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Simultaneous quantification of the organophosphorus pesticides dimethoate and omethoate in porcine plasma and urine by LC–ESI-MS/MS and flow-injection-ESI-MS/MS☆,☆☆

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ABSTRACT

Dimethoate is an organophosphorus toxicant used in agri- and horticulture as a systemic broad-spectrum insecticide. It also exhibits toxic activity towards mammalian organism provoked by catalytic desulfuration in the liver producing its oxon-derivative omethoate thus inhibiting acetylcholinesterase, initiating cholinergic crisis and ultimately leading to death by respiratory paralysis and cardiovascular collapse. Pharmaco- and toxicokinetic studies in animal models help to broaden basic understanding of medical intervention by antidotes and supportive care. Therefore, we developed and validated a LC–ESI-MS/MS method suitable for the simultaneous, selective, precise ($RSD_{intra\text{-}day}$ 1–8%; $RSD_{inter\text{-}day}$ 5–14%), accurate (intra-day: 95–107%; inter-day: 90–115%), and robust quantification of both pesticides from porcine urine and plasma after deproteinization by precipitation and extensive dilution (1:11,250 for plasma and 1:40,000 for urine). Accordingly, lower limits of quantification (0.24–0.49 μg/ml plasma and 0.78–1.56 μg/ml urine) and lower limits of detection (0.12–0.24 μg/ml plasma and 0.39–0.78 μg/ml urine) were equivalent to quite low absolute on-column amounts (1.1–2.1 pg for plasma and 2.0–3.9 pg for urine). The calibration range (0.24–250 μg/ml plasma and 0.78–200 μg/ml urine) was subdivided into two linear ranges ($r^2 \geq 0.998$) each covering nearly two orders of magnitude. The lack of any interfering peak in 6 individual blank specimens from plasma and urine demonstrated the high selectivity of the method. Furthermore, extensive sample dilution causing lowest concentration of potentially interfering matrix ingredients prompted us to develop and validate an additional flow-injection method (FI-ESI-MS/MS). Validation characteristics were as good as for the chromatographic method but sample throughput was enhanced by a factor of 6. Effects on ionization provoked by plasma and urine matrix from 6 individuals as well as in the presence of therapeutics (antidotes) administered in an animal study were investigated systematically underling in the reliability of the presented methods. Both methods were applied to porcine samples derived from an *in vivo* animal study.

1. Introduction

Dimethoate (O,O-dimethyl-S-methylcarbamoyl methylphosphorothioate, MW 229.3 g/mol, LD_{50} rat p.o. 358 mg/kg [1]) (Fig. 1A) belongs to the class of toxic organophosphorus compounds that is commonly used as a systemic broad-spectrum insecticide and acaricide for the protection of numerous crops and tobacco as well as to eradicate household pests [2–5]. In general, organophosphorus pesticides also exhibit low to moderate toxicity to mammals. Once dimethoate has entered the organism, it is enzymatically converted in the intestine wall and liver by monooxygenase CYP1A2 and CYP3A4 enabling desulfuration to its oxon-derivative omethoate (Fig. 1B) thus causing significantly enhanced neurotoxicity [6]. The acute toxic effect of omethoate (O,O-dimethyl-S-methylcarbamoyl methylthiophosphate, dimethoxon, MW 213.6 g/mol, LD_{50} rat p.o. 25 mg/kg [7]) is due to the inhibition of acetylcholinesterase (AChE, EC 3.1.1.7). The resulting dose-dependent symptoms of poisoning (cholinergic crisis) include, e.g. miosis, enhanced secretion of body fluids, and ultimately death by respiratory failure and cardiovascular collapse [8–11].

