Research Article

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Investigating a human pesticide intoxication incident: The importance of robust analytical approaches

<https://doi.org/10.1515/chem-2021-0193>

received November 3, 2020; accepted December 22, 2020

Abstract: A human intoxication incident attributed to pesticide abuse was investigated using cutting-edge analytical methodologies. An LC-ESI-MS/MS method, based on a hybrid solid-phase extraction protocol (hybrid-SPE), was applied for the detection and quantification of several pesticides and metabolites in human biological fluids. Concomitantly, an UHPLC-HRMS method was applied to investigate potential metabolites, assisted by a complementary GC-MS method to elucidate the presence of plausible pesticides co-formulants. The LC-ESI-MS/MS method exhibited acceptable mean recoveries at the lower limit of quantification (LLOQ) and three additional levels, varying from 85 to 106% for all analytes and matrices. In serum, urine, and gastric fluid samples, the suspect compounds, namely chlorpyrifos and myclobutanil, predominated. Gastric fluid samples contained the highest concentrations of chlorpyrifos (39,800 ng/mL) and myclobutanil (18,800 ng/mL), while the neonicotinoid imidacloprid was also quantified, below 30 ng/mL.

Notwithstanding, the UHPLC-HRMS analysis unveiled several metabolites of chlorpyrifos and myclobutanil. In parallel, GC-MS analysis, corroborated the presence of several co-formulants in gastric fluid samples, exemplified by *m*- and *o*-xylene, and cyclohexanone. Overall, three analytical methods were implemented to elucidate the chemical causality of a human intoxication incident. The presence of suspected active substances, one additional, and several metabolites and co-formulants were documented.

Keywords: pesticides, metabolites, LC-ESI-MS/MS, UHPLC-HRMS, biological fluids

1 Introduction

The pesticide intoxication incidents are reported worldwide and hold a significant share among human poisoning cases, especially in developing countries [1]. It is estimated that over 150,000 people die each year due to pesticide intoxication, despite the significant efforts toward pesticide restriction policies related to poisoning [2,3]. Suicide attempts usually by ingestion of pesticide formulations are observed, in particular, in rural communities. The main route of accidental or intentional acute pesticide poisoning is through ingestion and secondarily by inhalation of vapors. Poisoning incidents through dermal exposure to pesticides, including those with a fatal outcome, have been reported [4], but their contribution to the overall percentage is by far lower and usually occur when personal protective equipment is not at all or properly used by those involved in agricultural activities [5]. In addition, intoxication via contaminated food consumption should not be neglected [6].

Currently, exploitation of diagnostic biomarkers such as chemical markers in biofluids is sporadic leading to a knowledge gap on the record of human pesticides intoxication cases, thereby diminishing their importance and

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projection. If this knowledge gap was to be covered it could be considered as the scientific basis to minimize and prevent poisoning incidents through raising awareness activities, especially in rural communities and triggering the need for development of appropriate legislation aimed at restricting access of the general public to highly hazardous pesticides [7].

The European Commission has published a notice on the guidance for monitoring and surveying the impact of pesticide use on human health and the environment, which designates the necessity of robust biomonitoring data [8] covering aspects from human cohort studies to isolated intoxication incidents. Therefore, scientific data derived from incident reports, which not only include the description of the case, but also chemical analytical data, are of utmost importance. Among the pesticide superfamily, organophosphorus (OP) compounds are the predominant culprits behind most of these intoxications, even though the last decade measures have been taken to substitute highly toxic OPs with safer ones. Despite the efforts, banned pesticides (including some OPs) are still reported in European poison control centres [9,10].

Considering the extensive and sometimes rapid *in vivo* metabolism that several active substances undergo, and the toxic profile of metabolic products, it is imperative to include both the parent compounds and the potential metabolites in the analytical methods. Nevertheless, and since it is practically impossible to acquire all analytical standards even if they are available, the non-targeted analytical methods and metabolomics parallel elucidation of xenobiotics and metabolites have emerged as a pivotal tool in the investigation of such incidents, and the overall metabolic profiling of biological fluids [11,12]. Last but not least, the contingent presence of non-active ingredients of pesticide formulations, namely co-formulants, such as solvents, stabilizers, etc., in biological fluids also needs to be investigated. The latter stems from reports that documented an irritative potential or potentiation of toxicity of pesticides, such as OPs, or the known herbicide glyphosate in the presence of such chemicals [13–15].

In this work, we present the investigation of a pesticide intoxication incident, in which an 86-year-old female was hospitalized after suspected use of pesticides' formulations. A modern and universal sample preparation coupled with targeted LC-ESI-MS/MS and non-targeted UHPLC-HRMS analyses was implemented, managing to detect and quantify the alleged active substances and identify their metabolites in biological fluids. Finally, GC-MS analysis disclosed the presence of several co-formulants.

2 Materials and methods

An 86-year-old female was admitted to the hospital on day 1 (16 Oct 2019) after reportedly receiving organophosphates (chlorpyrifos) and an additional formulation with potential active ingredient myclobutanil (triazole fungicide). She had an altered level of consciousness and was gasping for air. She was intubated at the emergency department, and mechanical ventilation was initiated. Soon after the intubation, the patient went into cardiac arrest and was resuscitated immediately. After receiving vasopressors and pralidoxime, she was then transferred to the intensive care unit (ICU). On day 2, she received charcoal. The patient demonstrated a gradual improvement in her level of consciousness and her muscular strength. She did not demonstrate any evidence of miosis, bradycardia, salivation, nor did she need any atropine. Based on the above observation and after contacting poison control center, the infusion of pralidoxime was discontinued on day 3. In the evening of day 4, the patient demonstrated agitation and the next morning she had a sudden change in her level of consciousness along with bradycardia and evidence of miosis on physical examination. She gradually received 6 amps of intravenous atropine without a response, and the pralidoxime drip was restarted. This resulted in an improvement in her clinical condition later at the same night. Finally, at day 15, she was discharged upon further improvement.

