Rapid Multimethod for Verification and Determination of Toxic Pesticides in Whole Blood by Means of Capillary GC–MS

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Abstract

A rapid and single multimethod was developed to determine substances of different pesticide classes in whole blood in the event of acute human intoxications, as required by EU Commission Directive 96/46. The method was validated by an in-house and an independent laboratory validation. Whole blood is hemolyzed and then deproteinized. After extraction of the supernatant, blood levels are determined by gas chromatography–mass spectrometry. The method, which can be performed within 120 min, covers 15 active substances (8 organophosphate pesticides, 2 carbamates, 3 pyrethroids, 1 azole, and 1 organochlorine pesticide) classified as toxic or very toxic. These compounds can be identified down to concentrations between 100 and 1000 ng/mL by comparison of their mass spectra to those in a commercial pesticide mass spectra library. Using the standard addition method, they can be quantitated down to concentrations between 30 and 200 ng/mL. These limits of quantitation are considered to be sufficient in comparison to respective LD_{50} values.

Introduction

EU Commission Directive 96/46 section 4.2.5 requires analytical methods for the determination of residues in body fluids and tissues for pesticides classified as toxic or very toxic (1). The methods required are intended to support clarification of acute human intoxications as well as control of the extent of clinical detoxification.

The intention of European authorities is to require individual methods (2). However, multimethods will be more useful to clinical pathologists, as the source of intoxication and the nature of the poisonous substance is not always clear during clinical treatment.

Multimethods for determination of pesticides in body fluids have already been published previously. They cover individual

groups of active substances, for example, organophosphate pesticides (3,4), pyrethroids (5,6), and rodenticides (7), or use LC–MS (8), which has not been fully established in clinical laboratories.

This paper therefore proposes the use instead of a multimethod covering as many toxic or very toxic pesticides as possible, but at the expense of non-inclusion of metabolites which may contribute to the residue definition in food of crop and animal origin. Rodenticides were not included, as intoxications can be easily proved by the determination of the thromboplastin-time and multimethods for these actives already exist.

Blood and excreta are the preferred matrices for toxicological analysis. In excreta, however, the nature and concentration of residues are unpredictable without knowledge of the toxicant and its toxicokinetics. Consequently, whole blood is the body compartment with by far the highest probability of finding toxic pesticides as such in quantitatable concentrations. Because of the high lipophilicity of most toxic pesticides, high concentrations can be expected in the erythrocytes. Limits of quantitation (milligrams per kilogram whole blood) of at least 1% of the LD_{50} values (milligrams per kilogram body weight) should provide sufficient sensitivity when assuming equal distribution of the toxicant over the whole body as a first approximation of realistic blood levels.

Materials and Methods

Reference material

Bendiocarb (99.5% w/w), deltamethrin (98.8% w/w), endosulfan (99.3% w/w), fluquinconazole (99.4% w/w), heptenophos (91.4% w/w), pyrazophos (99.7% w/w), tralomethrin (99.2%), and triazophos (41.0% w/w) were obtained from AgrEvo GmbH (Frankfurt, Germany). Azinphos-methyl (97.6%), β-cyfluthrin (97%), fenamiphos (97.1%), fenthion (96.5%), methamidophos (94.3%), methiocarb (99.4%), and parathion-methyl (98.6%) were purchased from Labor Dr. Ehrenstorfer-Schäfers (Augs-

burg, Germany). Bromophos-methyl, PESTANAL®, was obtained from Riedel-de Haën AG (Seelze, Germany).

Solvents

The quality of all solvents should be suitable for pesticide analysis (e.g., Riedel-de Haën PESTANAL)

Equipment

The equipment used to perform this method included the following: disposable filtration columns with approximately 3 mL kieselguhr (e.g., Isolute HM-N, Handels-GmbH, Bad Homburg, Germany); test tubes for hematology prefilled with the anticoagulant K-EDTA (e.g., Saarstedt); test tubes with caps, 10-mL volume, and graduated at 200-µL and 500-µL levels; ultrasonic bath (e.g., Bandelin Sonorex RK 100); vacuum-manifold (e.g., Supelco Visiprep); vortex mixer for test tubes; centrifuge for test tubes; heated drying block with nitrogen flow (e.g., Techne); capillary GC (HP 5890) with an MSD (HP 5970) and autosampler (HP 7673 A) equipped with programmed temperature vaporization (PTV, e.g., Gerstel) and an HP 5-MS 30-m × 0.25-mm i.d. fused-silica capillary column coated with 0.25 µm 95% dimethyl-5% phenyl-silicone.

