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Review

Separation methods for amino group-possessing pesticides in biological samples

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Abstract

The separation methods for pesticides include liquid–liquid extraction, solid-phase extraction and solid-phase microextraction, gas chromatography (GC), GC–mass spectrometry (MS), GC–MS–MS, high-performance liquid chromatography (LC), LC–MS and LC–MS–MS. This review deals with each technique commonly used for extraction, chromatographic separation and detection of amino group possessing pesticides, such as diazines, triazines, carbamates, dinitroanilines and chloroacetanilides in biological samples. The methods presented for analysis of the pesticides in complicated biological matrices seem to be easily applicable to surface or groundwater in environmental chemistry.

Keywords: Reviews; Pesticides; Amino groups

Contents

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1. Introduction

Several hundred compounds are available for use as pesticides. The pesticides include insecticides, herbicides, germicides, fungicides, rodenticides, nematocides and acaricides. On a worldwide basis, intoxications attributed to these pesticides have been estimated to be as high as 3 million cases of acute and severe poisoning annually, with as many or more unreported cases and with some 220 000 deaths [1]. In clinical and forensic toxicology, the identification and quantification of a chemical(s) are essential in the case of victims whose cause of death is considered due to or related to intoxication by pesticides. The main interest in these fields is focused on methodologies, with regard to how rapidly, accurately and sensitively chemicals can be detected. This review deals with separation and chromatographic procedures commonly used for detection and quantitation of amino group possessing pesticides in biological matrices, such as whole blood, plasma, urine and tissues. These techniques can be easily applied to analyses of pesticides in clean surface water in environmental chemistry.

2. Strategies for extraction and detection of pesticides in biological samples

Biological samples such as whole blood, plasma, serum, urine or tissues are complicated multi-component mixtures. Toxic substances are usually present in these samples at low concentrations. Thus extraction, clean-up or specific isolation procedure for a target compound from samples is necessary prior to detection by various methods. Liquid–liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME) are being used as extraction techniques in the field of analytical toxicology [2]. In addition, gas chromatography (GC) and high-performance liquid chromatography (LC), GC–mass spectrometry (MS) and LC–MS are widely used for analyses of the compounds in biological samples. In this section, advantages and disadvantages of these techniques are mentioned briefly.

2.1. Extraction methods

The conventional LLE is useful for screening tests of unknown pesticides, because a non-volatile chemical to be analyzed is not lost during the procedure, but is contained in at least one of the fractions separated according to its physicochemical properties. Organic solvents such as dichloromethane, ethyl acetate, hexane and diethyl ether are popular, because they are capable of extracting compounds with a wide range of polarities. The cost of materials for LLE is generally lower than for SPE and SPME. However, the LLE often suffers from emulsion formation, inclusion of impurities, such as phospholipids and their decomposition products, and low recovery of the analytes. Such a massive use of organic solvents is not desirable from a viewpoint of environmental pollution.

SPE is a useful tool for isolation, concentration and purification of analytes from complicated matrices. The advantages of the use of SPE are that the analytical procedure is much simpler and that much cleaner extracts and higher recoveries can be ex-

pected. In addition, SPE also avoids the emulsion formation often encountered in LLE. The non-polar octadecyl (C_{18}) bonded silica is the most common sorbent packing material. Methanol or acetonitrile, mixed or not mixed with water is recommended for elution of target compounds from the cartridges according to the manufacturer's manual. However, we used a mixture of chloroform with methanol or isopropanol (9:1, v/v), or chloroform only, for elution of compounds to be analyzed and got good results; backgrounds were generally cleaner with the chloroform mixture than with the methanol or acetonitrile (or their water mixtures) [3–5]. The chloroform mixture also made evaporation time much shorter, backgrounds cleaner and recoveries much better. Extrelut columns can be also used for extraction of pesticides from biological samples [6,7].

SPME is a new technique developed by Pawliszyn and co-workers in 1990 [8,9]. The extraction, concentration and sample introduction steps are integrated into a single step by this method. It is accomplished on a fused-silica fiber coated with an immobilized stationary phase, such as polydimethylsiloxane (PDMS), polyacrylate or PDMS–divinylbenzene, for extraction without use of any organic solvent. The choice of fiber coatings and extraction type (headspace or direct immersion) is the first step for this method. As the next step, the conditions of contents in a vial should be optimized. The salt additives, pH, extraction temperatures and the time of incubation are important parameters for achieving the best efficiencies of extraction. The advantages of SPME are that the procedure is simpler and faster than those using LLE and SPE, and much cleaner extracts can be obtained for blood and urine samples, and that SPME is more suitable for automated analyses [10]. A disadvantage is that partitioning rates to the stationary phases coated on the fibers are quite different according to different compounds. The net extraction efficiencies by SPME are generally much lower than those by LLE and SPE. Nevertheless, it is gaining popularity because of its good quantitativeness and reproducibility. SPME methods generally give higher chromatographic response and much lower detection limits, as compared with the conventional extractions. This is because the entire amount of an analyte extracted by SPME is introduced into a GC capillary column, while only a small fraction (1–5%) of the final extract solution obtained by LLE or SPE is introduced into a GC port.