2.1 Materials–chemicals

Certified chemical standards were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Sigma Aldrich (Seelze, Germany). Mass-labelled internal standards (imidacloprid-d4, chlorpyrifos d-10, carbendazim-d3, and dimethoate d-6) used for the quantification of the target analytes in the LC-ESI-MS/MS method were obtained from Sigma Aldrich (Seelze, Germany).

Standard solutions were prepared in acetonitrile and methanol in volumetric flasks and kept at -18°C away from the light. Acetonitrile, methanol, ammonium formate, and formic acid of LC-MS grade were obtained from Fisher Scientific (Hampton, NH, USA). Dichloromethane (DCM) was of pesticide residues grade and was obtained from Sigma Aldrich (Seelze, Germany). Sodium sulfate anhydrous (Na_2SO_4), and sulfuric acid (purity >95%) were obtained from Fisher Scientific. Hybrid SPE–phospholipid cartridges (30 mg, 1 mL) were purchased from Supelco (Bellefonte, PA, USA). All reagents and chemicals were of analytical grade.

2.2 Samples

Serum and urine used as control samples were obtained from the stock reserved at Benaki Phytopathological Institute for biomonitoring studies performed *in-house*. With regard to real samples analyzed in this study, urine samples collected at day 1 (no 1) and day 7 (no 2), blood and gastric fluid samples (before (no 1) and after (no 2) gastric lavage) that had been collected during day 1 (all samples were kept in freezer) were sent to the analytical laboratory at day 7.

2.3 Liquid chromatography

2.3.1 Sample preparation

Sample preparation was envisaged by the work of Honda et al. [16]. Briefly, serum, urine, and gastric fluid (after being stored at -20°C) were thawed at room temperature. Then, 0.25 mL of the fluid was transferred to an Eppendorf tube, diluted with 0.75 mL of MeOH containing 1% ammonium formate (w/v), the mixture was hand-shaken for 10 s, and centrifuged at 4,500 rpm for 5 min. Consequently, the supernatant was loaded on a pre-washed (with 1.5 mL of MeOH containing 1% ammonium formate (w/v)) Hybrid-SPE cartridge. The eluate was collected, filtered, and directly injected into the LC-ESI-MS/MS and UHPLC-HRMS systems.

2.4 Gas chromatography

2.4.1 Sample preparation

Sample preparation was based on a liquid-liquid extraction (LLE) bibliographic protocol [17] with minor amendments, using DCM as extraction solvent. Briefly, 1 mL of the biological fluid was mixed with 1 mL sulfuric acid (1 M) and 0.1 g of Na_2SO_4 . The mixture was vortexed for 1.5 min (complete salt dissolution was observed). Then, 4 mL of DCM was added, and the mixture was agitated for 2 min (with a magnetic stirrer) and vortexed for an additional 1 min. Consequently, the sample was centrifuged for 4 min at 4,000 rpm, the DCM layer was collected, dried over Na_2SO_4 , and a portion of it (3 mL) was evaporated to dryness under a gentle stream of nitrogen. The dry residue was reconstituted in 30 μL of DCM, filtered, and then 1 μL injected to the GC-MS system.

2.5 Chromatographic conditions

2.5.1 LC-ESI-MS/MS system and operating conditions

Same chromatographic and operating conditions (see Supplementary material) with previous work of our group were encompassed [18].

2.5.2 Annotation of chlorpyrifos and myclobutanil metabolites by UHPLC-HRMS

For investigation of the chemical profiling and the metabolism of the samples, the Q-Exactive Orbitrap platform (Thermo Fisher Scientific, San Jose, CA, USA) connected to a Dionex Ultimate 3000 UHPLC system (Thermo Scientific™ Dionex™, Sunnyvale, CA, USA) was employed. A Hypersil Gold UPLC C18 (2.1×150 mm, $1.9 \mu\text{m}$) reversed-phased column (Thermo Fisher Scientific, San Jose, CA, USA) was used. Sample analysis was carried out in both positive (ESI+) and negative (ESI-) ion mode. Eluents A (ultrapure water with 0.1% formic acid) and B (acetonitrile) were used in a gradient mode of 30 min as follows: 0 to 21 min: 95% A: 5% B, 21 to 24 min: 5% A: 95% B, 24 to 30 min: 95% A: 5% B. The flow rate was 0.22 mL/min and data acquisition was performed on a mass range of 100–1,000 Da on profile mode. The conditions for the HRMS for both negative and positive modes were set as follows: capillary temperature, 350°C ; spray voltage, 2.7 kV; S-lense Rf level, 50 V; sheath gas flow, 40 arb. units; aux gas flow, 5 arb. units; aux. gas heater temperature, 50°C . The resolution for full-scan analysis was set on 70,000, whereas for the data-dependent acquisition mode, the resolution was 35,000, allowing for MS/MS fragmentation of the three most intense ions. Stepped normalized collision energy was set at 35, 60, and 100. The column temperature was kept at 40°C , while the sample tray temperature was set at 4°C . Data analysis was performed using Compound Discoverer 2.1 software (Thermo Fisher Scientific™ – San Jose, CA, USA). The detection of the metabolites was in accordance with the data from literature applying m/z tolerance of 5 ppm and included the composition rules of H/C, NOPS/C, RDBE, and the isotopic pattern.