An electronic mass spectra library for pesticides (e.g., Hewlett Packard HPPEST) was also used.

Chromatographic conditions

The carrier gas was helium (99.996%), and the column inlet pressure was 85 kPa. The temperature program was as follows: 45°C (2.66 min) to 170°C at 40°C/min, to 220°C at 4°C/min, and to 280°C at 20°C/min (15.72 min). PTV occurred as follows: splitless mode at 40°C to 280°C at 6°C/s (2 min), then open split valve until end of chromatography. The injection volume was 1 µL. The coupling to MS was a closed interface at 285°C

MS conditions

Full scan mode. Ions with m/z 50 to m/z 400 were monitored. The windows for ion-extraction were as follows: deltamethrin/tralomethrin, $t_R - 4.50$ min to $t_R + 0.50$ min; β-cyfluthrin, $t_R - 4.50$ min to $t_R + 1.00$ min; all other active substances, $t_R - 0.50$ min to $t_R + 0.50$ min.

Single ion monitoring (SIM) mode. Sampling time/mass was 100 ms; for bromophos-methyl it was 300 ms. The windows for ion-extraction were as follows: $t_R - 4.50$ min to $t_R + 0.50$ min for deltamethrin and tralomethrin and $t_R - 0.50$ min to $t_R + 0.50$ min for all other active substances with t_R being the retention time of the respective active substance.

Preparation of the stock solution of the internal standard

Exactly 4.0 mg bromophos-methyl was weighed into a 10-mL volumetric flask and made up to the mark with toluene. One-hundred microliters of this solution was transferred by means of a microliter syringe into another 10-mL volumetric flask and make up with toluene as before. This stock solution of the internal standard has a content of 4 ng/µL. It must be protected from light and stored at +4°C ± 3°C. Under these conditions, the storage stability of the stock solution is at least two months.

Extraction procedure

1. Exactly 1 mL whole blood was pipetted into a test tube. After it was capped, the test tube was inserted into the ultra-

sonic bath. Ultrasonic vibration was applied for approximately 15 min in order to hemolyze the erythrocytes.

2. Acetone (1.5 mL) was added by pouring it down the inner wall of the test tube to avoid mixing with the hemolyzed blood. The third step was performed immediately after the completion of the second step.

3. Blood and acetone were mixed vigorously on a vortex mixer for approximately 20 s, which led to a complete deproteinization.

4. Samples were centrifuged at approximately 3000 cycles/min ($\cong 17 \times g$) for 30 s.

5. A disposable kieselguhr column was connected to the vacuum manifold, the valve was opened, and the pale yellow supernatant of the centrifuged sample was transferred onto the column by means of a Pasteur pipette. The sample was allowed to penetrate into the kieselguhr and then left to stand for approximately 30 min.

6. During that time, 5 mL of the eluent I (2 volumes ethyl acetate and 1 volume dichloromethane) was added to the remaining precipitate from the blood and stirred (e.g., by means of the tip of the Pasteur pipette). The test tube was recapped, and ultrasonic vibration was applied for another 5 min. By completion of this procedure, all precipitates should have been removed from the inner wall of the test tube.

7. The tube was centrifuged, and the supernatant was transferred onto the disposable kieselguhr column, taking care to ensure that the aqueous supernatant obtained after the first centrifugation has had enough time (approximately 30 min) to penetrate into the kieselguhr.

8. When the eluent reached the lower end of the column, the valve (in the vacuum-manifold) was closed and allowed to stand for a minimum of 5 min.