2.2. Chromatographic methods

GC is most commonly used for separation of thermostable pesticides from biological samples. Various kinds of fused-silica capillary columns with bonded phases of different polarities are now commercially available. Combination of GC with detection methods such as flame ionization detection (FID) and nitrogen–phosphorus detection (NPD) are most popular; FID gives universal response to organic compounds, while NPD is selective for compounds containing nitrogen or phosphorus and gives much lower detection limits than FID. GC–NPD is thus very suitable for detection of amino group possessing pesticides. Electron-capture detection (ECD) is used for sensitive determination of the compound containing halogen or nitro group(s) in a molecule. Surface ionization detection (SID) first introduced by Fujii and Arimoto [11] in 1985, gives high sensitivity and specificity for compounds having tertiary amino groups in their structures.

Although GC has an excellent capability of separating a mixture of molecules, the final identification should be made by MS. Three modes of GC–MS operation are generally available: positive ion electron impact, positive ion chemical ionization and negative ion chemical ionization. Recently, a sophisticated technique using GC–MS–MS has been reported to enable analysis of pesticides and their metabolites at trace levels in the presence of many interfering impurities [12–15].

LC is the method of choice for highly polar, thermolabile and/or high-molecular mass compounds, which are not amenable to GC. LC is used with ultraviolet (UV), fluorometric (FL) and refractive index detection (RID). For LC columns, reversed-phase sorbents such as C_{18} or CN are usually used. LC–MS and LC–MS–MS are classified based on their interface and ionization methods. Thermospray ionization, electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and particle beam are used as interfaces between LC and MS at present.

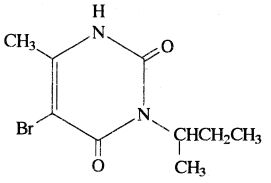
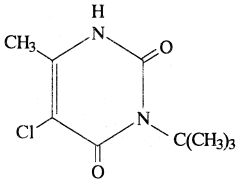
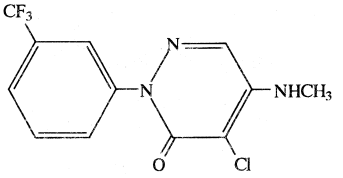
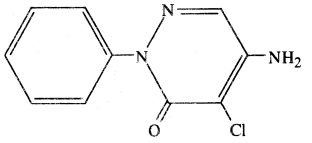
3. Diazines

Diazines are widely used as herbicides in agriculture. Pollution of a variety of crops, groundwater and soil by diazines is now being recognized as one of the serious environmental problems [16,17]. The structures and names of four diazines mentioned here are shown in Table 1. To our knowledge, only one report deals with LLE and SPE methods for diazines from human body fluids before GC appeared [18].

3.1. Liquid–liquid extraction

Four diazines, terbacil, bromacil, norflurazon and pyrazon (alternate name, PAC), were easily extracted from 1 ml of human whole blood, plasma and urine samples by LLE with 2 ml of diethyl ether [18]. Recoveries of terbacil, bromacil, norflurazon and pyrazon from the body fluids were 90–96 (except for plasma), 79–89, 72–92 and 32–76%, respectively. However, there were many intense impurity peaks in a wide range for whole blood and plasma samples by use of GC–FID; only terbacil in plasma was not measurable owing to interference by these impurities.

Table 1
Chemical structures of diazines

Compound	Structure
Bromacil	
Terbacil	
Norflurazon	
Pyrazon	

3.2. Solid-phase extraction

SPE with a Bond Elut C_{18} cartridge was used for extraction of terbacil, bromacil, norflurazon and pyrazon from human whole blood, plasma and urine samples [18]. One-milliliter plasma or urine containing four diazines was mixed with 4 ml of distilled water; in the case of whole blood, a 1-ml sample was mixed with 9 ml of distilled water for complete hemolysis. Each sample solution was poured into a Bond Elut C_{18} cartridge (sorbent mass 200 mg, Varian, Harbor City, CA, USA), which had been pretreated with 10 ml chloroform–methanol (9:1), 10 ml of methanol and 20 ml of distilled water. They were finally eluted from the cartridge with 3 ml of chloroform–methanol (9:1). The organic layer was evaporated to dryness under a stream of nitrogen and the residue was dissolved in methanol for GC analysis. Recoveries of all compounds, which had been added to whole blood, plasma and urine, was >91, >89 and >91%, respectively.

3.3. Gas chromatography

Lee et al. [18] reported the comparison of gas chromatographic separation for terbacil, bromacil, norflurazon and pyrazon in SPE extracts obtained using a non-polar DB-1 fused-silica capillary column (30 m×0.32 mm I.D., film thickness 0.25 μm, J&W, Folsom, CA, USA) with that obtained using an intermediately polar DB-17 fused-silica capillary column (30 m×0.32 mm I.D., film thickness 0.25 μm). Separation of four diazines from each other and from impurities was much better with the DB-17 capillary column. GC–FID gave intense peaks for each compound with low background noises. The calibration curves for the four compounds extracted from the body fluids with Bond Elut C_{18} cartridges were linear in the range of 0.16–10 μg/ml, with detection limits of 0.12–0.14 μg/ml for whole blood and plasma, and 0.11–0.12 μg/ml for urine.

4. Triazines

Triazines are used worldwide as herbicides for control of grassy and broadleaf weeds in the cultivation of corn, sugarcane, sorghum and other crops as well as in nonagricultural situations. As shown in Table 2, most of commercial triazines contain at least three nitrogen atoms and alkylamino group(s).

4.1. Liquid–liquid extraction

Pommery et al. [19] reported that atrazine and propazine could be extracted from 2 ml of human plasma with 6 ml of dichloromethane before LC analysis. Recoveries of atrazine in plasma at 6.25 and 100 ng/ml were 72 and 88%, respectively; those of propazine at the same concentrations were 82 and 98%, respectively.