Swallowing of pesticides is typical for accidental or intentional poisoning especially for committing suicide causing more than 200,000 death per year [3,12–16]. The optimization of standard

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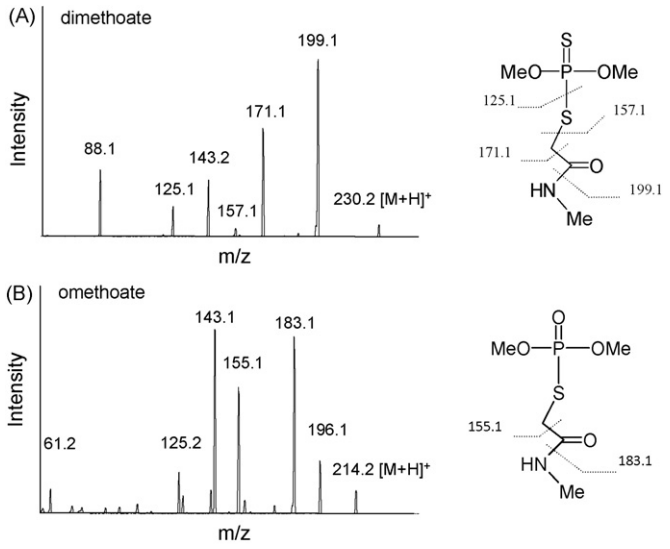

Fig. 1. MS/MS spectra of dimethoate and omethoate generated by ESI and CID. A: dimethoate; B: omethoate. Fragment spectra were obtained by positive electrospray ionization and dissociation by collision with nitrogen in a triple quadrupole machine (API 4000 QTrap). Cleavage sites (dotted lines) were deduced and assigned in analogy to the fragments of dimethoate described before [30,38].

drug regimen [12–14,17,18] in terms of required drug concentrations, period of administration and the structure of active pharmaceutical ingredient still represents a challenge in toxicological and pharmacological research [12,18].

Corresponding pharmaco- and toxicokinetic studies require robust bioanalytical methods that allow precise and accurate quantification of pesticides in body fluids, e.g. plasma and urine.

For detection of dimethoate and omethoate in soil and vegetables, capillary electrophoresis with MS detection after ionization by inductively coupled plasma (CE-ICP-MS) or UV-detection (CE-UV) has been rarely used [19,20]. In contrast, residues of both pesticides in different specimens (olive oil, olives, juice, urine and plasma) have often been quantified by more traditional gas chromatographic (GC) separation coupled to diverse detection techniques, e.g. electron ionization mass spectrometry (GC–EI-MS) [3,13,21,22], flame photometric detection (GC-FPD) [5,8,23] and nitrogen-phosphorus detection (GC-NPD) [21,24]. Nevertheless, GC techniques are not favourable for compounds that are polar, non-volatile or thermally labile as evident for both dimethoate ($\log P$ 0.78; vapour pressure at 25 °C: 1.1×10^{-3} Pa; decomposition close to melting point at 43–45 °C) [25] and omethoate ($\log P$ −0.74; vapour pressure at 25 °C: 3.3×10^{-3} Pa; decomposition at boiling point at 135 °C) [26]. In addition, the use of GC typically requires water-free sample injection causing more laborious and time-consuming sample preparation steps.

As reviewed recently, the most promising alternative current technique for the analysis of organophosphorus compounds overcoming the drawbacks mentioned above is based on liquid separation combined with electrospray ionization followed by tandem-mass spectrometric detection (LC–ESI-MS/MS) [9]. However, so far these procedures have mostly been applied to non-body fluids, e.g. leaves [27], fruit juice [28], vegetables [29,30], olive oil [22,30] or water [31]. Only in some cases these procedures were used quantitatively for urine [32] or animal tissues [33].

Therefore, we developed a LC–ESI-MS/MS and a much faster flow-injection procedure (FI-ESI-MS/MS) to quantify dimethoate and omethoate in plasma and urine. To the best of our knowledge this is the first time that both compounds were measured simultaneously by the referred techniques in biological specimens from porcine origin.