Table I. Preferred Fragments and Limits of Detection in Full-Scan Mode

Active substance	t_{RR}*	Mass fragments[†] (m/z)	LOD (ng/mL)
Bromophos-methyl (internal standard)	1.000	331	–
Methamidophos	0.421	**95**; 94; 141	200
Heptenophos	0.578	**124**; 215; 250	100
Bendiocarb	0.642	**151**; 166; 223	100
Parathion-methyl	0.850	**263**; 125; 109	100
Methiocarb	0.908	**168**; 153; 225	100
Fenthion	0.954	**278**; 125; 109	100
α-Endosulfan	1.125	**243**; 241; 339	700
Fenamiphos	1.165	**303**; 260; 217	300
β-Endosulfan	1.253	**195**; 267; 237	700
Triazophos	1.299	**161**; 162; 313	100
Azinphos-methyl	1.435	**160**; 132; 77	1000
Pyrazophos	1.476	**221**; 232; 373	100
Fluquinconazole	1.535	**340**; 341; 342	100
β-Cyfluthrin	1.587	**163**; 165; 226	500
Deltamethrin/ tralomethrin	1.812	**253**; 251; 181	1000

* Abbreviations: t_{RR}, relative retention time; LOD, limit of detection.
[†] Preferred mass fragment in bold type.

9. During that time, 5 mL n-hexane (eluent II) was added to the remaining precipitate from the blood. The sample was stirred, and ultrasonic vibration was applied. Centrifugation was performed as described.

10. A test tube was placed under the column, the valve in the vacuum-manifold was opened to obtain a flow of approximately 1 drop/s, and the eluate was collected.

11. The n-hexane obtained in the ninth step was poured onto the column, and the eluate was collected in the same test tube. The volume of the combined eluates should be between 7.5 mL and 8.0 mL. If it was less, weak vacuum was applied in order to obtain this amount.

12. Two-hundred microliters of toluene was added to the combined eluates, which may be slightly turbid, and the test tube was transferred to the drying block, which has been preheated to 50°C. The combined eluates were evaporated under a nitrogen flow to a remainder of 200 µL, but evaporation to dryness was avoided.

13. Fifty microliters of the stock solution of the internal standard was added, then made up with toluene to approximately 500 µL. Once a clear solution was obtained, it was mixed for a short time on a vortex mixer. If an autosampler was used, the solution was transferred to a capped injector vial. One microliter was used for GC–MS.

Identification of active substances

The lead ions summarized in Table I (typical fragmentation ions, preferred ions in bold type) were extracted in multiple channels from full scan. If peaks at the relative retention times (t_{RR}) for the active substances investigated were present, the background was subtracted from an averaged full-scan mass spectrum and a library search was performed (e.g., in HPPEST). Figure 1 shows the mass spectra of all active substances tested during method development. The retention time of the internal standard is approximately 15.6 min. As tralomethrin decomposes to deltamethrin in the GC injector, these two pyrethroids cannot be distinguished.

Detection and quantitation of blood levels in SIM mode

After identification in full-scan, three characteristic fragments of the pesticide identified and one mass fragment of the internal standard bromophos-methyl (Table II) should be monitored in SIM. Quantitation was performed by the ratio of the peak height of the active substance (a.s.) relative to that of the internal standard (IS). For quantitation, the preferential fragments typed in bold in Table II should be taken. A confirmation of identity was possible from the intensity ratio of the three characteristic fragments after subtrac-

tion of the background. Differences up to ± 10% of the tabulated ratios were still tolerable. The use of a correction factor F allowed the concentration of the toxicant to be approximated. Approximation was performed according to Equation 1.

$$C\ [ng/mL] \cong F \times \frac{Peak\ height_{a.s.}}{Peak\ height_{int.std}} \qquad \text{Eq. 1}$$

Exact quantitation should be performed using the standard addition procedure. For that a second blood sample (1 mL) is spiked with a known amount of the active substance dissolved in acetone. The spiked sample is processed as described. Quantitation is performed by the difference between the peak-height ratios of the spiked and the unspiked sample (Equation 2)

$$C\ [ng/mL] = \frac{P_{rel,0} \times C_1}{P_{rel,1} - P_{rel,0}} \qquad \text{Eq. 2}$$

