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Determination of carbofuran, carbaryl and their main metabolites in plasma samples of agricultural populations using gas chromatography–tandem mass spectrometry

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Abstract A gas chromatography–tandem mass spectrometric (GC-MS/MS) method has been developed for the determination of carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate), carbaryl (1-naphthyl-N-methylcarbamate) and their main metabolites in human blood plasma. Optimization of the isolation of the compounds from plasma matrix included the precipitation, denaturation and digestion of plasma proteins. Derivatization was achieved by the use of trifluoroacetic acid anhydride and was optimized for temperature, time and volume of derivatization agent. In the proposed method, a mild precipitation technique was applied using β -mercaptoethanol and ascorbic acid in combination with solid-phase extraction technique using Oasis HLB (Hydrophobic Lipophilic Balance) cartridges for further clean up of samples. Carbamate linkage was not hydrolyzed to its phenol product, but both carbamate phenol and ketones were transformed into trifluoroacetyl derivatives in order to become volatile compounds and were determined using tandem mass spectrometry. The linearity of the method was shown for nine concentrations in the range of 0.50–250 ng mL⁻¹ in fortified plasma aliquots. Limits of detection (LODs) for all compounds ranged from 0.015–0.151 ng mL⁻¹. Inter-day and intra-day assays (RSD) for all com-

pounds, at three concentration levels of 2.5, 25 and 100 ng mL⁻¹ ($n=3$) in fortified plasma samples were less than 18%. Accuracy (% E_r) was calculated at three concentration levels, 8, 80 and 160 ng mL⁻¹ ($n=3$), and ranged from -12.0 to 15.0%. Matrix effect was evaluated so mean recoveries were calculated for all compounds and ranged from 81–107%. Specificity for the use of this method to biological monitoring studies was achieved including four main metabolites of CF, 1-naphthol and 2-naphthol from the naphthalene metabolism pathways, and both the parent compound of carbofuran and carbaryl. The proposed method was applied to plasma samples of pesticide users.

Keywords N-methylcarbamates · Tandem mass spectrometry · Biological monitoring · Biomarkers of exposure · Human plasma samples

Introduction

One of the major types of worldwide pollutants is the pesticides. Pesticide use has increased over the last two decades in agriculture, but also in households and in other areas such as golf courses. This broad use of pesticides, among other chemicals, may be necessitated by modern civilization, but it is also of concern to hygienists, epidemiologists, physicians and other health officials due to likely adverse health effects caused by the exposure of humans to these chemicals. Pesticides include a variety of compounds such as insecticides, nematocides, and herbicides. Since the middle of last century when pesticide use started, humans have gained many benefits from them, but over the years have forgotten that they were deliberately developed to harm living species [1, 2].

Preventive medicine considers that it is extremely important to determine residues of pesticides and their mixtures in food, air and water to estimate the magnitude of human exposure. According to the latest report, released from the U.S. Department of Health and Human Services of the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, concentrations of environmental chemi-

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cals in blood or urine reflect exposures from one or more sources, and they are not the same as those in air, water, food, soil or dust. Since the mid 1960s, it has been well known that blood or urine measurements of an individual do not indicate that these chemicals can cause a disease. In order to estimate what blood or urine levels result in a disease, CDC suggests that other factors should be taken into consideration such as the duration of exposure, absorption, distribution and metabolism rates of each compound [3]. Environmental chemicals can be a possible threat for human health only when they have entered the body (by inhalation, ingestion or dermal contact), been absorbed into the blood and distributed in the body in order to be (1) excreted, (2) metabolized and excreted, (3) stored-equilibrated with concentrations in blood and slowly excreted or (4) undergo a combination of these processes [4]. Therefore, the development of new advanced analytical techniques to detect pesticides and their metabolites in the relevant biological matrix has emerged, especially in the last 5 years as health incidents to pesticide-affected populations have increased [5].

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) (CF) and carbaryl (1-naphthyl-N-methylcarbamate) (CRB) are two N-methylcarbamates used widely in households and agriculture. Their toxicity is related to acetylcholinesterase (AChE), and therefore, determination of AChE activity in red blood cells and pseudocholinesterase (PchE) activity in serum or plasma have been the most reliable and widely used biological indicators of human exposure [6] and poisoning [7]. Inhibition of AChE in nervous tissues from carbamate exposure is labile, with short duration and is reversible, in contrast with that produced by organophosphates (OPs). Since a cumulative inhibition of cholinesterase activity does not occur with repeated exposures, carbamates have not yet been well documented in long-term exposure studies, but only in acute and poisoning cases [8, 9].

CF and CRB, like other contemporary insecticides, were designed to have biological half-lives in the order of a few hours to a couple of days after oral intake. Therefore they do not circulate in the bloodstream for long periods, and they don't accumulate in tissues. Their metabolism is rather complex, and there are still several metabolites from plant metabolic routes that have not been identified in humans. The metabolism and excretion of CF has been well studied in rats, mice, insects, cows and goats [10–13].

The known pathways of CF metabolism shown in Fig. 1 comprise an oxidation route at the benzylic carbon that yields to 3-hydroxycarbofuran (3HO CF), which yields 3-hydroxycarbofuran phenol and 3-keto carbofuran-7-phenol (CFphenol-3-keto) after hydrolysis. According to California Environmental Protection Agency (CAEPA), 3HO CF is likely to be oxidized in animals, producing the metabolite 3-keto carbofuran (CF-3-keto). Another pathway of metabolism, proposed by CAEPA to be the most common one in mammals and insects, is the hydrolysis of CF directly to carbofuranphenol (7-phenol) [14].

Even though 3HO CF and CF-3-keto were defined as the most common metabolites in mammals [14] after CF oral

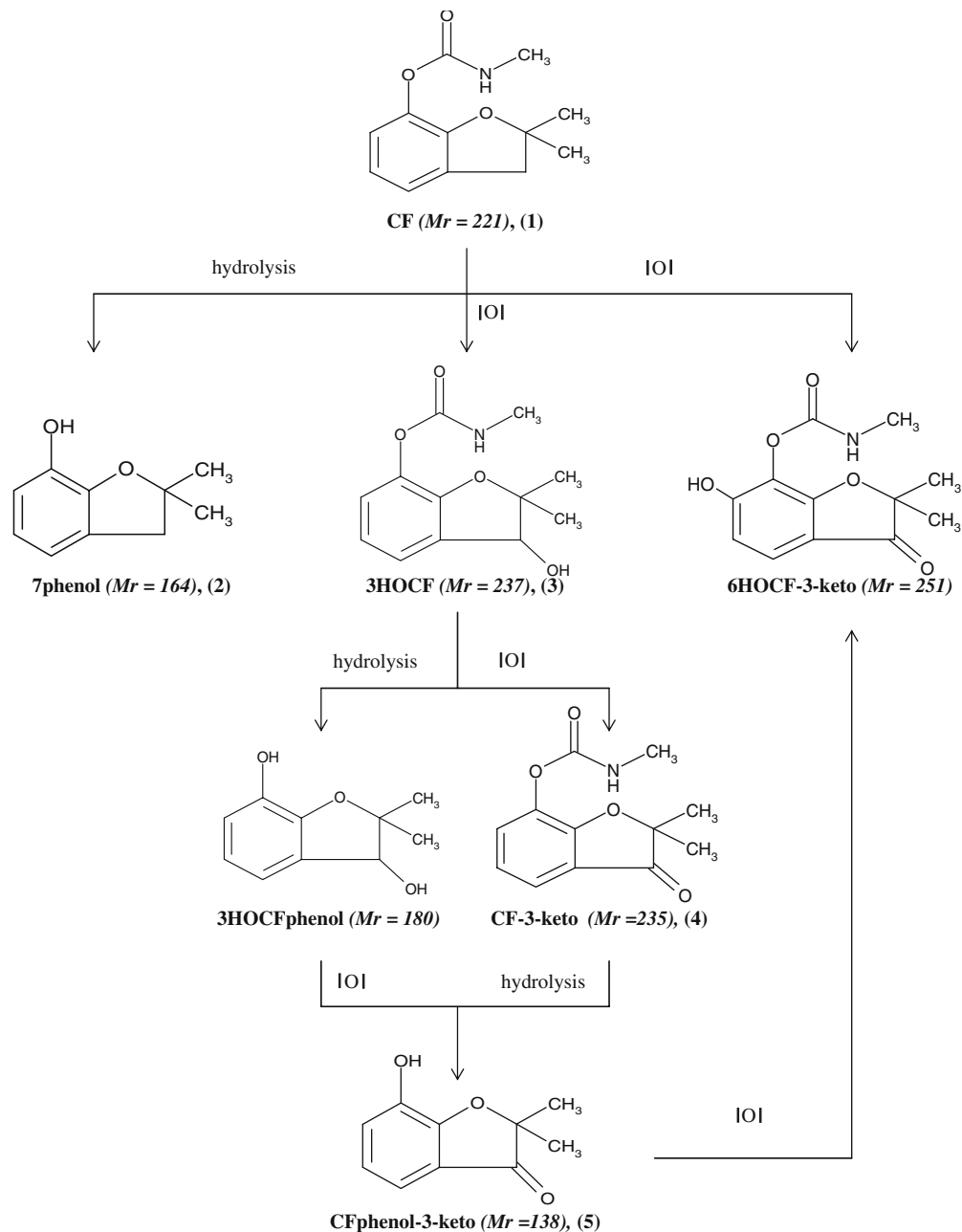
intake, the Third National CDC Report on Human Exposure to Environmental Chemicals suggests measurement of 7-phenol for estimating exposure to CF and three more carbamates, those of benfuracarb, furathiocarb and carbosulfan [3, 15]. Although in the United States only CF is registered for use, in European countries, Greece included, all the above-mentioned carbamates are registered for use. The metabolite of 7-phenol, therefore can not be considered specific enough to estimate possible CF exposure, especially when it has been reported that CF is also the main metabolite of furathiocarb in rats, which probably hydrolyzes to 7-phenol as well [16].