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN, gradient grade), water (LiChrosolv) and formic acid (FA, Uvasol) were purchased from Merck (Darmstadt, Germany). Dimethoate (CAS-No. 60-51-5) and omethoate (CAS-No. 1113-02-6) were delivered by Dr. Ehrenstorfer (Augsburg, Germany) in a purity of 99% and 97%, respectively. Porcine EDTA-plasma used for blank samples and standards was generated from fresh pig blood from a local slaughterhouse. Porcine urine used for blank samples and standards was taken from study pigs prior to poisoning and therapeutic treatment. For sample dilution a mixture comprising of HPLC solvent A–solvent B (80:20, v/v) was used (80:20-mix). Pralidoxime (2-PAM) chloride (CAS-No. 51-15-0) and atropine (free base, CAS-No. 51-55-8) were delivered by Sigma–Aldrich (St. Louis, MO, USA) in a purity of 99.6% (HPLC) and ≥98% (TLC), respectively.

2.2. HPLC and ESI-MS equipment

The HPLC system consisted of two pumps, an autosampler, column oven, and controller from Perkin Elmer, Rodgau-Jügesheim, Germany (PE 200 series) that was coupled to an electrospray ionization mass spectrometer (API 4000 QTrap, Applied Biosystems, Darmstadt, Germany) via a 10-port valve (model EHMA, Vici Valco Instruments, Houston, TX, USA). HPLC system and mass spectrometer were controlled by the Analyst 1.4.2 software (Applied Biosystems) and used for LC–MS/MS and flow-injection analysis.

2.3. LC–ESI-MS/MS analysis

Chromatography was performed at 30 °C with a flow rate of 1 ml/min on an Atlantis T3 C18 column, 5 μm, 150 mm × 4.6 mm I.D. (Waters, Eschborn, Germany) protected by a poly(ether etherketone)/poly tetrafluoroethylene (PEEK/PTFE) filter, 5 μm (Chromatographie-Handel Müller, Fridolfing, Germany). Solvent A (0.1%, v/v, FA in water) and solvent B (ACN/water 80:20, v/v; 0.1%, v/v, FA) were applied as mobile phase. Following an equilibration period of 2 min under starting conditions a 100 μl sample volume was injected and separated in gradient mode: time [min]/B[%]: 0/25; 3/45; 3.5/60; 6/60; 6.2/85 (analytical run). Subsequently, a washing step was performed to clean the injection system and column (washing step). Within one washing step three volumes of a neat solvent (ACN/water 80:20, v/v; 70 μl each) were injected during the following gradient program: time [min]/B[%]: 0/85; 5/85; 5.5/25; 6/25. Mass spectrometric detection of the analytical run was monitored in the positive multiple reaction mode (MRM) from 1.7 to 6.0 min after injection by switching the 10-port valve from waste to the mass spectrometer. The following settings were used to detect dimethoate and omethoate: ionization spray voltage 3000 V, curtain gas 1.72×10^5 Pa (25 psi), heater gas (GS1) 4.83×10^5 Pa (70 psi), turbo ion spray gas (GS2) 4.14×10^5 Pa (60 psi), gas temperature (TEM) 700 °C, entrance potential (EP) 10 V, and dwell time 50 ms. Gas pressure (nitrogen) for collision-activated dissociation (CAD) was adjusted to medium setting. Pesticide specific settings for transition, collision energy (CE), declustering potential (DP) and collision cell exit potential (CXP) were as follows: dimethaote m/z 230.2 → m/z 199.1 (CE 15 V, DP 46 V, CXP 14 V) and omethaote m/z 214.2 → m/z 183.1 (CE 17 V, DP 41 V, CXP 12 V). All samples were measured in duplicate.

2.4. Flow-injection-ESI-MS/MS analysis (FI-ESI-MS/MS)

Sample volumes of 100 μl were injected without using a chromatographic column to be directly transferred into the mass spectrometer at an isocratic flow of 1 ml/min (solvent A–solvent B 50:50, v/v). Dimethoate and omethoate were monitored within a 1-min-period after injection under the same MRM conditions as described above for the LC–ESI-MS/MS method with the exception of the dwell time which was set to 200 ms. All samples were analyzed by duplicate measurement.