4.2. Solid-phase extraction

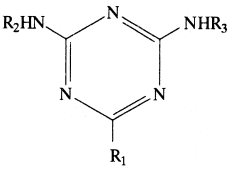
SPE was successfully used for extraction of eight triazines, simazine, atrazine, propazine, cyanazine, ametryn, prometryn, prometon and metribuzin, from human serum and urine samples with Sep-Pak C_{18} cartridges [20]. One-milliliter of serum or urine containing eight triazines was mixed with 4 ml of distilled water. Each sample solution was poured into a Sep-Pak C_{18} cartridge (sorbent mass 360 mg, Waters, Milford, MA, USA). It was then washed with 20 ml distilled water followed by 3 ml chloroform–methanol (9:1) or 3 ml chloroform only to elute the compounds from the cartridge. Recoveries of all compounds from serum and urine samples were more than 60% for both chloroform and chloroform–methanol (9:1). Evaporation time was shorter for the chloroform only than for the chloroform–methanol (9:1) mixture. Therefore, the use of chloroform as elution solvent was recommended for SPE of triazines from human body fluid samples.

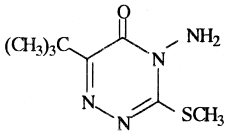
4.3. Solid-phase microextraction

Recently, we have established a detailed procedure of headspace SPME for the eight triazine herbicides from human whole blood and urine samples (to be published).

Four different SPME fiber coatings, PDMS, polyacrylate, PDMS–divinylbenzene and Carbowax–divinylbenzene (Supelco, Bellefonte, PA, USA), were

Table 2
Chemical structures of triazines



Compound	R_1	R_2	R_3
Ametryn	SCH_3	CH_2CH_3	$CH(CH_3)_2$
Atrazine	Cl	CH_2CH_3	$CH(CH_3)_2$
Cyanazine	Cl	CH_2CH_3	$CCN(CH_3)_2$
Prometon	OCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$
Prometryn	SCH_3	$CH(CH_3)_2$	$CH(CH_3)_2$
Propazine	Cl	$CH(CH_3)_2$	$CH(CH_3)_2$
Simazine	Cl	CH_2CH_3	CH_2CH_3
Metribuzin			

evaluated for extraction efficiencies of eight triazines; the non-polar PDMS fiber showed the highest efficiencies for all compounds in the headspace SPME. To a 0.5-ml whole blood sample containing triazine herbicides, were added 1.5 ml of distilled water, 1 g of $CaCl_2$ dihydrate and a small PTFE-coated stirring bar in a 7.5-ml glass vial. In the case of urine, a 1-ml sample containing triazines was mixed with 0.5 g Na_2SO_4. The vials were rapidly sealed with silicon-septum caps and placed on an aluminum block heater for heating and stirring. After heating at 95°C for 5 min, the needle of the SPME device pierced the septum of the vial, and a PDMS fiber was exposed in the headspace of the vial to allow adsorption of the compounds at the same temperature for 30 min. The fiber was withdrawn from the vial and immediately inserted into an injection port of a gas chromatograph for desorption of the analytes. Extraction efficiencies for all compounds were 0.21–0.99% for whole blood, except for cyanazine (0.06%). For urine, the extraction efficiencies for prometon, propazine, atrazine, prometryn and ametryn were 13.6–38.1%, and those of simazine, metribuzin and cyanazine were 1.35–8.73%.

4.4. Gas chromatography

Kumazawa et al. [20] reported that separation of simazine, atrazine, propazine, cyanazine, ametryn, prometryn, prometon and metribuzin, from each other and from impurities, was satisfactory with use of a DB-1 capillary column (30 m×0.32 mm I.D., 0.25 μm film thickness) after SPE with Sep-Pak C_{18} cartridges. The detection limits for the eight triazines on gas chromatograms using FID and NPD were 0.2–1.4 μg and 20–60 ng/ml serum or urine, respectively; NPD gave a sensitivity more than 10–20-times higher than FID.

After headspace SPME for the eight triazine herbicides in human whole blood and urine samples, detection of the compounds was achieved by GC–NPD with a DB-17 capillary column (30 m×0.32 mm I.D., film thickness 0.25 μm) (to be published). All compounds were separated from each other and gave sharp peaks. The blank chromatograms gave impurity peaks; but no interfering peaks appeared around the test peaks. The regression equations for the compounds extracted from whole blood were linear in the range of 0.01–1 μg/0.5 ml, with an *r* value of >0.995, for prometon, propazine, atrazine, prometryn and ametryn, and 0.02–1 μg/0.5 ml, with an *r* value of >0.994, for simazine, metribuzin and cyanazine. The detection limits of the compounds were 2.8–9.0 ng/0.5 ml for whole blood.

4.5. Liquid chromatography

Liquid chromatographic separation for atrazine and propazine extracted from human plasma by LLE was performed on a Hypersil ODS C_{18} column (125 mm×4.6 mm I.D., particle size 5 μm, Chromasciences, France) with a pre-column (20 mm×4 mm I.D.) which contained the same stationary phase [19]. The mobile phase was water–methanol (40:60, v/v). The detection system used was UV at a wavelength of 254 nm. The calibration curves for both compounds in plasma were linear in the range of 6.25–400 ng/ml, with an *r* value of 0.996. The detection limits of atrazine and propazine were reported to be 6 and 4.3 ng/ml, respectively. The method was applied to human plasma samples obtained from an atrazine-poisoned patient.