CRB metabolism in humans has not been investigated. The main metabolite from the hydrolysis route, 1-naphthol (1N), has been used in several biological monitoring techniques in order to estimate possible CRB exposure [17–20], even though it can not be used specifically for CRB exposure because 1N is also the main metabolite of naphthalene and napropamide [21, 22] (Fig. 2). Although the naphthalene metabolite of 2-naphthol (2N) can be used to trace the possible source of 1N, there is still no reported method for monitoring both the CRB parent compound and 1N and 2N.

Considering that carbamate metabolism produces a number of polar molecules containing hydroxyl groups that serve as sites for secondary conjugated reactions with glucuronic acid and sulfuric acid in mammals, only four analytical methods in human urine samples have been reported over the past 10 years, but none of them has included both carbamates and their main metabolites. Hill et al. used a gas chromatographic-tandem mass spectrometric method (GC-MS/MS) to measure both 7-phenol and 1N in human urine, but still neither CF nor CRB was measured [17]. Shealy et al., in 1996, improved Hill's method without including CF and CRB parent compounds [18]. In 2000, Martinez Vidal et al. and Parrilla Vazquez et al. reported two methods using liquid chromatography with ultraviolet (UV) and mass spectrometric (MS) detectors, but they measured only CF and its main metabolite of 3HO CF among with aldicarb and two of its metabolites in human urine [23, 24].

Understanding human exposure to carbamates requires information on the concentration of these environmental chemicals in the general population and on their pharmacokinetics, therefore not only techniques for urinary metabolites are needed. In 2002, Barr et al. reported a multi-analyte method for determining contemporary pesticides in serum and plasma samples using high-resolution mass spectrometry, including CF, 7-phenol and 1N but not CRB or the rest of CF metabolites or 2N for naphthalene exposure [25]. Blood samples are needed when detailed information for a pesticide metabolism is not well known and when two or more compounds are producing the same metabolite in urine excretions as in the case of CF and CRB. The determination of an intact parent pesticide in blood instead of an excreted metabolite is much more important, since it provides accurate information as to the pesticide to which one was exposed and especially provides information that one was not exposed directly to the metabolite of a pesticide through inhalation or food intake. Furthermore, blood samples, in

Fig. 1 Proposed CF metabolism pathways in mammals [14]



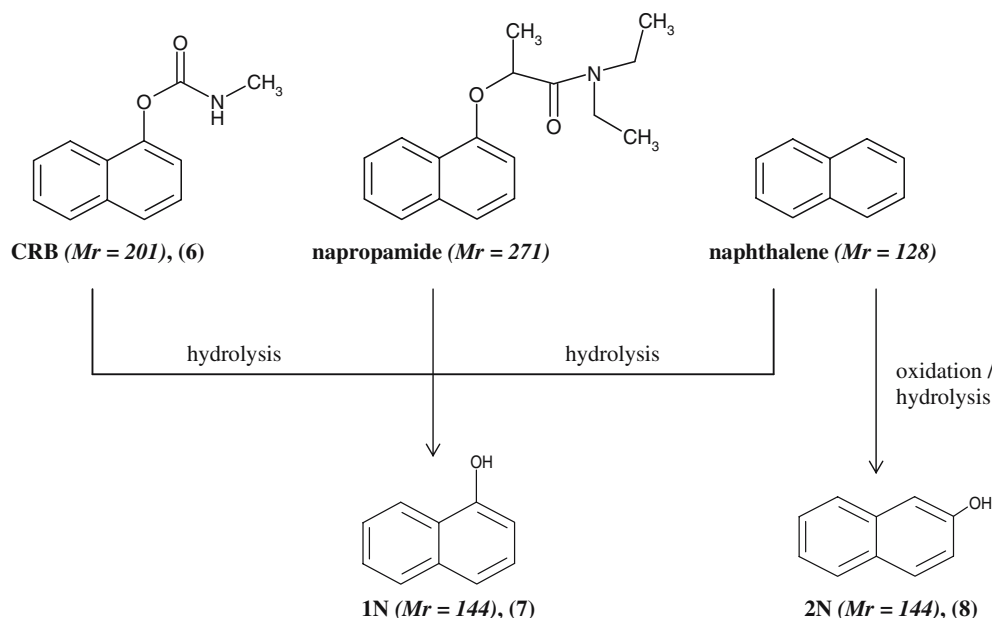
contrast to urine samples, do not need correction of dilution to allow population comparisons, since blood concentration does not vary substantially based on daily water intake. One of the major advantages in blood sample analyses is that the maximum concentrations for most pesticides have been defined directly after exposures, indicating a specific time for sampling in contrast to urine samples [25].

The above-mentioned advantages of analytical methods measuring specific analytes in serum and plasma samples are often countered by disadvantages such as the venipuncture procedure for obtaining the samples, which is especially difficult in large-scale studies or those with children, or the low toxicant concentrations that require extremely sensitive analytical techniques. Nonetheless, analytical techniques for several pesticides and/or their

metabolites have been reported in blood (whole blood, serum or plasma) samples [26–28], but no method has been reported for both CF and CRB.

The proposed GC-MS/MS method provides for the first time an analytical tool for the determination of CF, CRB and their main metabolites in human blood plasma samples. The method employs a simple protein-removal pretreatment of samples and the isolation of the compounds by means of a solid-phase extraction technique based on OASIS HLB cartridges. It has the advantage that it includes four metabolites of CF, and 1N for CRB in the presence of 2N from the naphthalene metabolic pathway in order to achieve higher specificity than methods previously published. The validation of this quantitative method in terms of sensitivity, accuracy, precision and recovery are presented. This method

Fig. 2 Hydrolysis of CRB and napropamide to 1N and oxidation/hydrolysis of naphthalene to 1N and 2N



is suitable to be employed in human exposure studies to estimate skin and inhalation exposure of subjects, in order to provide specific data for public health matters.

Experimental

Chemicals and reagents

Carbofuran (CF, purity 99%), carbofuranphenol-3-keto (CFphenol-3-keto, purity 99%), carbofuran-3-keto (CF-3-keto, purity 99%), 3 hydroxy-carbofuran (3HO CF, purity 98%), 2,3 dihydro-2, 2 dimethylbenzofuran-7-ol (7-phenol, purity 99%), carbaryl (CRB, purity 99.5%), 1-naphthol (purity 99.5%), 2-naphthol (purity 99.5%) were obtained from ChemService (Westchester, PA, USA). The internal standards, isoprocarb [2-(1-methylethyl)phenyl methylcarbamate, ISPR, purity 99%) (IS₁) was obtained from ChemService (Westchester, PA, USA), while N-ethyl-p-methane-3-carboxamide (CBXD) (IS₂) (purity >99%) was obtained from Acros Organics (New Jersey, USA) All organic solvents were of HPLC analytical grade and were obtained from Merck (Darmstadt, Germany). Tetrahydrofuran (THF), triethylamine (TEA), ammonium acetate and acetic acid (purity >99%) were obtained by Riedel de Haan, Germany. Water used in all experiments was deionized using a Barnstead/Thermolyne infinity apparatus (Dubuque, IA, USA) preceded by reverse osmosis with BWT UO 1000 K (BWT Wassertechnik, Schriesheim, Germany). Water, prior to its use, was vacuum-filtered using Titan Membrane Filters (nylon, 0.20 μm porous) obtained from Scientific Resources.