2.5. Preparation of samples and standards

2.5.1. Pesticide stock solutions

Initial solutions of both pesticides were prepared separately in acetonitrile at 2 mg/ml. Equal volumes of both solutions were combined to generate the stock solution (1 mg/ml each), which was stored at −20 °C being stable for at least several months [32].

2.5.2. Plasma samples and blank

A 50 μl volume of porcine plasma was precipitated by the addition of 100 μl ACN at ambient temperature (step 1, sample dilution 1:3). Following vigorous shaking and centrifugation at 12,000 × *g* 100 μl of the supernatant were diluted with 500 μl of the 80:20-mix (step 2, sample dilution 1:18). An aliquot of 30 μl of step 2 was mixed with additional 720 μl of 80:20-mix (step 3, sample dilution 1:450). At least, 30 μl of step 3 were diluted by adding 720 μl of the 80:20-mix (step 4, sample dilution 1:11,250) to be measured in duplicate either by LC–ESI-MS/MS or by FI-ESI-MS/MS.

2.5.3. Plasma standards and quality control samples

Nine porcine plasma standards for an external calibration curve ranging from 250 μg/ml (Std A) to 0.98 μg/ml (Std I) were produced by serial dilution (1:2; 100 μl + 100 μl) of plasma concomitantly spiked with dimethoate and omethoate. Standard A was prepared by adding 50 μl of the pesticide stock solution to 150 μl plasma. Preparation by precipitation and dilution of supernatant was performed by the standard protocol as described above (sample dilution 1:11,250).

Quality control samples (QCs) containing dimethoate and omethoate concomitantly were prepared in porcine plasma at four concentration levels (1.0, 5.0, 40.0 and 100.0 μg/ml) and stored as aliquots of 50 μl at −80 °C prior to use. All QCs were prepared freshly for each run following the procedure above.

2.5.4. Urine samples and blank

A 50 μl volume of porcine urine was diluted with 200 μl 80:20-mix (step 1, sample dilution 1:5), followed by a second dilution step mixing 50 μl of step 1 with 950 μl 80:20-mix (step 2, sample dilution 1:100). Subsequently, a 50 μl aliquot of step 2 was additionally mixed with 950 μl 80:20-mix (step 3, sample dilution 1:2000). Finally, a 50 μl volume of step 3 was diluted again with 950 μl 80:20-mix (step 4, sample dilution 1:40,000) to be analyzed either by LC–ESI-MS/MS or by FI-ESI-MS/MS.

2.5.5. Urine standards and quality control samples

Eight porcine urine standards covering a concentration range from 200 μg/ml (Std A) to 1.56 μg/ml (Std H) were generated by serial dilution (1:2; 100 μl + 100 μl) of urine concomitantly spiked with dimethoate and omethoate. Std A was prepared by adding 50 μl of the pesticide stock solution to 200 μl urine. Preparation by dilution was carried out by the standard procedure described above (sample dilution 1:40,000).

QCs containing dimethoate and omethoate were prepared in porcine urine at three concentration levels (5.0, 50.0 and 150.0 μg/ml) and stored as aliquots of 50 μl at −80 °C prior to use. All QCs were prepared freshly for each run following the procedure above.

2.6. Characteristics of LC–ESI-MS/MS and FI-ESI-MS/MS performance

Investigations to characterize and validate the quantitative procedures were performed for both matrices (porcine plasma and urine) and for both methods (LC–ESI-MS/MS and FI-ESI-MS/MS) separately. Below we describe the principle procedures to determine the quality criteria.

2.6.1. Linear range, lower limit of quantification and detection, precision, and accuracy

Standards in porcine plasma and urine additionally including lower concentrated standards (down to 0.122 μg/ml plasma and 0.195 μg/ml urine) were prepared ($n = 5$) and analyzed as described above to determine the linear range.