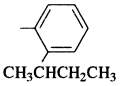
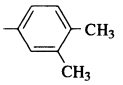
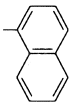
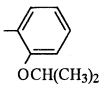
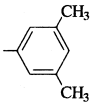
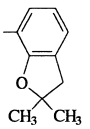
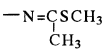
5. Carbamates

Carbamate pesticides are widely used as insecticides and herbicides because of their relatively low toxicity for mammals. Accidental or suicidal cases due to carbamate poisoning have increased accordingly with the increase in the use of the pesticides [21–26]. They are derivatives of neutral esters of carbamic acid (CH_3-NH-CO-O-R). Many of the insecticidal carbamates of commercial significance are phenyl carbamates. Their chemical structures are shown in Table 3. The toxic activity of carbamates in mammals is associated with inhibition of cholinesterases (EC 3.1.1.7 and 3.1.1.8).

5.1. Liquid–liquid extraction

A number of reports for carbamates have appeared addressing the usefulness of LLE before chromatographic separation and detection; organic solvents such as ethyl acetate and diethyl ether were accept-

Table 3
Chemical structures of carbamate pesticides

Product name	Alternate name(s)	IUPAC	CH_3-NH-CO-O-R R=
BPMC	Fenobucarb Bassa	*o*-*sec*-Butylphenyl-*N*-methylcarbamate	
MIPC	Isoprocarb Mipcin	*o*-Cumenyl-*N*-methylcarbamate	
MPMC	Xylylcarb Meobal	3,4-Xylyl-*N*-methylcarbamate	
MTMC	Metolcarb Metacrate	*m*-Tolyl-*N*-methylcarbamate	
NAC	Carbaryl Sevin	1-Naphthyl-*N*-methylcarbamate	
PHC	Propoxur Baygon	*o*-Isopropoxyphenyl-*N*-methylcarbamate	
XMC	Macbal Cosban	3,5-Xylyl-*N*-methylcarbamate	
Carbofuran	Furadan Curaterr	2,2-Dimethyl-7-coumaranyl-*N*-methylcarbamate	
Methomyl	Lannate Nudrin	*S*-Methyl-*N*-(methylcarbamoyl-oxy)thioacetimidate	

able for extraction of carbofuran, furathiocarb, benfuracarb, carbaryl or propoxur from various kinds of biological samples [27–31].

Klys et al. [31] reported extraction of carbofuran from mother's blood and fetus's tissues (the liver, kidney and brain) in a carbofuran-poisoned pregnant woman by LLE with methylene chloride before GC–NPD. Hill, Jr. et al. [12] also reported that carbamate metabolites could be extracted from human urine with use of laboratory robotics and ^{13}C-labeled internal standards before GC–MS–MS; targets for analysis were 2-isopropoxyphenol as propoxur metabolite, 1-naphthol as carbaryl metabolite, and carbofuranphenol as the metabolite from carbofuran, benfuracarb, carbosulfan and furathiocarb. After hydrolysis of urine sample, the metabolites were extracted with 1-chlorobutane–ethyl ether (8:2).

DeBerardinis, Jr. and Wargin [27] optimized the

method including extraction of carbaryl and its hydrolysis product 1-naphthol from human whole blood before LC–FL. Spiked whole blood (0.25 ml) containing napropamide as internal standard was hemolyzed with distilled water (0.25 ml) and extracted with 2.5 ml of ethyl acetate.

Pirimicarb (2-dimethylamino-5,6-dimethylpyrimidin-4-yldimethylcarbamate) was introduced in 1969 as a selective insecticide [32]. The major urinary metabolites are 2-dimethylamino-5,6-dimethyl-4-hydroxypyrimidine (DDHP), 2-methylamino-5,6-dimethyl-4-hydroxypyrimidine (MDHP) and 2-amino-5,6-dimethyl-4-hydroxypyrimidine (ADHP). Hardt and Angerer [33] reported that these hydroxypyrimidines could be extracted with 5 ml of diethyl ether–acetonitrile (1:1) from 5 ml of human urine. After derivatization with pentafluorobenzyl bromide for the extracts, LLE was made again with heptane. The derivatives were analyzed by GC–MS. The recoveries for DDHP, MDHP and ADHP were >81%.

5.2. Solid-phase extraction

Miyazaki et al. [6] reported that an Extrelut column could be used for extraction of methomyl from human blood, serum, urine and tissue samples before GC–MS. The sample homogenized with NaOH solution was poured into the Extrelut column (10×1 cm I.D., Merck, Darmstadt, Germany), and ethyl acetate was used as elution solvent. Recoveries of the pesticide added to blood and liver were 96.2 and 93.8%, respectively.

MTMC, MPMC, XMC, MIPC, BPMC, PHC, NAC, carbofuran and methomyl in human whole blood, plasma, urine and tissues (the liver, kidney and brain) were extracted by use of Sep-Pak C_{18} cartridges [34]. One-milliliter of body fluid (urine, plasma or whole blood) containing carbamates was mixed with 9 ml of distilled water and poured into the cartridge. It was then washed with 10 ml of distilled water; finally 3 ml of chloroform was passed through it to elute the pesticides. The eluate was evaporated to dryness under the stream of nitrogen, and the residue was dissolved in acetonitrile for GC analysis. To extract carbamates from tissues, each 1 g tissue was put into 4 ml distilled water containing carbamates and minced with scissors. The mixture was homogenized after addition of 7 ml of 0.4 *M* perchloric acid solution. The homogenate was then centrifuged, the clear supernatant was poured into the cartridge and the following procedure was exactly the same as described in the body fluids. The recoveries of carbamates added to whole blood, plasma and urine were close to 100%, and those for tissues were >60%.