Proteinase K (EC 3.4.21.64), β -mercaptoethanol and ascorbic acid were obtained from Sigma (St. Louis, MO, USA). Vacutainer tubes (3 mL, green top-EDTA) were supplied by Clinica (Athens, Greece).

Trifluoroacetic acid anhydride (TFAA) was gas chromatographic grade (purity >99.9%) and was supplied by Merck (Darmstadt, Germany). Oasis HLB (Hydrophilic Lipophilic Balance) 3 cm³ (60 mg) cartridges were obtained from Waters Associates (Milford, MA, USA). Potassium phosphate buffer (10 mM) was prepared by diluting with water a mixture of 39/61 v/v of KH₂PO₄ (0.100 M) and K₂HPO₄ (0.100 M), respectively. Phosphate buffer pH was adjusted to 7.00 \pm 0.02 with the use of NaOH or H₃PO₄.

Standard and working solutions

Stock standard solutions of all substances were prepared at 1 mg mL⁻¹ by dissolving the compounds in tetrahydrofuran (THF). A working standard solution of 0.100 mg mL⁻¹ of each substance was made into 1:10 and 1:100 dilutions by adding THF. The solutions of ISPR and CBXD were treated the same way, but separately from the other compounds. All samples were stored daily at -35 °C.

Blood samples

Venous blood samples of five applicants who volunteered to take part in our research were collected within 8 h after the application of each compound. Subjects were 23–68 years old and had applied the two insecticides at two different periods of time (April and July 2004), without taking any precautions. Carbofuran was applied by spraying with hoses in April and all applicators were exposed to it from 0.5–2 h. Carbaryl was applied by hand in July for 2–8 h. All application conditions and personal habits were checked with a questionnaire completed with personal interview. An aliquot of each blood sample was taken in a 3-mL Vacutainer tubes (green top-EDTA) and was centrifuged immediately at 3,300 g at room temperature to provide plasma. A pre-

exposed plasma sample from each volunteer was also supplied. The rest of the blood sample was taken in tubes with clot activators to provide serum for measuring liver and renal function of each individual. Renal and liver function for all five of them was estimated by measuring the appropriate indicators at the beginning of the study and during the research using serum samples in an organized local microbiology laboratory. Plasma samples were frozen at $-27\text{ }^{\circ}\text{C}$ and were transferred to the laboratory of analysis, where they were stored at $-70\text{ }^{\circ}\text{C}$ until the gas chromatographic–tandem mass spectrometric (GC-MS/MS) analysis.

Preparation of plasma extracts

Plasma aliquots were brought to $-35\text{ }^{\circ}\text{C}$ 1 day before analysis, and the day of the analysis they were brought quickly to ambient temperature in a water bath ($37\text{ }^{\circ}\text{C}$) with shaking. After thawing, $200\text{ }\mu\text{L}$ of plasma was transferred to a sterile 1.5-mL Eppendorf tube (Sarstedt, Germany). For developing the method each control plasma sample was enriched with the appropriate volume of working solution. Fortified control plasma samples were prepared daily and IS_1 solution was added before the precipitation procedure. In order to have the same amount of THF in volunteers' samples for analysis, $20\text{ }\mu\text{L}$ of THF solvent was added instead of the volume of working solution that was added in control samples.

A mild precipitation and denaturation technique was applied to every sample. Aqueous β -mercaptoethanol ($5\text{ }\mu\text{L}$, 200 mM) along with $100\text{ }\mu\text{L}$ of aqueous ascorbic acid (10% w/v) were added to samples. Samples were vortexed for 30 s and were put in sonics (ice-cold water) for 15 min . This procedure was carried out twice, and then samples were centrifuged at $5,000\text{ g}$ at $4\text{ }^{\circ}\text{C}$ for 30 min .

After centrifugation the supernatant layer was loaded onto the Oasis HLB cartridge, which was preconditioned with 1 mL of methanol and equilibrated with 1 mL of phosphate buffer. Remaining denatured proteins were further removed from each sample with three wash-out steps. First 1 mL of phosphate buffer was applied, followed by 1 mL of NH_4OH aqueous solution (5% vol. 1 M NH_4OH) and 1 mL of phosphate buffer. Cartridges were left out to dry under vacuum for 30 min . Pesticides were eluted with $2\times 1\text{ mL}$ of diethyl ether into one Eppendorf and the eluate was evaporated under a gentle steam of nitrogen at room temperature, to approximately $150\text{ }\mu\text{L}$. The extract was transferred to a $200\text{ }\mu\text{L}$ PCR Eppendorf (Eppendorf, Germany) and was evaporated with the same technique to dryness. Extraction efficiency was evaluated from the ratio of peak relative areas for all compounds adjusted by IS_1 to the peak relative areas of standards adjusted with IS_1 as well. The dry extract was enriched with IS_2 , which was also evaporated to dryness.

Derivatization

The extraction-evaporated residues were redissolved in $20\text{ }\mu\text{L}$ of TFAA and $10\text{ }\mu\text{L}$ of a 0.02% v/v solution of TEA

in THF made daily. Solution was vortexed and derivatization was allowed to proceed at $50\text{ }^{\circ}\text{C}$ for 30 min . During the procedure at 15 min , the derivatization agent was doubled. Splitless injections of $2\text{ }\mu\text{L}$ of the derivatization mixture were used for the development, validation and application of the method to real samples. The derivatization efficiency was evaluated from the ratio of peak relative areas of each compound adjusted to IS_2 to the adjusted relative peak areas of the standards.

Chromatography

Chromatographic separation of all compounds was achieved using Trace GC 2000 gas chromatographic equipment (Thermo Electro, San Jose, CA, USA). Samples ($2\text{ }\mu\text{L}$) were injected manually using a Hamilton syringe (Hamilton $10\text{ }\mu\text{L}$, Bonaduz, Switzerland) in splitless mode (1.0 min delay) into a crosslinked 5% diphenyl-dimethyl polysiloxane capillary column, $30\text{ m}\times 0.25\text{ mm I.D.}\times 0.25\text{ }\mu\text{m}$ film thickness (AT-MS5, Alltech Associates, Illinois, USA). The capillary column was connected with a universal connector to 2 m pre-column (crosslinked 5% diphenyl-dimethyl polysiloxane AT-MS5 capillary column of the same I.D. and film thickness (Alltech Associates, Illinois, USA).

The oven program was initially maintained at $75\text{ }^{\circ}\text{C}$ (hold for 1.5 min), afterwards it was programmed to initially increase temperature at a rate of $15\text{ }^{\circ}\text{C min}^{-1}$ to $135\text{ }^{\circ}\text{C}$ (hold for 2.0 min), increase further up to $138\text{ }^{\circ}\text{C}$ at a rate of $2\text{ }^{\circ}\text{C min}^{-1}$, then at a rate of $10\text{ }^{\circ}\text{C min}^{-1}$ up to $175\text{ }^{\circ}\text{C}$ (hold for 1.0 min), at a rate of $20\text{ }^{\circ}\text{C min}^{-1}$ up to $191\text{ }^{\circ}\text{C}$ and finally applying a rate of $25\text{ }^{\circ}\text{C min}^{-1}$ up to $300\text{ }^{\circ}\text{C}$ (hold for 4.0 min) in order to clean up the column from plasma residues. Helium (purity 99.999%) was used as a carrier gas at a constant flow of 1.0 mL min^{-1} . Injector temperature was adjusted to $200\text{ }^{\circ}\text{C}$, while the transfer-line temperature was set at $300\text{ }^{\circ}\text{C}$.