Lowest concentrated standards were measured in triplicate at 5 days to elaborate LOQ and LOD. The LOQ was defined as the concentration not exceeding either 20% RSD or 80–120% accuracy. The LOD was set to be the lowest concentrated standard allowing unambiguous qualitative analyte detection in all replicates.

Precision and accuracy of the method were assessed by intra- and inter-day validation over 5 non-consecutive days using QCs. The intra-day accuracy and precision were evaluated by processing the differently concentrated QCs in 5 replicates ($n = 5$). The concentrations of the QCs were calculated from daily calibrations curves. The inter-day accuracy and precision were determined by analyzing QCs in triplicate at 5 days. Accuracy was calculated as the relative ratio between the determined concentration and the nominal value. Relative standard deviation was used as a measure of precision. Means and standard deviations of concentrations were calculated by external calibration with the peak area method.

2.6.2. Selectivity of LC–ESI-MS/MS and FI-ESI-MS/MS for plasma and urine analysis

Selectivity of both methods for both porcine matrices was investigated by analyzing blank plasma and blank urine of 6 different individuals following the standard procedure described above.

2.6.3. Ruggedness

The ruggedness of the method, describing the influence of slightly varying parameters during analysis, was characterized by changing four selected parameters of the standard protocol (variant A) to slightly lower (variant B) and slightly higher settings (variant C). Ionization spray voltage was changed from 3.0 to 2.85 and 3.15 kV, HPLC flow from 1.0 to 0.95 ml/min and 1.05 ml/min, gas spray (GS1/2) from $4.83 \times 10^5/4.14 \times 10^5$ Pa (70/60 psi) to $4.55 \times 10^5/3.93 \times 10^5$ Pa (66/57 psi) and $5.1 \times 10^5/4.34 \times 10^5$ Pa (74/63 psi), and declustering potential from 46/41 V (dimethoate/omethoate) to 43/38 V and 49/44 V.

Plasma standards (Std G, 3.91 μg/ml and Std C, 62.5 μg/ml) as well as urine standards (Std G, 6.25 μg/ml and Std C, 100 μg/ml) were analyzed using the LC–ESI-MS/MS and FI-ESI-MS/MS parameter conditions of variants A, B, and C in triplicate and were quantified by a calibration curve measured under standard conditions (variant A). Resulting precision and accuracy were evaluated.

2.6.4. Recovery and matrix effects of plasma and urine in LC–ESI-MS/MS

Recovery and effects on the ionization efficacy of both pesticides analyzed from porcine plasma and urine by LC–ESI-MS/MS were investigated according to a procedure described earlier [34,35]. In brief, three sets of calibration curves were prepared differing

in their preparation and matrix composition: set 1 was prepared from plasma spiked prior to precipitation and further dilutions according to the relevant standard protocol, set 2 was made by spiking the supernatant of precipitated blank plasma, and set 3 was made from neat solvent not containing any compounds of the plasma matrix. Comparison of the slopes of the three linear calibration curves allowed the calculation of both averaged recovery (set 1/set 2) and effects on the ionization process (set 2/set 3). In addition, differing plasma sample dilutions (1:9, 1:90, 1:900, and 1:9000) and urine sample dilutions (1:100, 1:1000, 1:10,000, 1:20,000, 1:40,000, and 1:80,000) were analyzed to characterize matrix concentration-dependent effects on ionization.

2.6.5. Matrix effects of plasma and urine in FI-ESI-MS/MS

To investigate the effect of porcine urine-derived matrix compounds on the ionization efficacy of dimethoate and omethoate a series of standard curves was produced. Each curve comprised of 5 standards ranging from 0.625 to 10 ng/ml in the readily diluted injection solution but was prepared either in neat solvent or in 8 different urine dilutions: 1:100, 1:1000, 1:5000, 1:10,000, 1:20,000, 1:40,000, 1:80,000, and 1:160,000. Corresponding experiments were performed for prepared plasma matrix used in dilutions of 1:100, 1:1000, 1:5000, and 1:10,000.