Kawasaki et al. [7] developed SPE with a Extrelut column for eight carbamates, isoprocarb, metolcarb, fenobucarb, xylylcarb, XMC, ethiofencarb, propoxur and carbaryl, in human serum before LC–MS with an APCI interface. A 1.5-ml volume of sample containing eight pesticides plus 1.5 ml of 0.2 *M* phosphate buffer (pH 7.0) were poured into the column, and 15 ml of dichloromethane was used as elution solvent. After evaporation of the eluate, the residue was dissolved in 150 μl of 50% methanol in water, and a 100-μl aliquot was subjected to LC–MS analysis. The recoveries of the pesticides added to serum were reported to be >93%.

5.3. Solid-phase microextraction

A simple extraction of xylylcarb, XMC, isoprocarb, fenocarb, propoxur and carbofuran from human body fluids by headspace SPME with a PDMS fiber was reported by Seno et al. [35]. To 100-μl of whole blood placed in a 7.5-ml vial, were added 900 μl of distilled water and 0.5 g of sodium chloride. For urine, 1-ml of the sample was put into the 7.5-ml vial containing 0.5 g of sodium chloride. The fiber was exposed to the headspace of the vial at 70°C for 30 min. Extraction efficiencies of six carbamates extracted from whole blood were 0.5–1.2%; those from urine were 2.9–9.0%.

5.4. Gas chromatography

A sensitive GC–NPD method for quantitative determination of carbofuran in forensic samples was described by Klys et al. [31]; they used a non-polar SPB-1 wide-bore capillary column (30 m×0.75 mm I.D., film thickness 2 μm, Supelco). The calibration curves for the pesticide extracted from blood, the liver, kidney and brain were linear in the range of 1–5 μg/g. The concentration of carbofuran in blood of the mother was 2.6 μg/g; those in the kidney, liver and brain of the fetus were 1.4, 2.5 and 0.3 μg/g, respectively.

Similarly, the use of NPD for detection of furathiocarb from human blood in fatal poisoning cases was reported by Lee et al. [29]. Their calibration curve was linear in the range of 0.5–50 μg/ml for blood with carbaryl as internal standard using a slightly polar DB-5 MS fused-silica capillary column (15 m×0.25 mm I.D., film thickness 0.25 μm). The fatal blood levels of furathiocarb due to its ingestion were 0.1–21.6 μg/ml. They also determined benfuracarb and carbofuran (a metabolite of benfuracarb) in blood or urine in fatal benfuracarb poisoning cases with the same capillary column [30]. The concentrations of benfuracarb and carbofuran were 0.30–2.32 and 1.45–1.47 μg/ml of blood, respectively. Benfuracarb was not detected in urine, but carbofuran was (0.53–2.66 μg/ml).

Suzuki et al. [34] simultaneously determined MTMC, MPMC, XMC, MIPC, BPMC, PHC, NAC, carbofuran and methomyl in human whole blood, plasma, urine and tissues (the liver, kidney and brain) by GC–FID with a slightly polar SPB-5 wide-bore capillary column (10 m×0.53 mm I.D., film thickness 1.5 μm). The chromatogram gave intense peaks for each compound with low background impurities. Detection limits of each carbamate on the gas chromatograms were 0.5–1.0 μg/ml for the samples.

Seno et al. [35] reported GC–FID determination of xylylcarb, XMC, isoprocarb, fenocarb, propoxur and carbofuran in human body fluids after headspace SPME. They used a slightly polar RTX-35 fused-silica capillary column (30 m×0.32 mm I.D., film thickness 0.25 μm, Restek, Bellefonte, PA, USA). The chromatogram gave intense peaks for each compound with low background noises. The calibration curves for the six pesticides extracted from whole blood were linear in the range of 0.4–10 μg/ml for isoprocarb and fenobucarb, and 2–10 μg/ml for XMC, xylylcarb, propoxur and carbofuran. The detection limits were 100–500 ng/ml for whole blood, and 10–50 ng/ml for urine.

5.5. Gas chromatography–mass spectrometry and gas chromatography–tandem mass spectrometry

Mass fragmentographic analysis of methomyl in human blood, serum, urine and tissue samples was described by Miyazaki et al. [6]. After trimethylsilyl derivatization with trimethylchlorosilane–hexamethyldisilazane (1:9) of SPE extracts, the derivatives were chromatographed with a non-polar HiCap-CBP 1 wide-bore capillary column (12 m×0.53 mm I.D., film thickness 1 μm, Shimadzu, Kyoto, Japan). The pesticide was quantitated by selected ion monitoring in the positive ion chemical ionization mode. The calibration curves for the pesticide extracted from distilled water were linear in the range of 0.1–6 μg/ml with a detection limit of 0.01 μg/g.

Carbamate metabolites, 2-isopropoxyphenol, 1-naphthol and carbofuranphenol in human urine, after derivatization with tetrabutylammonium hydrogen sulfate for LLE extracts, were analyzed by GC–MS–MS [12]. Chromatographic separation was carried out with a slightly polar DB-5 capillary column (30 m×0.25 mm I.D., film thickness 0.25 μm). Positive chemical ionization with methane was used to produce quasi-molecular ions $[M+H]^+$ for the compounds. Each M+H ion was selected as a precursor and submitted to collision-induced dissociation with argon to produce a product ion to be used for quantitation. The calibration curves were linear for each metabolite in the range of 1–50 ng/ml, except for 1-naphthol. The detection limit for all compounds was 1 ng/ml.