Mass spectrometry

Mass spectrometric analyses were performed on a GCQ Plus system (Thermo Electro, San Jose, CA, USA), interfaced with the Trace GC 2000, which consisted of a cylindrical ion trap (IT) mass spectrometric detector equipped with an electron impact (EI)/chemical ionization (CI) source. Full-scan and tandem mass spectral data were acquired in the positive ion mode at electron energy of 70 eV , in the mass range of $50\text{--}500\text{ Da}$. Ion source temperature was adjusted to $220\text{ }^{\circ}\text{C}$ and multiplier voltage to $1,500\text{ V}$. Delays of 6.00 and 6.60 min were programmed for the filament and multiplier in the acquisition of the full scan chromatographic and tandem mass spectra. Chromatographic retention time and full scan mass spectra were used for the identification of the compounds, while the most abundant ion from full-scan mass spectra was employed as a precursor ion for the quantitation tandem MS analyses. Quantitation of all compounds in the plasma matrix was performed using extracted ion chromatograms (XIC) of the one or two most

abundant ions from the respective full-scan MS/MS spectra. The trifluoroacetyl derivative molecular ion $[M^+]$, i.e. m/z 240 for 1N and 2N, m/z 274 for CFphenol-3-keto and m/z 317 for CF, was the precursor ion for the collision-induced dissociation (CID) process. For the rest of the compounds, the most abundant ion was used as the precursor ion: the $[M-CH_3CNO]^+$ ion, for CRB, 3HOFCF and CF-3-keto derivatives, at m/z 240, 372 and 274, respectively. For ISPR and CBXD derivatives, the $[M-CF_3]^+$ ion at m/z 217 and 238, respectively, was used, while for 7-phenol the $[M-CH_3]^+$ ion was used. The scanned mass range of the tandem MS spectra was set to 2 Da higher than the mass of the selected precursor ion. These mass spectral data are summarized in Table 1. For all compounds the excitation q value was 0.30, excitation time was set at 8 ms and isolation width at 1.00 min. Instrument was tuned twice every day during an operation of 12 h, once at the beginning of the daily analysis and once after 6 h of operation of the instrument. The acquisition system used was Xcalibur 1.2 software.

Method validation

Calibration equations were calculated by using the peak relative areas of the analytes adjusted by the internal standard of CBXD (IS₂). Analytical procedure was validated according to [29, 30]. Linear regression was used for the fitting of calibration equations for nine concentration points (ranging from 0.5–250 ng mL⁻¹) after sample pretreatment in fortified plasma samples with mixture of all the compounds. In the beginning of developing the method, the matrix effect was evaluated and was found to be inconsiderable, so mean recoveries from the ratio of the slope of the calibration curves of fortified plasma samples to standards' equations were calculated. Precision, accuracy, limit of detection (LOD) and quantification (LOQ) were calculated in fortified control plasma samples. Stock solution stability and post-preparative stability studies were conducted for the stock and working solutions, and the fortified samples respectively. For the validation of the

assay, three freeze-and-thaw cycles were conducted in two stages of sample analysis, at concentrations of 2.5, 25 and 100 ng mL⁻¹. RSD values for fortified samples before any preparation were found to be in the acceptable range of 0.4–18%, but chromatography proved bad for quantitative analysis. A freeze-and-thaw stability study of samples fortified after the deproteinization procedure showed great losses for hydroxy compounds. Short- and long-term stability studies were conducted for the fortified samples. RSD values were in the range of 1.1–12%, higher values have been noted at lower concentrations.

Results and discussion

General considerations of plasma sample deproteinization

Blood plasma is a complex mixture composed of 90% v/v water and 7% w/w heterogeneous proteins such as albumin, fibrin, globular proteins and hemoglobin. The rest of the plasma mixture contains inorganic electrolytes (salts), glucose, urea, amino acids, vitamins, hormones, fatty substances (lipids) and waste products of metabolism. Even though it is a highly aqueous solution, the amount of protein included causes serious problems in the isolation of compounds from the matrix. One of the most familiar problems is the binding of compounds with plasma proteins, which lowers the recovery efficiency. Precipitation techniques under strong acidic or alkali conditions, such as perchloric, hydrochloric, trichloroacetic or sodium hydroxide, or trimethylamine often release compounds from proteins (pH-dependent reactions) but very often this pH disruption of the sample can adversely affect the chemical stabilities of the compounds such as ureas, esters, glucuronides or carbamates [31]. Aggregation of plasma proteins can be achieved with addition of organic solvents such as methanol, acetone, AcN and others, but most of the time these methods provide low recoveries, and they lack precision. Other agents such as polyethylenglycol (PEG), which has shown considerable success in aggregation of proteins, is difficult to remove

Table 1 Tandem MS parameters for the TFAA derivatives of CF, CRB, their metabolites and two internal standards

| Compound | I.D. Number | Derivative M_r | Rt (min) | E.V. ^a | Range (m/z) | Precursor ion | Product ions ^b | Ions used for quantification |
|-----------------|--------------------|------------------|----------|-------------------|-------------|-----------------|---------------------------|------------------------------|
| CF | (1) | 317 | 14.57 | 1.15 | (110–320) | M^+ | 260*, 245, 147 | 260 |
| 7 phenol | (2) | 260 | 7.02 | 0.70 | (100–250) | $[M-CH_3]^+$ | 217, 199*, 175, 147 | 199, 175 |
| 3HOFCF | (3) | 429 | 14.80 | 0.80 | (135–375) | $[M-CH_3CNO]^+$ | 258, 243*, 144 | 258, 243 |
| CF-3-keto | (4) | 331 | 15.31 | 1.02 | (110–280) | $[M-CH_3CNO]^+$ | 273, 259*, 176, 148 | 273, 259 |
| CFphenol-3-keto | (5) | 274 | 7.92 | 0.95 | (100–280) | M^+ | 273, 259*, 176, 148 | 273, 259 |
| CRB | (6) | 297 | 16.05 | 1.00 | (100–245) | $[M-CH_3CNO]^+$ | 143*, 115, 240 | 143, 115 |
| 1N | (7) | 240 | 8.95 | 1.05 | (90–245) | M^+ | 143*, 115 | 143, 115 |
| 2N | (8) | 240 | 9.57 | 1.10 | (100–245) | M^+ | 143*, 115, 212 | 143, 115 |
| ISPR | (IS ₁) | 217 | 11.69 | 1.40 | (90–220) | $[M-CF_3]^+$ | 151*, 149, 199, 179 | 151, 149 |
| CBXD | (IS ₂) | 307 | 11.51 | 1.10 | (100–250) | $[M-CF_3]^+$ | 147*, 137 | 147, 137 |

^aExcitation voltage

^bIons marked with an *asterisk* are the most abundant ones

from the samples [32]. Denaturation techniques such as temperature effect, mechanist energy or low salt concentrations rarely find application in analytical techniques since most compounds are adversely affected. Proteolysis and fibrinolysis are two enzymatic procedures (serine, cysteine, aspartic or metallo proteinases) commonly used in proteomics and biotechnology, but so far these techniques have not been investigated in methods for the determination of smaller-molecular-weight compounds, except for a reported method for the determination of polychlorobiphenyls (PCBs) in blood [33].

In the proposed method for determining CF, CRB and their keto- and phenol-type metabolites (compounds that are easily adversely disrupted by light, heat and pH-dependent effects), several of the above-mentioned techniques were investigated, in order to achieve a cleaner sample from the proteins (Table 2). The effectiveness of each method was calculated using the ratio of peak area ratio of each compound vs. that of IS₁ after the sample treatment to the peak area ratio of the standard vs. that of IS₁ under the same pretreatment methods.

Addition of trifluoroacetic acid (TFA) or acetic acid (CH₃COOH) and formic acid (HCOOH) induced the hydrolysis of CRB into its main metabolite 1N. Strong alkali conditions with addition of NaOH or TEA resulted in the breakdown of carbamate linkage for CF and CF-3-keto, which respectively yielded 7-phenol and CFphenol-3-keto. Addition of methanol (MeOH), acetonitrile (AcN) and ethylacetate (AcEt) in quantities of 10, 50, and 100 µL, respectively, were successful in precipitating proteins, but recoveries of our compounds ranged from 0–45%. Even if it is not proven that the compounds produce complexes with the plasma proteins causing their aggregation, it is known that organic-aqueous conditions disrupt the stability of carbamates [34]. It should be taken into consideration that the organic solvent added to a plasma sample could elute or disrupt the compounds during the solid-phase extraction procedure. On the other hand, saturated salts such as (NH₄)₂SO₄ or Na₂SO₄ resulted in very poor recoveries, while during pretreatment, samples produced emulsions.

Dilutions (1:1) of plasma samples with ammonium acetate buffer (pH=4.4, 6.0 and 7.0) at 0.1 and 1 M were tested, but proteins were not satisfactorily removed from the sample during the solid-phase extraction procedure, and recovery efficiencies were low. Our efforts to denature the plasma proteins did not include the temperature effects of heating the sample, since carbamates in aquatic solutions degrade at temperatures higher than 37 °C. Detergents such as sodium dodecyl sulfate (SDS) were also tried out with no success.

