principles of Environmental Sampling, 2nd ed., American Chemical Society, Washington, DC, 1996.
[96] D. Barcelo, M.C. Hennion, Anal. Chim. Acta 338 (1997) 3.
[97] P. Dzygiel, P. Wieczorek, J. Chromatogr. A 889 (2000) 93.
[98] B.M. McMahon, N.F. Hardin, Pesticide Analytical Manual, Vol. 1, US Food and Drug Administration, Washington, DC, 1994, Chapter 3.
[99] J. Fillion, R. Hindle, M. Lacroix, J. Selwyn, J. AOAC Int. 78 (1995) 1252.
[100] J.S. McConnell, L.R. Hossner, J. Agric. Food Chem. 33 (1986) 1075.
[101] R.J. Hance, Pestic. Sci. 7 (1976) 363.
[102] T. Watanabe, T. Sano, Hum. Exp. Toxicol. 17 (1998) 35.
[103] K. Koyama, Koyama, K. Goto, Toxicol. Lett. 95 (1998) 149.
[104] C.J. Miles, L.R. Wallace, H.A. Moye, J. Assoc. Off. Anal. Chem. 69 (1986) 458.