GC–MS analysis of DDHP, MDHP and ADHP as urinary metabolites of pirimicarb was demonstrated by Hardt and Angerer [33] after derivatization with pentafluorobenzyl bromide. The derivatives were chromatographed with a slightly polar HP-35 fused-silica capillary column (60 m×0.32 mm I.D., film thickness 0.25 μm, Hewlett-Packard, Palo Alto, CA, USA) and detected in the positive ion electron impact mode. The detection limits for DDHP, MDHP and ADHP defined as a signal-to-noise ratio of 3 using each molecular ion were 0.5, 1 and 4 ng/ml, respectively. By use of this method, they tried to measure DDHP, MDHP and ADHP in urine obtained 3.75–8.25 h after exposure to pirimicarb of workers employed in agriculture and fruit plantation; the three metabolites were found in urine samples in concentrations up to 60 ng/ml.

5.6. Liquid chromatography and liquid chromatography–mass spectrometry

LC–FL was used for detecting carbaryl and its hydrolysis product 1-naphthol from human whole blood [27]. Both compounds were chromatographed

by use of a C_8 column (250 mm×4.6 mm I.D., particle size 5 μm) with 0.13 *M* phosphate buffer (pH 6.2)–acetonitrile (2:3, v/v) as mobile phase. The effluent was exposed to an excitation wavelength of 285 nm and fluorescence intensity was measured above 340 nm. The regression equations for carbaryl and 1-naphthol extracted from whole blood were linear in the ranges of 10–500 and 12.5–525 ng/0.25 ml, respectively, with *r* values of >0.998.

The use of LC with RID for determination of carbaryl and propoxur in human blood, the lung and liver was reported by Sharma et al. [28]. They used a Zorbax CN column (250 mm×4.6 mm I.D., particle size 5 μm, Hewlett-Packard, Wilmington, DE, USA) and 20% ethyl acetate in isooctane as mobile phase. The method was also used for analysis of postmortem samples in propoxur poisoning cases; the concentration of propoxur was 470 μg/ml for blood.

Kawasaki et al. [7] developed LC–MS with an APCI interface for eight carbamates, isoprocarb, metolcarb, fenobucarb, xylylcarb, XMC, ethiofencarb, propoxur and carbaryl, in human serum. The pesticides were separated by use of a Nova-Pak C_{18} column (150 mm×3.9 mm I.D., particle size 4 μm, Waters) with 100 m*M* ammonium acetate buffer (pH 6.8)–methanol (6:4, v/v) as mobile phase. The detection limits obtained by selected ion monitoring for carbaryl, ethiofencarb, fenobucarb and propoxur were 12–33 ng/ml; those for isoprocarb, XMC and xylylcarb were 60 ng/ml.

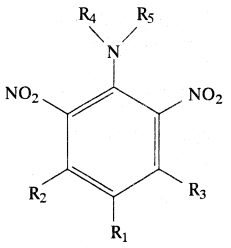
6. Dinitroanilines

Dinitroanilines are herbicides used on a wide variety of crops for the control of annual grasses and broadleaf weeds. Pollution of crops and groundwater by dinitroaniline herbicides is recognized as a serious environmental problem [36]. Although they are generally considered to have a very low degree of toxicity in mammals [37], there is also a possibility of suicidal and accidental ingestion of the herbicides. As shown in Table 4, most of commercial dinitroanilines contain a tertiary amino group and two nitro groups in their structures.

6.1. Solid-phase extraction

We reported that seven dinitroanilines, benfluralin, ethalfluralin, isopropalin, nitralin, pendimethalin, profluralin and trifluralin, could be extracted from human whole blood and urine samples by use of Sep-Pak C_{18} cartridges [38]. One-milliliter of urine

Table 4
Chemical structures of dinitroaniline herbicides



Compound	R_1	R_2	R_3	R_4	R_5
Benfluralin	CF_3	H	H	CH_2CH_3	$CH_2CH_2CH_2CH_3$
Ethalfluralin	CF_3	H	H	CH_2CH_3	$CH_2CCH_2CH_3$
Fluchloralin	CF_3	H	H	$CH_2CH_2CH_3$	CH_2CH_2Cl
Isopropalin	$CH(CH_3)_2$	H	H	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$
Nitralin	CH_3SO_2	H	H	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$
Pendimethalin	CH_3	H	CH_3	$CH(CH_2CH_3)_2$	H
Prodiamine	CF_3	NH_2	H	$CH_2CH_2CH_3$	$CH_2CH_2CH_3$
Profluralin	CF_3	H	H	CH_2—◁	$CH_2CH_2CH_3$