In this work, proteolysis/fibrinolysis of plasma proteins using proteinase K (PK), a non-specific serine protease was investigated. PK proved to be one of the best agents in cleaning the plasma samples of proteins. We investigated its proteolytic activity in comparison to recoveries of our method after SPE and derivatization procedures. Several conditions were studied, such as temperature, time of digestion, pH, activity (U/200 µL of plasma) and mixtures with other agents (Table 2). Conditions such as high temperatures

for short periods did not affect our compounds. Using 0.15 U of PK in 200 µL of fortified control plasma sample in the presence of 100 µL acetate buffer (0.01 M, pH=4.4) resulted in recoveries for 9 out of 10 compounds ranging from 71–107%. The disadvantage of this method was that CF-3-keto was irreversibly disrupted by the enzyme and recovery of CF-3-keto was only 5%, as shown in Table 2, so this technique could not be applied in our method.

Among the familiar techniques employed for precipitation, denaturation and digestion, a combination of two mild agents, one for precipitation and one for denaturation of proteins, was applied. Small amounts of THF were added as precipitating agent. THF could not exceed 10% v/v of the plasma sample, and afterwards we used β-mercaptoethanol (βME) to reduce the disulfide bonds of cross-linked proteins. Volume percentage of βME was investigated since at high concentrations our phenol-type compounds were affected, and the selected one was 2.6 M. Along with βME, ascorbic acid (AA) (mild reductant) was added at 3.3% v/v. Concentrations of these two mild agents (βME and AA) were investigated in volumes of plasma samples larger than 200 µL, and results were negative.

Dilution of plasma samples with potassium phosphate buffer at 0.01, 0.1 and 1 M (1:1 v/v and 1:2 v/v) before sample treatment in order to stabilize pH of the sample lowered our recoveries to 20–50% for all compounds. pH of plasma samples after centrifugation in EDTA Vacutainer was found to be 9.50±0.12 for all measured samples.

Solid-phase extraction

Solid-phase extraction (SPE) technique has been widely used for carbamate isolation, concentration and purification from many different samples such as water [35, 36], soil [37], malt beverages [38], fruits and vegetables [39, 40] and honey [41]. SPE has also been applied to carbamate isolations from biological matrices such as urine [16, 23, 24], blood [25, 27, 42] and tissues or saliva [26] over the past years, while several different cartridge packing materials like aminopropyl [23, 43, 44], cyano [45], carbon [46] silica [45, 47] and polymeric [25, 27, 48, 49] have been investigated. Polymer-based SPE OASIS HLB cartridges have been primarily used in multi-residue samples and in difficult matrices such as biological ones, because of two key parameters of the polymer: the ability to remain wet during the procedure and the ability to retain or absorb analytes, increasing recovery efficiencies [50].

The proposed method for isolating the analytes from plasma supernatants employed OASIS HLB cartridges and has been optimized in nine steps: *equilibration*: 1 mL methanol followed by 1 mL of potassium phosphate buffer, *loading*: approximately 230 µL supernatant solution of fortified plasma sample after protein removal pretreatment, *first wash*: 1 mL potassium phosphate buffer (0.010 M, pH=7.01), *second wash*: 1 mL 5% v/v aqueous solution of NH₄OH (1.00 M), *third wash*: 1 mL potassium phosphate buffer (0.010 M, pH=7.01), *elution*: 2 mL (2×1 mL) diethylether, *evaporation*: under gentle nitrogen steam at

Table 2 Recoveries (% , single runs), for the eight compounds from fortified plasma samples at 50 ng mL⁻¹ under different precipitation conditions with protease PK and different chemical reagents

| PK | Conditions | Agent | Concentration | Volume, μL | pH | CF | 7-phenol | 3HO CF | CF-3- keto | CFphenol- 3-keto | CRB | 1N | 2N |
|-------|--------------------|-----------------------------------|-------------------|---------------|-----|-----|----------|--------|---------------|---------------------|-----|-----|-----|
| Units | Temp (°C), time | | | | | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) |
| 1.2 | 37, 16 h | | | | | 105 | 203 | 35 | 16 | 171 | 126 | 170 | 171 |
| 0.6 | 37, 16 h | | | | | 116 | 160 | 63 | 5 | 130 | 150 | 130 | 156 |
| 0.15 | 60, 90 min | | | | | 38 | 165 | 81 | 9 | 124 | 78 | 205 | 240 |
| 0.6 | 37, 16 h | (NH ₄)SO ₄ | Saturated | 80 | 6.5 | 134 | 78 | 104 | 1 | 178 | 107 | 170 | 370 |
| 3.0 | 37, 16 h | SDS | 1% | 200 | 6.0 | 60 | 270 | 36 | 2 | 159 | 39 | 289 | 64 |
| 0.6 | 37, 16 h | SDS | 1% | 80 | 6.0 | 112 | 118 | 25 | 13 | 120 | 154 | 197 | 210 |
| 0.6 | 37, 16 h | SDS | 1% | 200 | 6.0 | 86 | 80 | 64 | 5 | 150 | 73 | 124 | 280 |
| 0.3 | 37, 16 h | SDS | 1% | 200 | 6.0 | 94 | 109 | 84 | 5 | 128 | 78 | 364 | 82 |
| 0.3 | 65, 30 min | SDS | 1% | 200 | 6.0 | 106 | 148 | 74 | 1.3 | 144 | 88 | 261 | 82 |
| 0.3 | 65, 30 min | SDS | 1% | 800 | 6.0 | 83 | 218 | 44 | 0.7 | 83 | 68 | 173 | 60 |
| 0.6 | 37, 16 h | Acet. buffer | 0.1 M | 80 | 4.4 | 113 | 254 | 66 | 180 | 75 | 114 | 216 | 57 |
| 0.3 | 37, 16 h | Acet. buffer SDS | 0.1 M | 10 | 4.4 | 69 | 200 | 58 | 19 | 86 | 88 | 142 | 54 |
| | | | 1% | 200 | 6.0 | | | | | | | | |
| 0.15 | 37, 16 h | Acet. buffer | 0.1 M | 50 | 4.4 | 140 | 60 | 147 | 109 | 104 | 103 | 154 | 45 |
| 0.15 | 37, 16 h | Acet. buffer | 0.1 M | 100 | 4.4 | 126 | 49 | 87 | 176 | 99 | 119 | 254 | 38 |
| 0.15 | 37, 16 h | Acet. buffer | 0.1 M | 200 | 4.4 | 78 | 43 | 44 | 113 | 81 | 68 | 231 | 28 |
| 0.15 | 37, 16 h | Acet. buffer | 0.1 M | 400 | 6.0 | 94 | 119 | 60 | 5 | 230 | 54 | 259 | 50 |
| 0.15 | 37, 16 h | Acet. buffer | 0.01 M | 100 | 4.4 | 93 | 75 | 97 | 4 | 71 | 107 | 106 | 100 |
| | | Acet. buffer | 0.1 M | 200 | 5.0 | 62 | 42 | 37 | 101 | 51 | 70 | 106 | 83 |
| | | Acet. buffer | 0.1 M | 100 | 6.0 | 54 | 91 | 77 | 49 | 65 | 29 | 246 | 0 |
| | | Acet. buffer | 0.1 M | 200 | 6.0 | 23 | 50 | 39 | 15 | 86 | 95 | 124 | 0 |
| | | Acet. buffer | 0.1 M | 200 | 7.0 | 51 | 51 | 21 | 4 | 79 | 70 | 153 | 107 |
| | | βME, AA | 200 mM, 1%, | 20, 300 | | 85 | 19 | 62 | 124 | 68 | 86 | 63 | 3 |
| | | βME, AA | 200 mM, 10% | 10, 154 | | 57 | 100 | 55 | 66 | 67 | 44 | 256 | 8 |
| | | βME, AA, acet. buffer | 200 mM, 1%, 0.1 M | 20, 20, 20 | 4.4 | 98 | 106 | 68 | 96 | 74 | 73 | 0 | 0 |
| | | βME, AA, acet. buffer | 200 mM, 1%, 0.1 M | 20, 200, 20 | 6.0 | 105 | 141 | 66 | 109 | 139 | 87 | 263 | 0 |
| | | βME, AA, SDS | 200 mM, 1%, 1% | 20, 20, 200 | | 91 | 133 | 68 | 79 | 124 | 91 | 253 | 0 |
| | | βME, AA | 200 mM, 10% | 5, 300 | | 122 | 1 | 114 | 95 | 25 | 103 | 95 | 50 |
| | | βME, AA | 200 mM, 10% | 5, 150 | | 168 | 13 | 107 | 139 | 53 | 146 | 110 | 57 |
| | | βME, AA | 200 mM, 10% | 5, 100 | | 106 | 109 | 78 | 112 | 104 | 101 | 120 | 108 |
| | | βME, AA | 200 mM, 10% | 2.5, 150 | | 107 | 114 | 100 | 108 | 107 | 116 | 87 | 89 |
| | | βME, acet. buffer | 200 mM, 0.01 M | 5, 300 | | 106 | 37 | 87 | 55 | 66 | 84 | 212 | 56 |
| | | βME, acet. buffer | 200 mM, 0.01 M | 5, 90 | | 101 | 95 | 107 | 71 | 72 | 87 | 127 | 99 |

Other conditions as described in text

room temperature at an approximate volume of 150 μL, *evaporation to dryness*: transfer into smaller volume Eppendorf (as described earlier) and evaporate to dryness, *IS addition*: fortification with IS₂ in the amount of 40 ng and evaporate to dryness.