2.5.3 Gas chromatography-mass spectrometry analysis and operating conditions

The GC-MS analysis was performed on a Chromtech Evolution MS/MS triple quadrupole mass spectrometer built on an Agilent 5975 B inert XL EI/CI MSD system,

which was operated in full scan data acquisition mode. Samples were injected (injection volume was 1 μL) with a Gerstel MPS-2 autosampler using a 10- μL syringe. Separations were performed on an HP-5 ms UI, length 30 m, ID 0.25 mm, film thickness 0.25 μm (J&W Folsom, USA). Helium (99.9999% purity) was used as the carrier gas at a flow rate of 1.2 mL min^{-1} . The column oven temperature program initiated from 45°C, staying for 1 min, increased to 250°C at a rate of 5°C min^{-1} where it stayed for 5 min. The transfer line, manifold, and source of ionization temperatures were 300, 40, and 230°C, respectively. The electron multiplier voltage was set at 2,000 V. The total GC analysis was 47 min. Identified peaks in GC-MS (triplicate analysis) were verified by matching the acquired mass spectra with those in the commercial library of NIST 08.

2.6 Analytical method validation

Validation of the analytical method was based on the bioanalytical and forensic toxicology validation guideline documents [19,20]. SANTE guideline was also taken into account [21]. Validation parameters considered were linearity, sensitivity (lower limit of quantitation, LLOQ), selectivity, accuracy (recovery evaluation), precision, and matrix effect (ME). Linear range varied from 5 to 1,000 ng/mL containing at least six calibration levels, using internal standard calibration (preferably matrix matched calibrators). Internal standards use also corrected recovery and assisted the accurate quantification of some of the analytes. LLOQ was determined by injecting three replicates (three times each) of the lowest (non-zero) calibrator (5 ng/mL), evaluating the concordance with the detection/identification criteria. Selectivity was assessed by analyzing consecutive blank samples of biofluids, evaluating respective responses in the retention times of the analytes. LLOQ coincides in this study with the decision point concentration (for reporting positive analytes) of 5 ng/mL, verified in this study. Recovery was assessed at four concentration levels (1, 5, 50, and 200 LLOQ), using five independent replicates per level. Up to three MRM transitions were monitored for the active substances. Since blank (human) gastric fluid sample was not available (it required consent for gastroendoscopic examination from volunteers or patients, not pursued at the time of the incident), results were extrapolated using urine or serum matrix-matched standard solutions. In order to confirm suitability of this approach, an artificial mixture of hydrochloric acid (aqueous solution), potassium chloride, and

sodium chloride (the main components of gastric fluid) was prepared to mimic this fluid. Spiking this solution, subsequent extraction and analysis, did not exhibit substantial differences (in terms of analytical figures of merit) in comparison to the spiked urine or serum samples. Lastly, considering that the objective of this work is to present the findings of the specific incident, no data are presented for the rest of the active substances validated with this methodology (more than 70 substances and metabolites) of the analytical methods (will be presented in a forthcoming work of our group).

Ethical approval: The presented investigations were carried out following the ethics rules of the Declaration of Helsinki of 1975, and later amendments, as revised in 2013. More specifically, the ethical committee of Benaki Phytopathological Institute approved the sample preparation and subsequent chemical analysis of the mentioned biological fluids.

3 Results and discussion

The analytical method validation criteria fulfilled requirements of the respective guidelines. More specifically, for the tested biological fluids, linearity was acceptable with the lowest calibrator demonstrating concentrations $<\pm 15\%$ of the nominal concentrations (and correlation coefficients values, $r > 0.9989$ for all analytes derived from matrix-matched calibration curves). The LLOQs were established for chlorpyrifos, myclobutanil, and imidacloprid at 5 ng/mL. Trueness was assessed by the recovery study, verifying sufficient extraction of analytes from all types of biological fluids (recoveries were in the range of 85–106%, at all studied concentrations, RSD% 6–9.2%, see Table S1). Precision (inter and intraday, Table S1) was also acceptable displaying RDS% values up to 10%. For chlorpyrifos and myclobutanil, ME varied from –2.7 to 7.9%, respectively, since no significant interfering peaks were obtained, demonstrating the acceptable selectivity of the method. For imidacloprid, ME fluctuated from 8.3 to 14.8%. Carryover was far below the threshold value of 20% with regard to the LLOQ.

Consequently, the implementation of this analytical method in the gastric fluid, serum, and urine samples of the patient hospitalized revealed mainly the presence of chlorpyrifos and myclobutanil, yet, in far lower magnitude imidacloprid (see Table 1 for analytical results, and Figure S1a–d for MRM transitions in real samples). The results designate that the female patient was exposed to

high amounts of both active substances, also considering their biological half-life ($t_{1/2}$). The half-life for chlorpyrifos is 8.15 h, and for myclobutanil, it ranges from 0.6 to 0.9 for low dose and to 30.1 h for high dose [22,23].