In addition, the influence on ionization of one pesticide on the other was characterized by corresponding standard curves which were only prepared with single pesticides. The resulting slope was compared to that obtained from standards containing both pesticides. All standard solutions were analyzed in duplicate by FI-ESI-MS/MS.

2.6.6. Matrix effects of individual specimens and of antidotes

2.6.6.1. Effects of individual specimens. Plasma and urine blank samples from six individuals were spiked in triplicate ($n = 3$) with both pesticides in concentrations relevant for the animal study (16.7 μg/ml each in plasma and 20.8 μg/ml each in urine) prior to sample preparation and duplicate analysis by LC–ESI-MS/MS and FI-ESI-MS/MS following the standard procedure. Resulting peak areas were compared to those from reference samples ($n = 3$) containing the pesticides in neat solvent (80:20-mix).

2.6.6.2. Effects of antidotes. Blank plasma and urine samples were spiked with both pesticides (5 μg/ml each in plasma and 41.6 μg/ml each in urine) and were additionally spiked with antidotes used in the animal study (2-PAM and atropine) prior to analysis by LC–ESI-MS/MS and FI-ESI-MS/MS. Two different concentrations of therapeutics were tested: one lower similar to the maximum measured in the animal study and a second one being at least twice as high. Samples were spiked with single antidotes or in combination. In plasma 41.7 and 20.8 ng/ml were adjusted for atropine and 41.7 and 8.3 μg/ml for 2-PAM. Urine was spiked with 1.7 and 0.85 μg/ml atropine and 833 and 417 μg/ml 2-PAM. Spiking was done in triplicate and samples were measured in duplicate each. Resulting peak areas were compared to references ($n = 3$) containing the pesticides in neat solvent (80:20-mix) without any antidote.

2.6.7. Stability of dimethoate and omethoate samples

2.6.7.1. Thaw-and-freeze cycles. Plasma QC samples (5, 40 and 100 μg/ml in triplicate) and urine QC samples (5, 50, 150 μg/ml in triplicate) were stored for 24 h at −20 °C prior to complete thawing at room temperature. Subsequently, samples were re-frozen for 24 h and completely thawed again. This procedure was repeated 4 times in total prior to analysis by FI-ESI-MS/MS. Resulting peak areas were compared to those obtained from QC samples stored without thawing.

2.6.7.2. Stability in the autosampler. Stability of prepared plasma samples stored in the autosampler for 12 h at room temperature was investigated by preparing two QCs (5 and 40 μg/ml). In contrast to the standard procedure the last dilution step was performed by mixing 150 μl dilution from step 3 with 3600 μl 80:20-mix. Volumes derived from both QCs were divided into 12 aliquots of 300 μl each, set into the autosampler and analyzed alternately in duplicate by LC–ESI-MS/MS (two measurements per QC per hour). Resulting peak areas of both pesticides and their corresponding area ratio were plotted against the storage time.

2.7. Animal study

The study was performed in the UK under Home Office License number 60/3757 in male Göttingen minipigs (Ellegaard Göttingen Minipigs ApS, Dalmose, Denmark) after institutional ethics review. The animals were treated in accordance with the Animals (Scientific Procedures) Act of 1986.

Pigs were anaesthetized with isoflurane and mechanically ventilated. Central arterial and venous lines as well as urinary catheter were placed by cutdown. Animals were poisoned with 2.5 ml/kg body weight of an emulsifiable concentrate of dimethoate (400 g/l, EC 40, BASF plc, Cheadle Hume, UK) applied by gavage. Depending on clinical symptoms, the animals were treated following standard human treatment protocols with atropine and pralidoxime [36]. Blood samples were taken from the arterial line into a syringe at hourly intervals over a 12-hour period. Blood was immediately placed in a Sarstedt EDTA plasma tube which was spun at 2500 × *g* for 7 min. Plasma was taken off and placed at −22 °C prior to shipment on dry ice. Urine samples were taken at corresponding time points from the indwelling catheter. Urine was stored at and shipped under conditions as described for plasma samples.