Table 5
Summary of separation methods for amino group possessing pesticides

Chemical(s) analyzed	Sample	Extraction		Elution solvent	Analytical system	Column	Linearity	Detection limit	Ref.
		LLE	SPE						
Diazines									[18]
Bromacil, norflurazon, pyrazon and terbacil	Whole blood		Bond Elut C_{18}	Chloroform–methanol (9:1)	GC–FID	DB-17	0.16–10 μg/ml	0.12–0.14 μg/ml	
	Plasma		Bond Elut C_{18}	Chloroform–methanol (9:1)	GC–FID	DB-17	0.16–10 μg/ml	0.12–0.14 μg/ml	
	Urine		Bond Elut C_{18}	Chloroform–methanol (9:1)	GC–FID	DB-17	0.16–10 μg/ml	0.11–0.12 μg/ml	
	Whole blood	Diethyl ether			GC–FID	DB-17			
	Plasma	Diethyl ether			GC–FID	DB-17			
	Urine	Diethyl ether			GC–FID	DB-17			
Triazines									[20]
Prometon, propazine, atrazine, simazine	Serum,		Sep-Pak C_{18}	Chloroform	GC–NPD	DB-1		20–60 ng/ml	
prometryn, ametryn metribuzin and cyanazine	Urine		Sep-Pak C_{18}	Chloroform	GC–FID	DB-1		0.2–1.4 μg/ml	
Atrazine	Plasma	Dichloromethane			LC–UV	Hypersil ODS (C_{18})	6.25–400 ng/ml	6 ng/ml	[19]
Propazine	Plasma	Dichloromethane			LC–UV	Hypersil ODS (C_{18})		4.3 ng/ml	
Carbamates									
Carbofuran	Blood, kidney, liver, brain	Methylene chloride			GC–NPD	DB-5 MS	1–5 μg/g		[31]
Furathiocarb	Blood	Ethyl acetate			GC–NPD	DB-5 MS			[29]
Benfuracarb and carbofuran	Blood, urine	Ethyl acetate			GC–NPD	DB-5 MS			[30]
MTMC, MPMC, XMC, MIPC, BPMC, PHC, NAC, carbofuran and methomyl	Whole blood, plasma, urine liver, kidney, brain		Sep-Pak C_{18}	Chloroform	GC–FID	SPB-5		0.5–1 μg/ml	[34]
Methomyl	Serum, urine, liver, kidney brain		Extrelut	Ethyl acetate	GC–MS	HiCap-CBP 1	0.1–6 μg/g	0.01 μg/g	[6]
2-Isopropoxyphenol	Urine	1-Chlorobutane–ethyl ether (8:2)			GC–MS–MS	DB-5	1–50 ng/ml	1 ng/ml	[12]
Carbofuranphenol	Urine	1-Chlorobutane–ethyl ether (8:2)			GC–MS–MS	DB-5	1–50 ng/ml	1 ng/ml	
1-Naphthol	Urine	1-Chlorobutane–ethyl ether (8:2)			GC–MS–MS	DB-5	1–1000 ng/ml	1 ng/ml	
DDHP	Urine	Diethyl ether–acetonitrile (1:1)			GC–MS	HP-35	2–100 ng/ml	0.5 ng/ml	[33]
MDHP	Urine	Diethyl ether–acetonitrile (1:1)			GC–MS	HP-35	2–100 ng/ml	1 ng/ml	
ADHP	Urine	Diethyl ether–acetonitrile (1:1)			GC–MS	HP-35		4 ng/ml	
Carbaryl	Whole blood	Ethyl acetate			LC–FL	C_8	10–500 ng/0.25 ml		[27]
1-Naphthol	Whole blood	Ethyl acetate			LC–FL	C_8	12.5–525 ng/0.25 ml		
Carbaryl and propoxur	Blood, lung, liver	Diethyl ether			LC–RID	Zorbax CN			[28]
Carbaryl, ethiofencarb, fenobucarb, isoprocarb, metolcarb, propoxur, XMC and xylylcarb	Serum		Extrelut	Dichloromethane	LC–MS	Nova-Pak C_{18}	1–10 μg/ml	12–60 ng/ml	[7]

Table 5. Continued

Chemical(s) analyzed	Sample	Extraction		Elution solvent	Analytical system	Column	Linearity	Detection limit	Ref.
		LLE	SPE						
Dinitroanilines									[38]
Benfluralin, ethalfluralin, isopropaline, nitraline, pendimethalin, profluralin and trifluralin	Whole blood		Sep-Pak C_{18}	Chloroform–methanol (9:1)	GC–ECD	DB-1		2.4–4.5 pmol/ml	
	Urine		Sep-Pak C_{18}	Chloroform–methanol (9:1)	GC–ECD	DB-1		1.9–4.0 pmol/ml	
Chloroacetanilides									
Alachlor mercapturate	Urine		C_{18}	Methanol	LC–MS–MS	ODS-3	1–1000 ng/ml		[42]
Metolachlor mercapturate	Urine		C_{18}	Methanol	LC–MS–MS	ODS-3	10–250 ng/ml	3 ng/ml	[43]

containing seven dinitroanilines (300 pmol each) was mixed with 4 ml of distilled water. For whole blood, the 1-ml sample was mixed with 9 ml distilled water for complete hemolysis. Each sample solution was poured into the cartridge (sorbent mass 360 mg). It was then washed with 20 ml distilled water followed by 3 ml chloroform–methanol (9:1) to elute the compounds from the cartridge. Recoveries of the seven compounds were more than 91% for whole blood and urine samples.

6.2. Solid-phase microextraction

Guan et al. [39] have reported that benfluralin, ethalfluralin, fluchloralin, prodiamine, isopropalin, pendimethalin and profluralin can be extracted from human whole blood and urine samples by headspace SPME with a PDMS fiber. One-milliliter of urine spiked with the herbicides was mixed with 0.28 g of anhydrous Na_2SO_4 and preheated at 70°C for 10 min; a PDMS fiber was exposed to the headspace of the vial at same temperature for another 30 min. In the case of whole blood, 0.5 ml of it was diluted with 0.5 ml of distilled water and treated at 90°C in the same way. Extraction efficiencies of the pesticides from whole blood and urine were 3.2–7.2 and 17–58%, respectively.