Marques et al. reported blood chlorpyrifos levels in a poisoned individual at approximately 52 ng/mL [24]. In a 1993 report regarding chlorpyrifos poisoned persons, the range of serum concentrations varied from 52.6 to 2485.7 ng/mL, which designate that the serum finding of the present study falls within this range [25]. Higher concentrations in serum were reported by a Spanish group in 2004 (at 5,400 ng/mL); yet, in the same work, the gastric fluid concentration was determined at 9,400 ng/mL [26], 3–4 times lower than that in the present case report. Myclobutanil was detected in two serum samples of orchard workers (previous work of our group) at much lower levels [27], although the two cases are not comparable. To emphasize on the severity of this incident, the total amount of chlorpyrifos in gastric fluid is estimated taking into account the highest concentration of chlorpyrifos analytically determined in gastric fluid as worst case (i.e. 39,800 ng/mL), the weight of the patient (70 kg), and the human adult production of 1,500 mL gastric fluid on a single day [28]. The total amount of chlorpyrifos in gastric fluid was estimated to be at least 852.9 $\mu\text{g kg}^{-1}\text{ bw}$, which is 170.6 times higher than the acute reference dose (ARfD) of 5 $\mu\text{g kg}^{-1}\text{ bw}$ for chlorpyrifos [29]. For myclobutanil, the respective calculations resulted in exceedance of the ARfD of myclobutanil by 1.3 times [30]. It is acknowledged that this is a rough estimation since the amount detected in gastric fluid is

expected to be lower than the actual ingested amount considering that both chlorpyrifos [31] and myclobutanil [30] are readily bioavailable due to rapid and complete absorption via the oral route.

The prevalence of chlorpyrifos and myclobutanil metabolites was assessed using UHPLC-HRMS analysis. For chlorpyrifos, 3,5,6-trichloro-2-pyridinol (TCPy), the principal metabolite found in the plasma, and chlorpyrifos oxon (diethyl(3,5,6-trichloropyridin-2-yl) phosphate), both were annotated in gastric fluid samples (see Figures 1 and 2), and in the serum sample with mass error values (D (ppm)) below 0.6 ppm. Chlorpyrifos oxon detection is another indication of the high concentration of the parent compound, taking into account its fast *in vivo* conversion to TCPy. The dichloro metabolite (3,6-dichloro-2-pyridinol) was annotated in one gastric fluid sample (no 2). Chlorpyrifos-related dialkyl phosphates (DAPs) and the hydrolysis products such as diethyl phosphate (DEP) and diethylthiophosphate (DETP) were also annotated in both the samples of gastric fluid (see indicative LC-HRMS chromatogram, Figure S3 exhibiting D (ppm) far below the 5 ppm threshold (–0.65 to 0 ppm)). Both metabolites though are usually targeted in urine samples are reported in other biofluids as well (such as blood serum) [25,32]. On the whole, these outcomes verify the *in vivo* degradation of chlorpyrifos to several of its metabolites. Among them, the activated desulfurated oxon counterpart produced in the liver via cytochrome P450 is a potent acetylcholinesterase (AChE) inhibitor, since 99% of it binds irreversibly to blood AChE [33,34].

The other DAPs, such as dimethyl phosphate (DMP) and dimethyl thiophosphate (DMTP), were also annotated, but are not specific, and can be related to other OPs as well. Such findings might also be associated with dietary or environmental uptake related active substances.

Diethyl dithiophosphate (DEDTP) was annotated in one urine and one blood serum sample. In the same samples, both DMP and DEP were additionally detected. For myclobutanil, three metabolites were putatively identified, which are hydroxy (or dihydroxy) and keto derivatives of the parent compound (see Figure 3 for one of them).

Interestingly, both targeted and untargeted analyses (LC-HRMS chromatogram, Figure S2 D (ppm) for both gastric samples varied from –1.17 to –0.39) unveiled the presence (in gastric fluids) of relatively low concentrations of the neonicotinoid insecticide imidacloprid. Imidacloprid metabolites were not identified (both with LC-MS/MS and LC-HRMS). Overall, the metabolites in blood serum and gastric samples are presented in Table 2.

Table 1: Analytical results for biological fluid samples using LC-ESI-MS/MS targeted analysis

Sample	Compound	Concentration $\pm$ SD (ng/mL, $n = 3$) ^a
Urine no. 1	Chlorpyrifos	<5
	Myclobutanil	<5
Urine no. 2	Chlorpyrifos	<5
	Myclobutanil	<5
Blood serum	Chlorpyrifos	1,800 $\pm$ 37.4
	Myclobutanil	<5
Gastric fluid no. 1	Chlorpyrifos	39,800 $\pm$ 182.4
	Myclobutanil	18,300 $\pm$ 86.2
	Imidacloprid	23.2 $\pm$ 1.7
Gastric fluid no. 2	Chlorpyrifos	28,400 $\pm$ 98.8
	Myclobutanil	18,800 $\pm$ 59.7
	Imidacloprid	25.7 $\pm$ 2.1

^a LLOQs for the presented analytes at 5 ng/mL, SD: standard deviation (analysis of 3 distinct replicates of the sample).

Since pesticides' formulations contain co-formulants (i.e., solvents, surfactants, emulsifiers, inert materials) and adjuvants (such as polyethoxylated alkyl amines (POEA)), GC-MS analysis was implemented to possibly unveil such chemicals. Ignorance of them and their potential toxicity [14] can lead to falsification with regard to the safety of the commercial pesticide [35], and subsequently to the partial assessment of intoxication incidents, where pesticides formulations are implicated.

In this direction, such constituents were identified in gastric fluid samples (see representative GC-MS full scan TIC magnified chromatogram in Figure 4) after successful application of an LLE protocol and exemplarily depicted in Table 3 (for gastric fluid sample no. 1). The most abundant components were *m*- and *o*-xylene, and cyclohexanone. Quantification of the co-formulants was out of the scope of the presented study.