where $P_{rel,0}$ is peak-height ratio of the nonfortified sample, $P_{rel,1}$ is peak-height ratio of the fortified sample, and C_1 is concentration fortified (ng/mL).
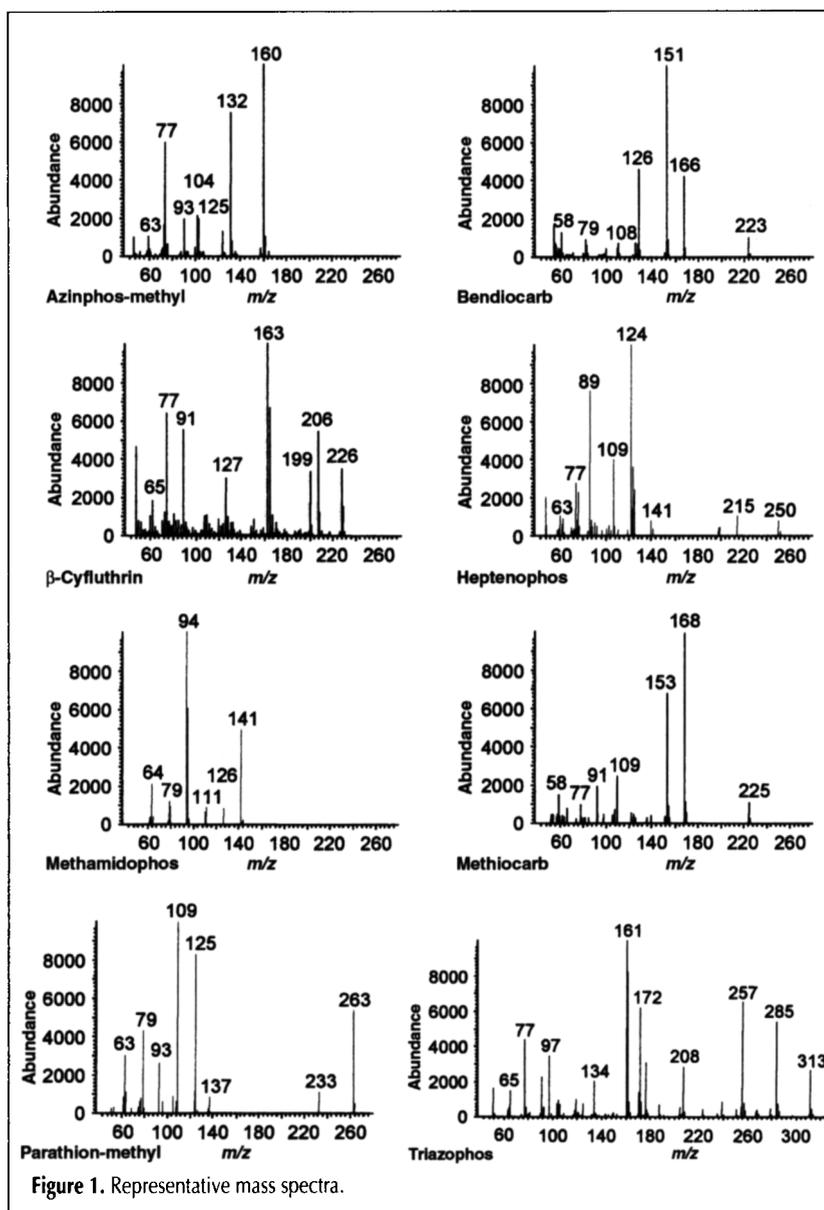