6.3. Gas chromatography

Capillary GC with four different detection methods (FID, NPD, ECD and SID) for dinitroanilines, benfluralin, ethalfluralin, isopropalin, nitralin, pendimethalin, profluralin and trifluralin, was reported [38]. The herbicides were well-separated with a DB-1 fused-silica capillary column (30 m×0.32 mm I.D., film thickness 0.25 μm). ECD was found most powerful in measuring all dinitroanilines containing nitro group in human body fluids, because it showed the highest sensitivity and the lowest background impurity peaks. In SID, ethalfluralin, trifluralin, benfluralin and profluralin, which contain halogen groups in their structures, showed much lower responses than isopropalin. Nitralin, which contains a sulfur group in its structure, showed the lowest sensitivity, suggesting a negative effect of the sulfur group for SID; pendimethalin, which has a secondary amino group, also showed very low sensitivity. However, only isopropalin could be detected by SID with sensitivity as high as that by ECD; relatively high sensitivity could be also obtained for isopropalin, pendimethalin and nitralin by use of NPD. The detection limits of all compounds by GC–ECD were 2.4–4.5 pmol/ml for whole blood and 1.9–4.0 pmol/ml for urine.

7. Chloroacetanilides

Chloroacetanilides, alachlor and metolachlor, have been used for more than 20 years as herbicides on soybeans, corn and other crops; they are one of the most widely used classes of pesticides [40]. Exposure to the pesticide mist for agricultural workers is a serious problem from a hygienic point of view. Chloroacetanilides are readily metabolized in human body [41]; thus their metabolites should be analyzed for forensic or hygienic purposes for these pesticides.

7.1. Solid-phase extraction

Driskell and Hill, Jr. reported SPE with C_{18} cartridges for the metabolites, alachlor mercapturate [42] and metolachlor mercapturate [43], in human urine. The samples were obtained from agricultural workers occupationally exposed to alachlor or metolachlor. Each metabolite in urine sample was extracted by the cartridge with methanol as elution solvent.

7.2. Liquid chromatography–tandem mass spectrometry

LC–MS–MS with APCI interface has been used for determination of alachlor mercapturate and metolachlor mercapturate in human urine samples [42,43]. The chromatographic separation was carried out with an ODS-3 column (250 mm×4.6 mm I.D., Whatman, Clifton, NJ, USA) with water–methanol (3:7, v/v) with 0.1% acetic acid as mobile phase at flow-rate of 1 ml/min. The mass spectrometer was set in the positive ionization MS–MS mode, and the compounds produced quasi-molecular ions $[M+H]^+$ by the first MS. Each quasi-molecular ion was subjected to collision-induced dissociation with argon to produce product ions, one of which was selected for measurements. The calibration curves for alachlor mercapturate and metolachlor mercapturate spiked to urine were linear in the range of 1–1000 and 10–250 ng/ml, respectively. The detection limit was 3 ng/ml for metolachlor mercapturate.

8. Conclusion

Summaries of application of separation methods to amino group possessing pesticides in biological samples are given in Tables 5 and 6. Since 1995, we have reported the SPME for forensic analysis of a number of compounds of medico–legal interest including these pesticides [10]; SPME has proved to be useful for extraction of amino group possessing pesticides from biological samples. The major chromatographic method for analysis of pesticides, which

Table 6
Summary of separation methods for amino group possessing pesticides by headspace SPME–GC

Chemical(s) analyzed	Sample	Additive[a]	Fiber[b]	Vial temperature (°C)	Pre-heat time (min)	Exposure time (min)	Detector	Linearity	Detection limit	Ref.
Triazines										
Prometon, propazine, atrazine, simazine, prometryn, ametryn, metribuzin and cyanazine	Whole blood	DW, $CaCl_2$	PDMS (100 μm)	95	5	30	NPD	0.01–1 μg/0.5 ml (simazine, metribuzin and cyanazine 0.02–1 μg/0.5 ml)	2.8–9.0 ng/0.5 ml	
	Urine	Na_2SO_4	PDMS (100 μm)	95	5	30	NPD	0.005–0.25 μg/ml	0.4–2.0 ng/ml	
Carbamates										[35]
Fenobucarb, carbofuran, isoprocarb, XMC, xylylcarb and propoxur	Whole blood	DW, NaCl	PDMS (100 μm)	70	10	30	FID	2–10 μg/ml (isoprocarb and fenocarb 0.4–10 μg/ml)	100–500 ng/ml	
	Urine	NaCl	PDMS (100 μm)	70	10	30	FID		10–50 ng/ml	
Dinitroanilines										[39]
Ethalfluralin, benfluralin, fluchloralin, prodiamine,	Whole blood	DW, Na_2SO_4	PDMS (100 μm)	90	10	30	ECD	1–60 ng/0.5 ml	0.5 ng/0.5 ml	
isopropalin, pendimethalin and profluralin	Urine	Na_2SO_4	PDMS (100 μm)	70	10	30	ECD	0.1–10 ng/ml	0.1 ng/ml	

[a] DW = Distilled water.
[b] PDMS = Polydimethylsiloxane.

has been frequently used during the last 20 years, is GC. There is a trend that pesticide analysis is now shifting from GC or GC–MS to LC–MS with various MS interfaces. Each interface has its own advantages and disadvantages; for analytical toxicology, APCI and ESI interfaces for LC–MS are becoming very popular, because of its quantitative nature, reproducibility, sensitivity and wide applicability. In this review, we have dealt with analytical methods for amino group possessing pesticides in complicated biological matrices. These methods seem easily applicable to surface or groundwater in environmental chemistry.

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