Among the GC-MS revealed chemicals, cyclohexanone was reported to play significant role in the potentiation of toxicity of another key OP, dimethoate, in a minipig model, after oral administration of an emulsifiable concentrate (EC) formulation [13]. Hence, the detection of cyclohexanone in the gastric fluid DCM extract (as one of the major components) can possibly lead to a similar hypothesis concerning the potentiation of chlorpyrifos toxicity. Similar conclusion can be drawn

for *m*- and *o*-xylene, since studies have shown xylene-induced toxicity in several organisms (indicatively see ref. [36]).

Ethylbenzene was also retrieved at lower relative amounts. No hazard is identified for the general population exposed to ethylbenzene via the oral route.

The substance has been assessed in the frames of the REACH (Regulation (EC) 1907/2006 for Registration, Evaluation, Authorisation and Restriction of Chemicals) and it is registered for use up to 1,00,00,000 tonnes per annum [37]. In the REACH dossier, hearing loss has not been considered a specific effect for ethylbenzene but has also been found with other aromatic solvents such as styrene, toluene, or xylenes. According to Gagnaire *et al.*, ethylbenzene led to maximal hearing impairment after about 4 weeks without further deterioration by prolongation of exposure [38].

For other benzene derivatives, such as 1,2,4-trimethylbenzene, there is EU-harmonized classification for serious eye irritation, skin, and respiratory irritation, and is harmful if inhaled [39]. An EU indicative occupational exposure limit value (OELV) of 20 ppm for a reference exposure period of 8-hours' time weighted average (TWA) via the inhalation route has collectively been set for trimethylbenzenes [40], while the need for more specific national values is recognized.

Figure 1: 3,5,6-Trichloro-2-pyridinol (TCPy) annotation in gastric fluid sample based on HRMS data in positive ionization mode.

Figure 2: Chlorpyrifos oxon annotation in gastric fluid sample using HRMS data in positive ionization mode.

Figure 3: (2R)-2-(4-Chlorophenyl)-5-oxo2-(1H-1,2,4-triazol-1-ylmethyl)hexanenitrile (myclobutanil metabolite) annotation in gastric fluid.

Chlorpyrifos is a controversial compound, including its degradation products [41]. In July 2019, the European Food Safety Authority (EFSA) was mandated by the

European Commission to provide a statement on the available outcomes of the human health risk assessment in the context of the peer review of chlorpyrifos [42].

Table 2: Annotation of the metabolites applying *m/z* tolerance of 5 ppm

Experimental <i>m/z</i>	<i>t_R</i> (min)	Molecular formula	<i>D</i> (ppm)					Annotation
			Urine	Urine	Serum	Gastric fluid no. 1	Gastric fluid no. 2	
349.9336	20.63	C ₉ H ₁₁ Cl ₃ NO ₃ PS	—	—	—	−0.86	−0.86	Chlorpyrifos
127.0155	2.70	C ₂ H ₇ O ₄ P	0	0	—	−0.79	0	Dimethyl phosphate
142.9926	13.16	C ₂ H ₇ O ₃ PS	—	—	—	0.70	0.70	Dimethyl thiophosphate
155.0468	2.70	C ₄ H ₁₁ O ₄ P	−0.65	0	—	−0.65	−0.65	Diethyl phosphate
171.0239	13.16	C ₄ H ₁₁ O ₃ PS	—	—	—	0	0	Diethyl thiophosphate
184.9854	12.96	C ₄ H ₁₁ O ₂ PS ₂	1.62	1.62	—	—	—	Diethyl dithiophosphate
197.9275	14.64	C ₅ H ₂ Cl ₃ NO	—	—	0.51	0	0	3,5,6-trichloro-2-pyridinol (TCPy)
333.9563	17.15	C ₉ H ₁₁ Cl ₃ NO ₄ P	—	—	—	−0.30	−0.30	Diethyl(3,5,6-trichloropyridin-2-yl)phosphate
163.9664	12.63	C ₅ H ₃ Cl ₂ NO	—	—	—	—	−1.22	3,6-dichloro-2-pyridinol
289.1215	16.69	C ₁₅ H ₁₇ ClN ₄	—	—	—	−1.38	−1.04	Myclobutanil
303.1007	13.58	C ₁₅ H ₁₅ ClN ₄ O	—	—	—	−0.33	0	(2 <i>RS</i>)-2-(4-Chlorophenyl)-5-oxo2-(1 <i>H</i> -1,2,4-triazol-1-ylmethyl)hexanenitrile
305.1164	15.73	C ₁₅ H ₁₇ ClN ₄ O	—	—	—	−0.65	−0.65	(2 <i>RS</i> ,5 <i>RS</i>)-2-(4-Chlorophenyl)-5-hydroxy-2-(1 <i>H</i> -1,2,4-triazol-1-ylmethyl)hexanenitrile
321.1113	11.22	C ₁₅ H ₁₇ ClN ₄ O ₂	0	—	—	—	—	(2 <i>RS</i> ,5 <i>RS</i>)-2-(4-Chlorophenyl)-5,6-dihydroxy-2-(1 <i>H</i> -1,2,4-triazol-1-ylmethyl)hexanenitrile
256.0593	10.62	C ₉ H ₁₀ ClN ₅ O ₂	—	—	—	−1.17	−0.39	Imidacloprid

Figure 4: Magnified full scan GC-MS total ion chromatogram (TIC) of gastric fluid DCM extract.