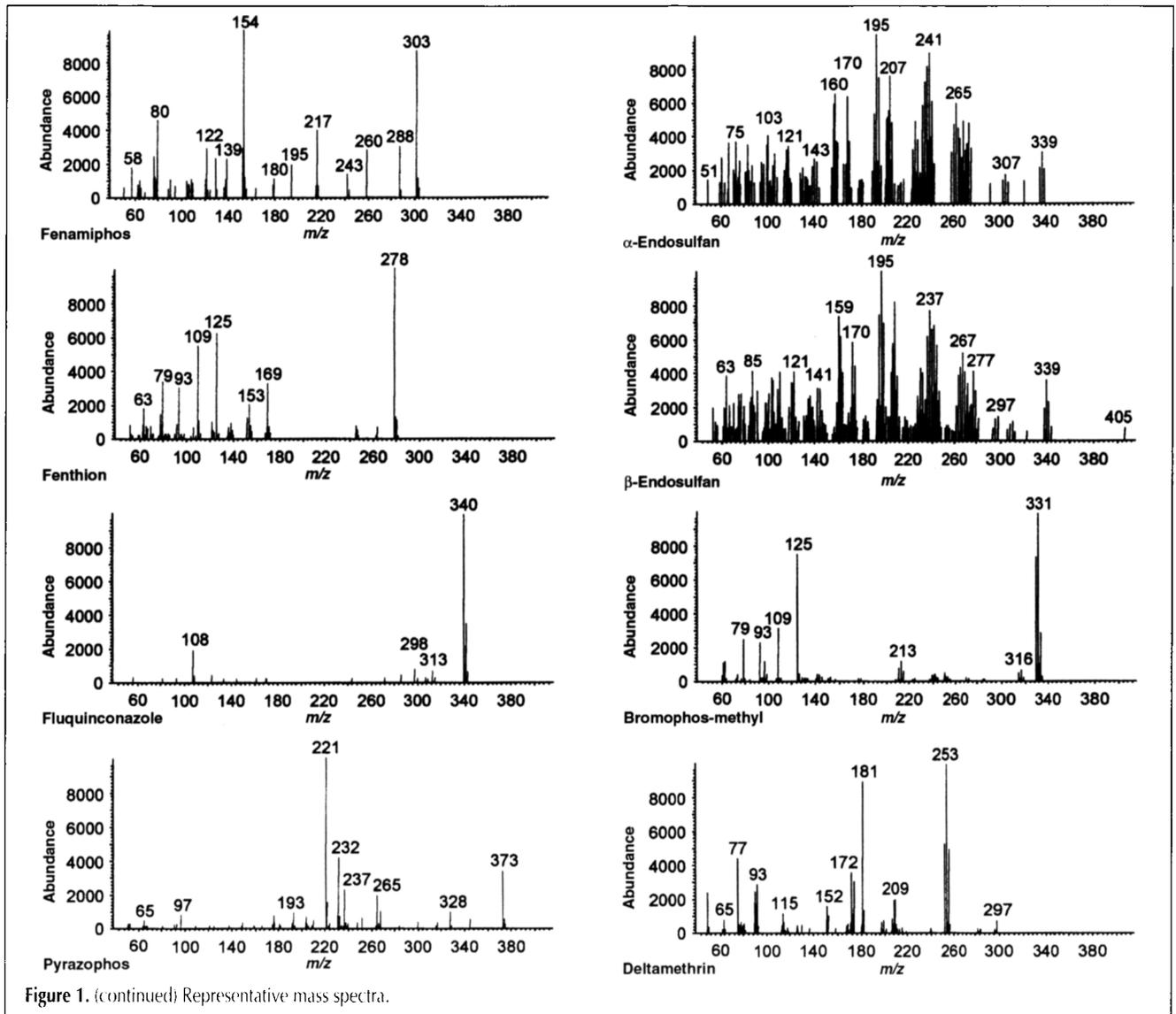**Figure 1.** Representative mass spectra.

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Figure 1. (continued) Representative mass spectra.

In-house method validation

One-milliliter aliquots of hemolyzed blood were each spiked with differing amounts of a stock solution containing all active substances in acetone—with the exception of tralomethrin, which decomposes to deltamethrin—to obtain concentrations of 25–4000 ng/mL of each active substance for validation of the SIM mode and quantitation of blood levels and 100–5000 ng/mL of each active substance for validation of the full-scan mode leading to mass spectra for identification.

One-milliliter aliquots were separately spiked with various amounts of a stock solution of tralomethrin in acetone.

From the spiked samples recorded in full-scan mode, mass spectra were taken for identification via the pesticide mass spectra library. The lowest concentrations that could be identified were defined as limits of detection in full-scan mode.

Limits of detection (LOD) and quantitation (LOQ) for the SIM mode were defined using the calibration curve procedure (9) based upon the analysis of replicated samples spiked over a range of concentrations (k = 5 spiked concentrations, m = 3 repetitions, error probability α = 5%).

Independent laboratory validation (ILV)

ILV of the method was performed by Bayer AG, Institute for Metabolism Research and Residue Analysis. Recoveries and precisions were determined for 14 active substances at three different fortification levels in the range from 50 ng/mL to 2000 ng/mL according to about 1× LOQ, 2× LOQ, and 10× LOQ in SIM mode. At the highest and at the next lower fortification level, mass spectra of each substance were taken to confirm LODs in full-scan mode.

Results and Discussion

Selection of pesticides for method development

The intention of this method is to identify and quantitate as many toxic pesticides as possible in blood in cases of acute intoxications, which was why active substances from several chemical classes were used for method development. The pesticides addressed are being purchased worldwide and are commonly available. A number of serious human poisonings with

the included pesticides are described in the literature (10–15). We checked, for each pesticide, whether the determination of the parent compound is appropriate to clarify an acute human intoxication. If it was not the case, the pesticide was not included.