During equilibration, and first and third washing steps of the extraction procedure, the water and 5% methanol suggested by the manufacturer were replaced with potassium phosphate buffer with constant pH value, since several percentages of methanol tested (0.02, 0.1, 0.5 and 1.0%) did increase the efficacy of the cleaning procedure, but they also simultaneously removed our analytes from the cartridge, decreasing the recoveries in most times to

0%. An extra washing step with acetate buffer (0.1 M, pH=5.0) to remove basic interferences increased the analyte recoveries (to 20%) for all the hydroxyl group compounds formed, but CF and CRB efficiencies were 50–70% lower.

Ammonium hydroxide (5% v/v) in water was used to elute acidic interferences during the second washing step, increasing the retention of basic interferences. Basic forms of our hydroxyl-group compounds probably imposed the need for the third step in order to reverse our basic forms to neutral in order to be eluted by the non-polar organic agent. It has been hypothesized that parent compounds of CF and CRB are not affected as they are relatively neutral.

Derivatization

The general strategy for optimization of the derivatization procedure needed in order to increase carbamate instability at higher temperatures and transform polar metabolites (hydroxyl group compounds) into thermolabile compounds with better chromatographic behavior included the following: (1) Find an appropriate solvent for the reaction, (2) select the appropriate derivatization agent, (3) select a base as catalyst as described in the literature [51] and test it with the solvents of the first and second steps, (4) derivatize all compounds separately, and (5) optimize the derivatization in terms of volumes, reaction time and temperature.

Solvents investigated for the optimization of the derivatization reaction were methanol, acetone, ethyl acetate (AcEt), AcN, THF and water. THF was proven to be the most suitable solvent. Solvents like water and methanol did not dissolve all the compounds so they were rejected. AcEt and AcN affected the derivatization reaction of CF-3-keto. Different bases have been tested as catalysts according to the literature, including pyrimidine, NaOH and TEA. Strong bases affected carbamates including those with a high percentage of suitable base and TEA. After careful evaluation a percentage of 0.02% of TEA in THF was selected as a catalyst. Following optimization of the derivatization agent solution with TFAA as derivatization agent [52], the reaction time and temperature were tested. Best derivatization efficiencies were achieved at 50 °C for 30 min. It should be noted that high temperatures above 60 °C (the boiling point of THF) were not tested. Derivatization procedure for longer than 2 h at lower temperatures such as 40 or 50 °C resulted in lower efficiencies than the selected ones. Volume of the derivatization reaction proved to be important. Time of derivatization was not enough for the mixture to be evaporated, but doubling the volume of the derivatization agent from approximately 30 to 60 μL , raised the reaction yield more than 50%, especially for the compounds with the carbamate linkage. A fraction (2 μL) of the approximately 60 μL of the derivatization solvent was used for the splitless injections.

GC-MS/MS analysis

The total ion chromatogram (TIC) and the respective extracted ion chromatograms (XICs) for the compounds' TFAA derivatives in a fortified control plasma sample are shown in Fig. 3a. The product ion mass spectra of the aforementioned derivatives are shown in Fig. 3b. The precursor ion fragmentation was optimized in order to yield specific ions for each compound, while maintaining 5–10% of the precursor ion's relative abundance. The selected values of the excitation voltages for the tandem MS analysis are shown in Table 1, along with the ions employed for the quantification. Among the extended fragmentation shown in the tandem mass spectra of the TFAA derivatives (Table 1), the two most abundant product ions were employed in order to achieve low LODs. The selected precursor ion was either the molecular

ion or an abundant fragment ion arising from the neutral loss of CH_3 , CF_3 or the N-methylisocyanate moiety, as has been described in detail for each derivatized compound in the [Experimental](#) section. It should be noted that the selected product ions for quantification (Table 1) were observed in the full-scan EI mass spectra as well, but their abundance in the tandem mass spectra was enhanced following the collision-induced dissociation process. In the full-scan mass spectrum of the CBXD (IS_2) there were signals at m/z 307 and 238 corresponding to the molecular ion and loss of CF_3 , respectively, while its tandem mass spectrum contained additional fragment ions at m/z 147 and 137 (Table 1). Occasional loss of the methyl group and neutral loss of a CO molecule were also observed in the tandem mass spectra. Both internal standards exhibited the same fragmentation pathways, since both were chosen to have alkyl-substituted nitrogen in an amide bond.

Method validation

Specificity of the proposed method was not only taken into consideration as a validation parameter. It was initially considered for the applicability of the method to real biological monitoring studies, and as mentioned earlier, more metabolites of CF than 7-phenol (carbofuran phenol) and the metabolite 2N of naphthalene were included for determination. As a validation parameter to be defined, specificity was achieved for each compound from the specific fragment ions of each full-scan tandem mass spectra and their characteristic relative abundances.

Stock solution stability for all compounds in the polar solvent of THF was investigated in order to avoid random errors in our measurements. Stored solutions of 1N and 2N in deep-freezing conditions below -30 °C were stable for 3 months at a time in concentrations above 50 and 200 ng mL^{-1} , respectively. The rest of the compounds were found to be stable under the same conditions for more than 6 months. When stock solutions were stored at 4 °C in dark-colored vials, they were stable for more than 3 months except the solutions of 1N and 2N, which were found to be stable under these conditions only for 1 week.

Post-preparative stability studies were conducted for three fortified samples at 2.5, 25 and 100 ng mL^{-1} . The post-preparative study was evaluated for 0.5 day and 1 day of analysis time, and the results showed that the samples could not be analyzed after 2 h at room temperature. Sample baselines were prohibitive for quantitation assays, especially at the lower concentrations. In contrast, short- and long-term stability studies (on the same day of analysis) at the same three concentrations suggested that samples that are being fortified after the sample preparation provide measurements with accepted accuracy and precision ($\text{RSD} < 18\%$), which can be misleading to some assay development.

During sample analysis, three freeze-and-thaw cycles were conducted in two stages. Three concentrations of fortified samples at 2.5, 25 and 100 ng mL^{-1} were prepared before sample preparation procedures occurred, and three more fortified samples at the same concentration levels were