Briefly, EFSA concluded that chlorpyrifos is toxic if swallowed and harmful in contact with skin. Inhibition of AChE was the critical effect after repeated oral administration of chlorpyrifos in experimental animals resulting in cholinergic overstimulation. Significant uncertainties were linked to neurodevelopment, since decrease in cerebellum height (corrected by brain weight) was observed in pups from the lowest dose of 0.3 mg/kg b.w./day in a developmental neurotoxicity study in rats. EFSA considered that these concerns were supported by available epidemiological evidence related to the development of neurological outcomes in children [43–46]. There was no evidence of carcinogenicity, but the genotoxic potential of chlorpyrifos was unclear. Concerns were raised on the possibility of chlorpyrifos to induce chromosomal

aberrations and DNA damage through oxidative stress or topoisomerase II inhibition; the latter was considered a molecular initiating event for infant leukemia based on the previous work of the EFSA Panel on Plant Protection Products and their Residues [47]. This mode of action is not supported by more recent research funded by EFSA [48] but more research would be needed before a definite conclusion can be drawn.

Concerning myclobutanil, its acute toxicity to mammals is far lower compared to chlorpyrifos, exhibiting an LD₅₀ oral at 1,600 mg kg⁻¹ in rats [49] (respective end-point value for chlorpyrifos is 66 mg kg⁻¹ [31]).

It is noted, however, that toxic effects after exposure to a mixture of pesticides may be influenced by interactions between individual components of the mixture.

Table 3: Chemical and relative composition of the DCM extract of gastric fluid sample (no. 1)

Peak number ^a	Analyte	Retention time (min)	RI ^b	Relative amount (%)
1	Ethylbenzene	4.94	855 (855)	5.43 ± 0.32
2	<i>m</i> -Xylene	5.18	866 (866)	30.13 ± 1.84
3	<i>o</i> -Xylene	5.78	887 (887)	30.88 ± 1.30
4	Cyclohexanone	5.90	894 (894)	17.02 ± 2.01
5	Propyl benzene	7.27	953 (953)	0.70 ± 0.07
6	<i>p</i> -Ethyl toluene	7.52	954 (954)	4.61 ± 0.46
7	1,3,5-Trimethyl benzene	7.70	972 (972)	2.00 ± 0.19
8	1,2,4-Trimethyl benzene	8.01	990 (990)	1.13 ± 0.06
9	1,2,3-Trimethyl benzene	8.42	1,013 (1,013)	5.07 ± 0.22
10	1-Methyl-3-propyl-benzene	9.26	1,037 (1,037)	1.12 ± 0.05
11	4-Chloro- <i>o</i> -xylene	10.38	1,075 (1,075)	1.91 ± 0.25

^a Peak number refers to the chromatogram presented in Figure 4. ^b RI, retention index on HP5-MS UI column (relative to *n*-alkanes), identification based on mass spectra comparison with the reference databases, and comparison with literature RIs (depicted in parentheses).

The mechanism behind these interactions may be linked to the toxicokinetic and/or toxicodynamic properties of each component. At doses exceeding dietary-relevant levels, as it is the case with this study of pesticide abuse, synergism may also occur. Compounds such as OPs and triazoles display synergistic effects in a plethora of organisms [50]. The azole moiety is known to inhibit several CYP450s isoforms, yet, its effect is diverse, depending on the insecticide with which it interacts. For example, with pyrethroids, the toxicity is often enhanced, yet with compounds (such as organophosphorothioates) whose oxon derivatives are the toxic counterparts, the triazoles, through the inhibition of CYP450 enzymes, block transformation to oxon derivatives, leading to a decrease in toxicity [50].

4 Conclusions

A developed and validated LC-ESI-MS/MS analytical method was applied for the detection and quantification of suspect pesticides in biological fluids collected from a documented pesticide intoxication incident from a Greek hospital. Significant levels of chlorpyrifos and myclobutanil were determined in gastric fluid samples, and in the serum sample, verifying the severity of the incident. UHPLC-HRMS unveiled the presence of key metabolites of both suspect active substances, which were more prolific in the gastric fluids, where the elevated concentrations were observed. The neonicotinoid imidacloprid was also identified in the gastric fluids. Lastly, GC-MS analysis unveiled the presence of several co-formulants in gastric fluids. Next steps of the herein presented analytical approaches include the incorporation in the LC-ESI-MS/MS, and UHPLC-HRMS methods of dialkylphosphates, and other metabolites not currently enrolled in the analytical scope, and their subsequent quantification, which is further important from a risk assessment perspective.

Acknowledgments: Authors thank Dr. Chris Anagnostopoulos for the assistance in the chemical analyses.

Funding: This research received no external funding.

Author contributions: K. M. and K. M. K.: conceptualization; K. M. K. and E. B.: methodology; K. M. K. and E. B.: software; K. M. K. and E. B.: validation; K. M. K.: formal analysis; K. M. K., E. B., E. M. K., and D. N.: investigation; K. M. and K. G.: resources; K. M. K., E. B., E. M. K., K. G.: data curation; K. M. K.: writing – original draft preparation;

K. M., K. M. K., E. B., D. N.: writing – review and editing; K. M. K. and E. B.: visualization; K. M. K.: supervision; K. M., K. M. K.: project administration; K. M.: funding acquisition. All authors have read and agreed the published version of the manuscript.

Conflict of interest: The authors declare no conflict of interest.

Data availability statement: All data generated or analysed during this study are included in this published article [and its supplementary information files].