Table II. Mass Fragments for Quantitation and Confirmation of Identity in SIM Mode

Active substance	Mass fragments* (m/z)	Relative intensity	Correction factor $F^\dagger$
Bromophos-methyl (IS)	**331**	–	–
Methamidophos	**141**	1.0	2500
	126	0.19	
	111	0.16	
Heptenophos	**250**	1.0	1700
	215	0.79	
	124	4.7	
Bendiocarb	**151**	1.0	210
	166	0.41	
	223	0.16	
Parathion-methyl	**263**	1.0	430
	125	0.53	
	109	0.68	
Methiocarb	**225**	1.0	2000
	168	6.9	
	153	4.9	
Fenthion	**278**	1.0	130
	125	0.15	
	109	0.14	
α-Endosulfan	**243**	1.0	3500
	241	1.4	
	339	1.0	
Fenamiphos	**303**	1.0	310
	260	0.26	
	217	0.22	
β-Endosulfan	**195**	1.0	2200
	237	0.92	
	267	0.75	
Triazophos	**161**	1.0	340
	162	0.75	
	313	0.32	
Azinphos-methyl	**160**	1.0	4000
	132	0.85	
	77	0.72	
Pyrazophos	**221**	1.0	240
	232	0.37	
	373	0.42	
Fluquinconazole	**340**	1.0	90
	341	0.20	
	342	0.34	
β-Cyfluthrin	**165**	1.0	1800
	163	1.6	
	226	0.74	
Deltamethrin/ tralomethrin	**253**	1.0	2200
	251	0.52	
	181	0.70	

* Preferred mass fragment in bold type.
† See equation 1.

Blood extraction

Three different methods were tested for blood extraction. Using solid-phase extraction with C_{18} material, poor recoveries were observed for methamidophos, deltamethrin, and β-cyfluthrin. Because of its high hydrophilicity, methamidophos was not retained by the sorbent. Binding to plasma proteins seemed to be responsible for low recoveries of the pyrethroids. Classical liquid–liquid extraction was inappropriate because of the formation of emulsions. The use of kieselguhr columns as a solid sorbent for the aqueous phase made it possible to avoid this phenomenon completely.

Co-elution of water was not observed under the described conditions. This may occur, if the kieselguhr column is overloaded by the aqueous phase. For this reason the lower part of the column should not yet be loaded after supernatant obtained from deproteinization of the blood has penetrated into the kieselguhr column. In this case the amount of acetone for deproteinisation can be reduced up to 1.2 mL (16).

The kieselguhr columns were cleaned prior to use. For this purpose 2 mL of water were drawn into the column. After 30 min, elution was performed with 5 mL of eluent I and 5 mL of eluent II. The aqueous phase was removed by drawing 10 mL of acetone through the column. The columns were dried by applying vacuum and drawing air through for several minutes. The cleaned columns can be stored at room temperature in a sealed container.

As mentioned previously, hemolyzation of whole blood was necessary to remove active substances from the erythrocytes. Normally distilled water or organic water-miscible solvents are used for this step. Both methods result in a dilution of the sample which requires increased amounts of kieselguhr and eluents in the subsequent extraction step. For these reasons, hemolyzation was performed by ultrasonic destruction of the blood cells.

A number of eluent systems were tested to obtain good recoveries for many pesticides. On the one hand, highly polar eluents were necessary to give a satisfactory recovery for methamidophos, while at the same time avoiding coextraction of water and blood components. Best results were observed with ethyl acetate/dichloromethane (2:1, v/v); n-hexane was suitable for all lipophilic pesticides. The eluent sequence polar–unpolar was used to push not more than 5 mL of the polar eluent through the column.

By applying an internal standard, exact adjustment of the final sample volume prior to GC analysis could be avoided.

Chromatography

The carbamates methiocarb and bendiocarb are sensitive to thermal decomposition in the GC injector. This effect was completely suppressed by optimizing the PTV program. Additionally the sample matrix supported the thermal stability of the carbamates. Unlike methiocarb and bendiocarb, tralomethrin decomposed always completely to deltamethrin. β-Cyfluthrin consists of two diastereomers which could not be separated. The isomer with the higher response factor was used for quantitation. The response factor of azinphos-methyl depends on the age of the chromatography column. For the determination of this active substance at a level near the LOQ, the use of a "new" column is recommended.