3. Results and discussion

Depending on the chemical structure defining, e.g. stability, reactivity, and lipophilicity, diverse organophosphorus pesticides may exhibit significant differences in their toxicological behaviour. Therefore, individual alterations in absorption, distribution, metabolization, and elimination demand for appropriate optimum medical treatment [3,5,10,12–15,37]. To characterize such properties of ingested dimethoate, the course of poisoning was elaborated and characterized in an animal study using minipigs. Analysis of relevant samples demanded rather selective than sensitive methods as obvious from typical concentrations of dimethoate and omethoate found in fatally poisoned patients ranging from 2 to 300 μg/ml plasma or urine [3,13–15]. Therefore, we developed, validated and applied two ESI-MS/MS-based techniques which allow simultaneous analysis of both pesticides in body fluids.

3.1. Mass spectrometric characterization of pesticides

First the mass spectrometric behaviour of dimethoate and omethoate was characterized during collision-induced dissociation (CID) of the protonated precursor ion after positive electrospray ionization in infusion experiments. Fig. 1 shows the fragment spectrum and the corresponding chemical structure of each pesticide. Dotted lines indicate cleavage sites of major fragments which were deduced according to assignments for dimethoate fragmentation presented by Mazotti et al. [30] and Thurman et al. [38] very recently. However, fragmentation of omethoate by ESI-CID has not been reported in the literature in detail so far. Therefore, we restricted fragment ion assignment to the both most obvious and commonly described signals (m/z 183.1 and m/z 155.1, Fig. 1B). Future mass spectrometric investigations should help to elucidate the relevant fragmentation pathways.

Transition to the most abundant product ion of each pesticide was elaborated by the automatic tuning mode of the mass spectrometer and used for quantification in the MRM mode: dimethoate (230.2 → 199.1, loss of methylamine; Fig. 1A) and omethoate (214.2 → 183.1, loss of methylamine; Fig. 1B).

3.2. Sample preparation

As reviewed recently, a couple of conventional (e.g. precipitation; solid-phase extraction, SPE; liquid–liquid extraction, LLE) and sophisticated modern methods for sample preparation (e.g. membrane-assisted solvent extraction, MASE; stir-bar-sorptive extraction, SBSE; molecularly imprinted polymers, MIP) have been introduced to extract organophosphorus compounds from samples of diverse origin prior to quantification [9]. For the analysis of plasma and urine the conventional techniques were predominantly applied so far. Therefore, we chose protein precipitation by the addition of ACN to deproteinize plasma as an easy, rapid, cheap, and effective alternative to the more laborious LLE or SPE procedures. As discussed in Section 3.3.2.1 recovery was nearly quantitative for both dimethoate (94 ± 3%) and omethoate (98 ± 2%). Due to high concentrations of both pesticides in plasma of poisoned pigs, subsequent dilution of the supernatant was necessary (1:11,250 in total) thereby optimizing analyte-concentration for measurement and minimizing potentially deteriorating effects of matrix compounds.

The dilution procedure was performed by four consecutive steps instead of a single one due to reasons of practicability although causing an increased number of manual working steps. Handling of larger volumes of the higher concentrated solutions (30 μl) appeared to be more comfortable and robust than transferring smallest volumes of about 1–2 μl, especially when considering the reduced viscosity of the ACN containing solvent. Furthermore, this procedure allowed to minimize the consumption of the solvent mixture used to dilute the sample: 1.94 ml (for a plasma sample) instead of 7.5 ml when performing one dilution step starting with 2 μl supernatant. At least, our procedure allowed using the 1.1 ml HPLC glass vials for each dilution step, which could easily be sealed with a screw cap and stored in small boxes.

Accordingly, preparation of less complex but also highly concentrated urine samples also merely comprised of several dilution steps to obtain a total dilution of 1:40,000 ready for measurement.