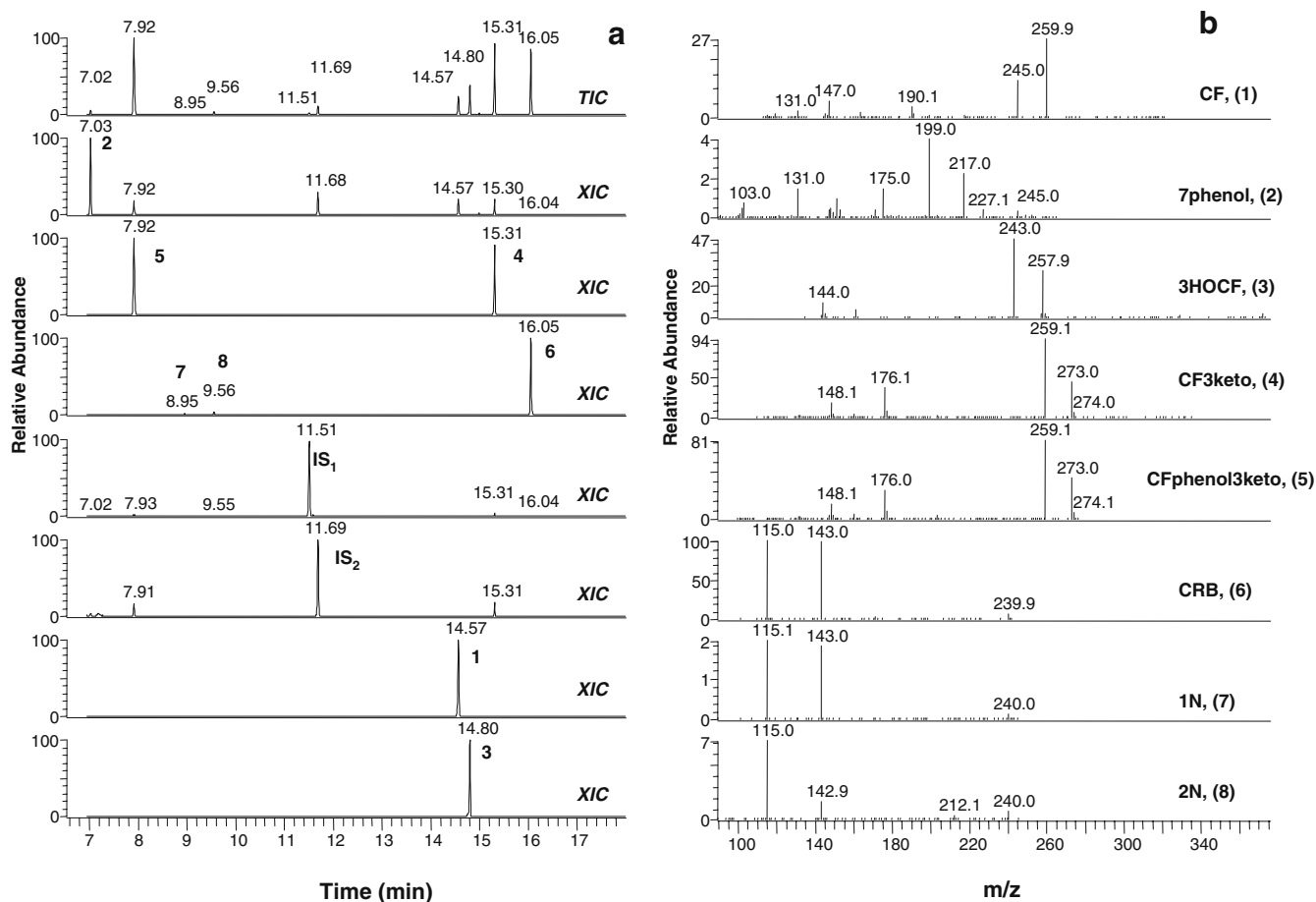


Fig. 3 a TIC and XIC of CF, CRB and their metabolites in fortified control plasma sample at the 50 ng mL⁻¹ level after GC-MS/MS analysis. b Product ion mass spectra of the derivatized compounds

prepared after the deproteinization procedure. The first procedure proved that after the third cycle only 3.0–8.0% loss was calculated for all compounds, but chromatography was extremely poor for quantitative analysis. During the second procedure hydroxy compounds were unstable and experienced 60–100% loss. SPE cartridges could not be frozen after the SPE procedure since losses of 20–100% were noticed for all compounds.

Calibration equations were calculated for nine different concentration levels for all compounds in fortified control plasma specimens (0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100 and 250 ng mL⁻¹), plotted against the corrected peak areas to IS₂. Three sets ($n=3$) for each concentration level were determined, and calibration equations were repeated on 3 different days. It should be noted that the linear regression model used was not weighted and the (0,0) point was neither included nor forced. The same procedure was followed for the calculated calibration equations from standard solutions, for the same linear range for one set of concentrations. Linear regression analyses provided slopes and intercepts (Table 3) from which concentrations of unknown samples could be determined, while the matrix effect could be estimated by comparing slopes and intercepts

normalized to CRB fragment ions. MS conditions: ion source temperature 220 °C, multiplier set at 1,500 V, excitation q 0.30, excitation time 8 ms, mass range 50–500 Da

of calibration curves of fortified samples to slopes and intercepts from the calibration curves of standards.

Since the matrix effect was found to be inconsiderable, recoveries for all compounds were determined from the ratio of the slopes from calibration curves of fortified control samples to standard samples. The calculated recoveries are shown in Table 3.

Sensitivity of the proposed method was evaluated from the analytical LOD, determined as $3s$, where s was defined as signal to noise (S/N) ratio in a fortified control plasma sample at the level of 0.5 ng mL⁻¹. Experimentally determined LOD in fortified plasma samples for all compounds was 0.1 ng mL⁻¹.

LOQ was determined as $10s$, but since it was much lower than the lowest concentration level of the calibration curves, we considered LOQ to be the lowest concentration level, 0.5 ng mL⁻¹ for all compounds.

The method accuracy was evaluated at three different concentration levels of 8, 80 and 160 ng mL⁻¹ for each compound, calculated as the percent systematic error (% E_r), defined as the agreement between the experimental (measured) arithmetic mean concentration value and the nominal (true) concentration value of the determinants in fortified drug-free control plasma samples. Accuracy data

Table 3 Validation parameters of linearity ($y=ax+b$), sensitivity and recoveries from fortified control plasma samples and standards

| Compound ID number | CF (1) | 7-phenol (2) | 3HO CF (3) | CF-3-keto (4) | CFphenol-3-keto (5) | CRB (6) | 1N (7) | 2N (8) |
|-------------------------------------------------------------|-----------|-----------------|---------------|------------------|------------------------|------------|-----------|-----------|
| Linearity, slope (a) (fortified samples) | 0.044 | 0.015 | 0.062 | 0.126 | 0.140 | 0.296 | 0.061 | 0.162 |
| Linearity, intercept (b) (fortified samples) | -0.086 | +0.110 | -0.155 | +0.630 | -0.429 | -0.911 | +0.240 | -0.883 |
| Coefficient of correlation (R^2) | 0.996 | 0.994 | 0.997 | 0.997 | 0.995 | 0.998 | 0.994 | 0.989 |
| LOD (ng mL ⁻¹) | 0.028 | 0.075 | 0.068 | 0.151 | 0.029 | 0.034 | 0.015 | 0.016 |
| LOQ (ng mL ⁻¹) | 0.094 | 0.250 | 0.225 | 0.503 | 0.095 | 0.115 | 0.050 | 0.053 |
| Linearity, slope (a) (standard solution) | 0.041 | 0.015 | 0.074 | 0.118 | 0.161 | 0.284 | 0.075 | 0.187 |
| Linearity, intercept (b) (standard solution) | +0.050 | +0.074 | +0.241 | +0.290 | -0.558 | +0.153 | -0.125 | -0.104 |
| Coefficient of correlation (R^2) | 0.999 | 0.989 | 0.997 | 0.998 | 0.999 | 0.999 | 0.991 | 0.988 |
| Recovery (%) (slope of fortified/slope of standard samples) | 107% | 96% | 84% | 107% | 87% | 104% | 81% | 87% |

Linearity range for all compounds was 0.5–250 ng mL⁻¹ for nine concentrations, ($n=9 \times 3=27$)

were determined in three replicates for each concentration on the same day of analysis.

Intra- and inter-day precision values were evaluated for three replicate sets at three concentration levels of the calibration curves for each compound (2.5, 25 and 100 ng mL⁻¹). Intra- and inter-day precision levels were defined by the relative standard deviation percentage and are summarized in Table 4, among with the accuracy % E_r .

Application to pesticide users

We have applied the proposed method to determine CF, CRB and their possible metabolites in plasma samples of five pest-control applicators and five non-occupationally-exposed humans of the same area (baseline area), while more samples are being scheduled to be measured in the near future. Different unspiked plasma samples ($n=40$) of people living in the urban area of Athens were investigated for pesticides and were found negative. For method development and validation, one control plasma from an urban citizen of Athens city, randomly selected, was used. Different unfortified control samples were measured in

between sample analyses to control for a possible column contamination.

Each farmer provided three blood samples: one before any pesticide use, one after CF application and one after CRB application. Representative XICs of an unfortified control plasma sample and two positive plasma samples of two different people during CF application and CRB application are reported in Fig. 4a–c.

In baseline samples none of the compounds was detected, indicating that there was no exposure to these particular pesticides or their metabolites (through inhalation, water or food consumption) in general in the area of investigation. CF, 7-phenol, 3HO CF, CF-3-keto, and CFphenol-3-keto were detected in 60, 100, 100, 20 and 80% of the samples, respectively (Table 5). Not all of CF metabolites were detected in all subjects, and CF was found in levels higher than LOQ in only one sample. Even though 3HO CF and 7-phenol were detected in all samples, they could not be quantitated in two samples (levels lower than LOQ). Any correlations investigated among people and their levels of detected metabolites proved unsuccessful.