In SIM-mode all active substances were entirely separated from matrix components (Figure 2). In full-scan mode only methiocarb, fenamiphos, and α-endosulfan interfered with fatty acids (Figure 3). In these cases extraction of preferential mass fragments facilitated taking full-scan mass spectra. Fatty acids as such (not in form of their methyl esters) were characterized by wide peaks in gas chromatograms.
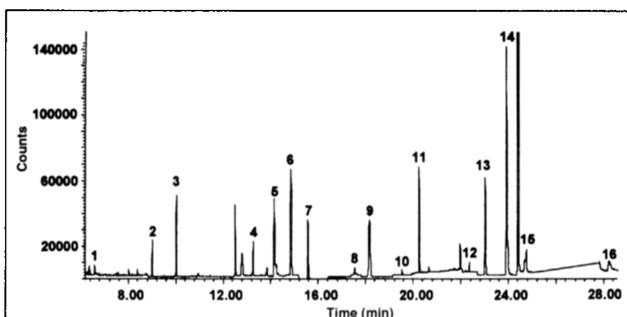
Sterols as major matrix components dominated the full-scan chromatograms of blood samples. Their low volatility had to be taken into consideration in optimizing the oven program.

The retention time of the internal standard was used as a basis for calculating relative retention times for each pesticide. The latter ones were constant even after a change of the chromatography column. The potential use of peak-height ratios to estimate blood levels of actives substances without prior calibration is a further benefit of the application of an internal standard.
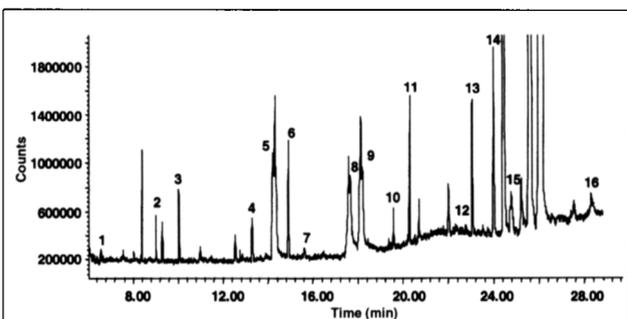
Precision, recoveries, and linearity

The method has an excellent precision, with standard deviations—averaged over the concentration range—between 4% and 8% (Table III).

Recoveries were between 74% for tralomethrin and 97% for

α-endosulfan and fluquinconazole. Because of its relatively high hydrophilicity, methamidophos had a recovery of only 51%, although it was constant over all concentration ranges, thus allowing respective correction of blood levels. This low recovery is the consequence of compromises necessitated by the development of the multimethod. The results of the in-house method validation were completely confirmed by the ILV.

Table III. Recoveries

Active substance	Mass fragment (m/z)	Recovery (IHV)*,† Mean (%)	SD (%)	Recovery (ILV)* Mean (%)	SD (%)
Methamidophos	141	51	5	40	4
Heptenophos	250	90	6	81	5
Bendiocarb	151	94	6	87	3
Parathion-methyl	263	88	4	89	2
Methiocarb	225	96	6	83	5
Fenthion	278	93	5	90	3
α-Endosulfan	243	97	7	90	3
Fenamiphos	303	94	6	93	3
β-Endosulfan	195	95	8	91	4
Triazophos	161	96	6	96	7
Azinphos-methyl	160	88	7	95	5
Pyrazophos	221	95	5	96	3
Fluquinconazole	340	97	7	94	3
β-Cyfluthrin	165	89	7	80	4
Deltamethrin	253	90	8	80	5
Tralomethrin	253	74	5	–‡	–‡

* n = 5 samples at each fortification level, mean of all levels.
† Abbreviations: IHV, in-house method validation; ILV, independent laboratory validation; and SD, standard deviation.
‡ not determined.