References

- [1] Kallel H, Charfeddine A, Khlaf-Bouaziz N, Hergafi L, Bahloul M, Ben Hamida C, et al. Curious fatal intentional poisoning case with organophosphate pesticide. *Med Sci Monit.* 2007;13(1):Cs1–3.
- [2] Eddleston M. Poisoning by pesticides. *Medicine.* 2020;48(3):214–7.
- [3] Kim Y, Min J, Lee SJ. Suicide overall and suicide by pesticide rates among South Korean workers: a 15-year population-based study. *Int J Environ Res Public Health.* 2019;16:23.
- [4] Han SK, Yeom SR, Lee SH, Park SC, Kim HB, Cho YM, et al. A fatal case of chlorfenapyr poisoning following dermal exposure. *Hong Kong J Emerg Med.* 2019;26(6):375–8.
- [5] Kumar A, Verma A, Kumar A. Accidental human poisoning with a neonicotinoid insecticide, imidacloprid: a rare case report from rural India with a brief review of literature. *Egypt J Forensic Sci.* 2013;3:123–6.
- [6] Kweyiga B, Ario AR, Bulage L, Harris J, Zhu BP. Fatal cases associated with eating chapatti contaminated with organophosphate in Tororo District, Eastern Uganda, 2015: case series. *BMC Public Health.* 2019;19:767.
- [7] Gunnell D, Knipe D, Chang SS, Pearson M, Konradsen F, Lee WJ, et al. Prevention of suicide with regulations aimed at restricting access to highly hazardous pesticides: a systematic review of the international evidence. *Lancet Glob Health.* 2017;5(10):E1026–37.
- [8] Commission-Notice. Guidance on monitoring and surveying of impacts of pesticide use on human health and the environment under Article 7(3) of Directive 2009/128/EC establishing a framework for Community action to achieve the sustainable use of pesticides (referred to as the Sustainable Use Directive); 2017.
- [9] Boucaud-Maitre D, Rambourg MO, Sinno-Tellier S, Puskarczyk E, Pineau X, Kammerer M, et al. Human exposure to banned pesticides reported to the French Poison Control Centers: 2012–2016. *Environ Toxicol Pharmacol.* 2019;69:51–6.
- [10] Slavica V, Dubravko B, Milan J. Acute organophosphate poisoning: 17 years of experience of the National Poison Control Center in Serbia. *Toxicology.* 2018;409:73–9.
- [11] Chen C, Fan Z, Xu H, Tan X, Zhu M. Metabolomics-based parallel discovery of xenobiotics and induced endogenous