CRB was detected at quantitative levels in all five farmers. 1N was detected in three samples but could be determined in only two samples. 2N was determined in three

Table 4 Validation parameters of inter-, intra-day precision (%RSD) and accuracy (% E_r) in fortified control plasma samples

| Compounds ID number | CF (1) | 7-phenol (2) | 3HO CF (3) | CF-3-keto (4) | CFphenol-3-keto (5) | CRB (6) | 1N (7) | 2N (8) |
|------------------------------|-----------|-----------------|---------------|------------------|------------------------|------------|-----------|-----------|
| Intra-day, RSD (%) ($n=3$) | | | | | | | | |
| 2.5 ng mL ⁻¹ | 15.0 | 3.4 | 5.6 | 9.0 | 9.1 | 6.9 | 12.0 | 15.0 |
| 25 ng mL ⁻¹ | 13.0 | 9.0 | 0.7 | 2.9 | 6.3 | 7.8 | 3.7 | 17.0 |
| 100 ng mL ⁻¹ | 14.0 | 5.2 | 11.0 | 9.3 | 9.3 | 12.0 | 0.4 | 6.1 |
| Inter-day, RSD (%) ($n=3$) | | | | | | | | |
| 2.5 ng mL ⁻¹ | 7.3 | 11.0 | 11.0 | 11.0 | 6.1 | 17.0 | 7.0 | 12.0 |
| 25 ng mL ⁻¹ | 9.1 | 3.2 | 7.3 | 12.0 | 8.6 | 9.7 | 9.9 | 11.0 |
| 100 ng mL ⁻¹ | 12.0 | 5.3 | 9.0 | 5.4 | 12.0 | 1.7 | 11.0 | 8.7 |
| Accuracy (% E_r) | | | | | | | | |
| 8 ng mL ⁻¹ | -3.0 | -12.0 | 6.0 | 12.0 | -9.0 | 10.0 | 6.5 | 4.4 |
| 80 ng mL ⁻¹ | -2.7 | -5.4 | 5.0 | -7.0 | 3.0 | 13.0 | 3.8 | 3.9 |
| 160 ng mL ⁻¹ | -1.5 | -7.2 | 15.0 | 6.0 | 1.0 | 9.0 | 2.9 | 1.9 |

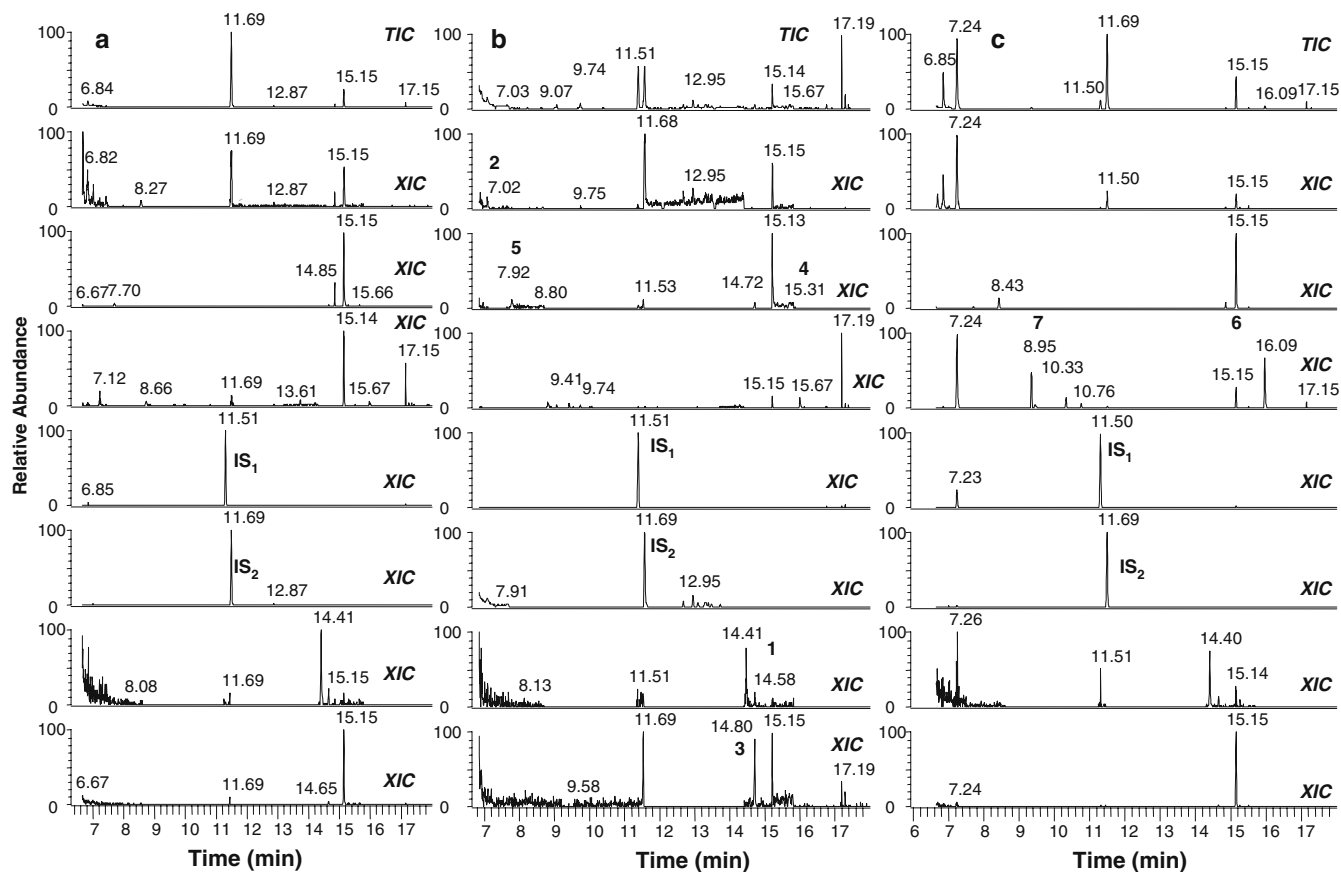


Fig. 4 TIC and XIC chromatograms from the GC-MS/MS analysis of **a** control plasma fortified with two internal standards of ISPR (IS₁) and CBXD (IS₂), **b** plasma sample from a CF applicator, and **c** plasma sample from a CRB applicator

samples as well, but it was not correlated to smoking habits of the subjects and no research has been reported yet on detectable levels of 2N in plasma samples from passive smoking, therefore these data can not be further evaluated (Table 5).

Conclusions

The aim of this research was to develop, validate and apply a sensitive and specific analytical method for the determination of CF, CRB and their main metabolites (7-phenol, 3HOFC, CFphenol-3-keto, CF-3-keto and 1N) in human

plasma. The proposed method employs a simple protein-removal pretreatment of samples and the isolation of the compounds by the means of a solid-phase extraction technique based on OASIS HLB cartridges. One internal standard was used to optimize the extraction efficiency of each compound and one to optimize derivatization technique and calculate validation parameters. Validation parameters complied with the international criteria for method development and validation in biological fluids.

The method has been applied to a suitable number of samples from pesticide users and urban area citizens in order to confirm the applicability and usefulness of our method, especially in the area of biomarker research and biomonitor-

Table 5 Concentrations of CF, CRB and their metabolites in five pesticide-user plasma samples during CF application (April 2004) and CRB application (July 2004)

| ID | CF (1) ng mL ⁻¹ | 7-phenol (2) ng mL ⁻¹ | 3HOFC (3) ng mL ⁻¹ | CF-3-keto (4) ng mL ⁻¹ | Cfphenol-3-keto (5) ng mL ⁻¹ | CRB (6) ng mL ⁻¹ | 1N (7) ng mL ⁻¹ | 2N (8) ng mL ⁻¹ |
|----------|----------------------------------|----------------------------------------|-------------------------------------|-----------------------------------------|-----------------------------------------------|-----------------------------------|----------------------------------|----------------------------------|
| Farmer 1 | ND | 16.2 | 1.38 | ND | 1.02 | 1.22 | ND | 5.60 |
| Farmer 2 | <LOQ | <LOQ | <LOQ | ND | ND | 4.96 | 14.4 | ND |
| Farmer 3 | ND | 22.9 | <LOQ | <LOQ | 1.11 | 5.70 | 3.28 | 9.43 |
| Farmer 4 | 3.04 | <LOQ | 5.67 | ND | 1.07 | 1.23 | ND | ND |
| Farmer 5 | <LOQ | 14.1 | 1.80 | ND | 1.17 | 1.39 | <LOQ | 5.50 |

LOQ Limit of quantification, ND not detected

ing studies such as low-, medium-, high-level ones, and incidental, residential or occupational research. Public health researchers rely on these two fields in order to gain reliable exposure data for preventing health effects and disorders related to xenobiotic substances such as pesticides.

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