Figure 2. SIM chromatogram of a whole blood sample spiked with 0.2 μg/mL of each of the active substances. Peak identification: 1, methamidophos; 2, heptenophos; 3, bendiocarb; 4, parathion-methyl; 5, methiocarb; 6, fenthion; 7, bromophos-methyl; 8, α-endosulfan; 9, fenamiphos; 10, β-endosulfan; 11, triazophos; 12, azinphos-methyl; 13, pyrazophos; 14, fluquinconazole; 15, β-cyfluthrin; and 16, deltamethrin.

Table IV. Limits of Detection, Limits of Quantitation, and Linearity in SIM Mode

Active substance	Mass fragment (m/z)	LOD* (ng/mL)	LOQ (ng/mL)	Concentration range (ng/mL)	Correlation coefficient (r)
Methamidophos	141	50	100	100–4000	0.993
Methiocarb	225	30	50	50–4000	0.999
Heptenophos	250	25	50	50–4000	0.999
Bendiocarb	151	25	50	50–4000	0.998
Parathion-methyl	263	40	50	50–4000	0.999
Fenthion	278	30	50	50–4000	0.999
α-Endosulfan	243	30	50	50–2000	0.998
β-Endosulfan	195	40	60	50–2000	0.998
Fenamiphos	303	20	30	50–4000	0.998
Triazophos	161	40	50	50–4000	0.998
Azinphos-methyl	160	30	40	50–4000	0.994
Pyrazophos	221	40	50	50–4000	0.996
Fluquinconazole	340	30	50	50–4000	0.993
β-Cyfluthrin	165	70	100	100–4000	0.993
Deltamethrin/ tralomethrin	253	130	200	200–4000	0.992

*Abbreviations: LOD, limit of detection; LOQ, limit of quantitation.

Figure 3. Total ion chromatogram in full-scan mode of a whole blood sample spiked with 2 μg/mL of each of the active substances. Peak identification: 1, methamidophos; 2, heptenophos; 3, bendiocarb; 4, parathion-methyl; 5, methiocarb; 6, fenthion; 7, bromophos-methyl; 8, α-endosulfan; 9, fenamiphos; 10, β-endosulfan; 11, triazophos; 12, azinphos-methyl; 13, pyrazophos; 14, fluquinconazole; 15, β-cyfluthrin; and 16, deltamethrin.
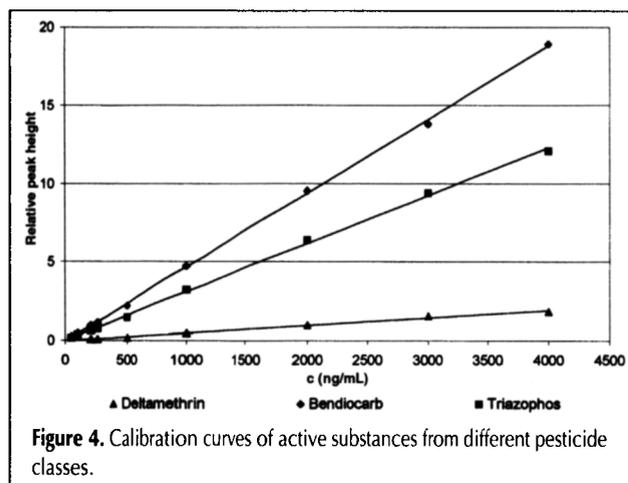
Figure 4. Calibration curves of active substances from different pesticide classes.

For all active substances tested, the method was linear within the tested concentration range with correlation coefficients (r) between 0.992 and 0.999 (Table IV and Figure 4).

Performance, LOD, and LOQ

The whole method has been optimized with regard to speed and practicability and can be easily performed within 120 min. It provides identification of the toxicants by their mass spectra at concentrations that can be expected to occur during acute intoxications. LODs in full-scan mode as defined previously are summarized in Table I. They were completely confirmed by the ILV.

The method also offers limits of quantitation of at least 1% of the LD_{50} values which are considered to be sufficient in cases of acute human pesticide poisoning. LODs and LOQs in SIM mode are summarized in Table IV. At the LODs, confirmation of identity was possible from the intensity ratio of three characteristic fragments (Table II). Recoveries and precisions determined in the ILV at the LOQ levels completely corresponded to the results of the in-house method validation. Mass spectrometry is the only established method for both identification and quantitation of substances. The use of other detection techniques, GC–ECD for organochlorines (17) for example, leads to LOQs much lower than 1% of the LD_{50} values but is not sufficient for undoubted identification.

Acknowledgment

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