- metabolic dysregulation in clinical toxicology. *Biomed Chromatogr.* 2019;33(2):e4413.
- [12] De Paepe E, Van Meulebroek L, Rombouts C, Huysman S, Verplanken K, Lapauw B, et al. A validated multi-matrix platform for metabolomic fingerprinting of human urine, feces and plasma using ultra-high performance liquid-chromatography coupled to hybrid orbitrap high-resolution mass spectrometry. *Anal Chim Acta.* 2018;1033:108–18.
 - [13] Eddleston M, Street JM, Self I, Thompson A, King T, Williams N, et al. A role for solvents in the toxicity of agricultural organophosphorus pesticides. *Toxicology.* 2012;294(2–3):94–103.
 - [14] Defarge N, Takacs E, Lozano VL, Mesnage R, Spiroux de Vendomois J, Seralini GE, et al. Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. *Int J Environ Res Public Health.* 2016;13:3.
 - [15] Langrand J, Blanc-Briset I, Boucaud-Maitre D, Puskarczyk E, Nisse P, Garnier R, et al. Increased severity associated with tallowamine in acute glyphosate poisoning. *Clin Toxicol (Phila).* 2020;58(3):201–3.
 - [16] Honda M, Robinson M, Kannan K. A rapid method for the analysis of perfluorinated alkyl substances in serum by hybrid solid-phase extraction. *Environ Chem.* 2018;15(1–2):92–9.
 - [17] Drabinska N, Mlynarz P, de Lacy Costello B, Jones P, Mielko K, Mielnik J, et al. An Optimization of liquid-liquid extraction of urinary volatile and semi-volatile compounds and its application for gas chromatography-mass spectrometry and proton nuclear magnetic resonance spectroscopy. *Molecules.* 2020;25:16.
 - [18] Kasiotis KM, Anagnostopoulos C, Anastasiadou P, Machera K. Pesticide residues in honeybees, honey and bee pollen by LC-MS/MS screening: reported death incidents in honeybees. *Sci Total Environ.* 2014;485–6:633–42.
 - [19] Bioanalytical-Method-Validation. Bioanalytical method validation: guidance for industry; 2018.
 - [20] SWGTOX. Scientific working group for forensic toxicology: standard practices for method validation in forensic toxicology. *J Anal Toxicol.* 2013;37:452–74.
 - [21] SANTE/11813/2017. Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed; 2017.
 - [22] WHO-Myclobutanil. <https://apps.who.int/pesticide-residues-jmpr-database/pesticide?name=MYCLOBUTANIL>
 - [23] WHO-Chlorpyrifos. <https://apps.who.int/pesticide-residues-jmpr-database/pesticide?name=CHLORPYRIFOS>
 - [24] Marques GLM, Siqueira AA, Minassa VS, Peres MD, Pelicao FS, Sampaio KN. Method validation for simultaneous determination of atropine, pralidoxime and 12 organophosphorus compounds in blood samples by means of high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). *J Chromatogr B Anal Technol Biomed Life Sci.* 2018;1097–8:44–53.
 - [25] Drevencar V, Vasilic Z, Stengl B, Frobe Z, Rumenjak V. Chlorpyrifos metabolites in serum and urine of poisoned persons. *Chem-Biol Interact.* 1993;87(1–3):315–22.
 - [26] Martinez MA, Ballesteros S, Sanchez de la Torre C, Sanchiz A, Almarza E, Garcia-Aguilera A. Attempted suicide by ingestion of chlorpyrifos: identification in serum and gastric content by GC-FID/GC-MS. *J Anal Toxicol.* 2004;28(7):609–15.
 - [27] Kasiotis KM, Kyriakopoulou K, Emmanouil C, Tsantila N, Liesivuori J, Souki H, et al. Monitoring of systemic exposure to plant protection products and DNA damage in orchard workers. *Toxicol Lett.* 2012;210(2):182–8.
 - [28] Gastric-Fluid. <https://www.britannica.com/science/human-digestive-system/Gastric-secretion>; 2020.
 - [29] Chlorpyrifos-EFSA. Conclusion on the peer review of the pesticide human health risk assessment of the active substance chlorpyrifos. *EFSA J.* 2014;12(4):34.
 - [30] Myclobutanil-EFSA. Conclusion on the peer review of the pesticide risk assessment of the active substance myclobutanil. *EFSA J.* 2010;8(10):85.
 - [31] Chlorpyrifos-EC. Review report for the active substance chlorpyrifos. SANCO/3059/99 – rev. 1.5; 2005 June 3.
 - [32] Drevencar V, Stengl B, Frobe Z. Microanalysis of dialkylphosphorus metabolites of organophosphorus pesticides in human blood by capillary gas-chromatography and by phosphorus-selective and ion-trap detection. *Anal Chim Acta.* 1994;290(3):277–86.
 - [33] Dourson M, Gadagbui B, Onyema C, McGinnis P. A commentary on some epidemiology data for chlorpyrifos. *Regul Toxicol Pharmacol.* 2020;113:104616.
 - [34] EPA. United States Environmental Protection Agency. Chlorpyrifos: revised human health risk assessment for registration review. *Off Chem Saf Pollut Prev*; 2014. Available from: <http://op.bna.com.s3.amazonaws.com/env.nsf/r%3FOpen%3Ddscz-9shl19>
 - [35] Mesnage R, Antoniou MN. Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. *Front Public Health.* 2017;5:361.
 - [36] Maliszewska J, Tegowska E. Toxicity of insecticide carrier solvent: effect of xylene on hemolymph biochemical parameters in *Blaberus giganteus* L. *Pol J Environ Stud.* 2018;27(5):2385–90.
 - [37] Ethylbenzene-2. ECHA Registration Dossier. <https://echa.europa.eu/registration-dossier/-/registered-dossier/15377/7/>
 - [38] Gagnaire F, Langlais C, Grossmann S, Wild P. Ototoxicity in rats exposed to ethylbenzene and to two technical xylene vapours for 13 weeks. *Arch Toxicol.* 2007;81(2):127–43.
 - [39] Alkylbenzenes. ECHA C&L Inventory. Harmonised classification – Annex VI of Regulation (EC) No 1272/2008 (CLP Regulation). <https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/8820>
 - [40] Trimethylbenzenes. EU. Indicative OELVs, Directive 2000/39/EC establishing a first list of IOELVs in implementation of Directive 98/24/EC, last amended by Directive 2019/1831/EU; 2019 Oct 31. <https://echa.europa.eu/legislation-obligation/-/obligations/100.042.803>
 - [41] Hazarika J, Ganguly M, Mahanta R. Molecular interactions of chlorpyrifos and its environmental degradation products with human sex hormone-binding globulin: an in silico study. *J Appl Toxicol.* 2019;39(7):1002–11.
 - [42] Chlorpyrifos-EFSA. Statement on the available outcomes of the human health assessment in the context of the pesticides peer review of the active substance chlorpyrifos. *EFSA J.* 2019;17(8):5809.
 - [43] Castorina R, Bradman A, Fenster L, Barr DB, Bravo R, Vedar MG, et al. Comparison of current-use pesticide and other toxicant urinary metabolite levels among pregnant women in the CHAMACOS cohort and NHANES. *Env Health Perspect.* 2010;118(6):856–63.

- [44] Marks AR, Harley K, Bradman A, Kogut K, Barr DB, Johnson C, et al. Organophosphate pesticide exposure and attention in young Mexican-American children: The CHAMACOS study. *Env Health Persp.* 2010;118(12):1768–74.
- [45] Sebe A, Satar S, Alpay R, Kozaci N, Hilal A. Organophosphate poisoning associated with fetal death: a case study. *Mt Sinai J Med.* 2005;72(5):354–6.
- [46] US-EPA. US EPA (United States Environmental Protection Agency), 2016. US EPA, Office of Chemical Safety and Pollution prevention. Memorandum on Chlorpyrifos: Revised Human Health Risk Assessment for Registration Review; 2016 Nov 3.
- [47] Ockleford C, Adriaanse PI, Berny P, Brock TCM, Duquesne S, Grilli S, et al. EFSA PPR panel (EFSA panel on plant protection products and their residues), 2017. Scientific opinion on the investigation into experimental toxicological properties of plant protection products having a potential link to Parkinson's disease and childhood leukaemia. *EFSA J.* 2017;15(3):325.
- [48] Rodríguez-Cortez VC, Menéndez P. Genotoxicity of permethrin and clorpyrifos on human stem and progenitor cells at different ontogeny stages: implications in leukaemia development. *EFSA supporting publication*; 2020. p. 35. EN-1866.
- [49] Myclobutanil-SITEM. <https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/478.htm>
- [50] Hernandez AF, Gil F, Lacasana M. Toxicological interactions of pesticide mixtures: an update. *Arch Toxicol.* 2017;91(10):3211–23.