For systematic characterization effects of different dilution factors on the pesticides ionization process as well as effects derived from individual specimens were determined as discussed below in Section 3.3.2.

3.3. Analysis by LC–ESI-MS/MS

Pesticide analytes were monitored in MRM mode to achieve optimum detection selectivity thereby favouring the simple and unselective sample preparation method. Chromatographic separation of the polar omethoate and the more lipophilic dimethoate was performed on a modified C18 column (Atlantis T3) highly appropriate for polar analytes. The ACN-gradient applied allowed elution of nearly non-retarded very polar or ionic matrix ingredients, e.g. salts, amino acids, carbohydrates (dead time 1.5 min) prior to the elution of omethoate (t_R 2.6 min) followed by dimethoate (t_R 5.5 min) (Fig. 2A). It was observed that dimethoate similar to other small molecules [39,40] adsorbs to contacted solid surfaces provoking contamination of, e.g. sample loop and injection needle. To minimize such effects, which might cause cross contamination of samples, the sample loop was permanently integrated into the eluent flow for the total run time of the chromatographic separation thereby being washed effectively. Whereas the inner surface of the injection needle was washed immediately after injection by a 4-times flushing step using aqueous 2-propanol (50:50, v/v) the outer surface remained untreated. Therefore, we performed a washing step subsequent to each analytical run which included 3 consecutive injections of a neat solvent (ACN/water 80:20, v/v) (Fig. 2B). Dipping the injection needle into this neat solvent helped to remove pesticide residues at the inner and outer surface of the injection needle. Fig. 2B demonstrates the absence of the polar omethoate (log P −0.74) [26] and the presence of the more hydrophobic dimethoate (log P 0.78) [25] in the washing step. The relative height of each dimethoate peak represents nearly 1.6% of the former analytical run shown in Fig. 2A.

In addition, the high content of organic modifier in the mobile phase (85%, v/v, solvent B) applied for this washing step allowed the elution of the more lipophilic matrix-derived compounds from the analytical column (e.g. peptides, remaining proteins, fatty acids and phospholipids) (Fig. 2B). However, following this procedure in initial experiments the extent of potential cross contamination and column pollution could be reduced even though not excluded. To achieve additional reduction of potential cross contamination, chromatographic runs injecting neat solvent were performed following highly concentrated standards prior to the analysis of samples with low concentrations. In addition, after each set of samples the outer surface of the injection needle was cleaned with a 2-propanol-moisted wipe thereby effectively removing any potential residues. Accordingly, blank samples for example measured after Std A or B were free of any detectable traces of both pesticides (Fig. 2C and E).

3.3.1. Characteristics of LC–ESI-MS/MS

The LC–MS/MS procedure was validated for the analysis of plasma and urine as applied to samples of the animal study in total dilutions of 1:11,250 and 1:40,000, respectively.

3.3.1.1. Linear range. As summarized in Table 1 the calibration ranges reached from 0.24 to 250 μg/ml in plasma and 1.56 to 200 μg/ml in urine corresponding to absolute on-column-amounts in the pg-range. Best linear regressions ($r^2 \geq 0.998$) were obtained when subdividing the ranges into a lower (l) and an overlapping upper range (u) both covering nearly two orders of magnitude providing optimum accuracy.

3.3.1.2. Precision (RSD) and accuracy (intra- and inter-day). Intra- and inter-day precision (RSD, 1–14% for both pesticides) and accuracy (90–115% for both analytes) were determined from replicate measurements of QCs covering the lower, middle and higher concentration range in plasma and urine. Despite relinquishment of internal standards precision and accuracy were of satisfying and suitable quality appropriate for analysis of *in vivo* samples (Table 1).

3.3.1.3. Selectivity, LOQ and LOD. Representative chromatograms of plasma and urine blank samples are shown in Fig. 2C and E indicating the absence of any interfering compound being detectable by the chosen MRM transitions. Therefore, the HPLC method is of high selectivity allowing pesticide detection without any endogenous interference of similar mass spectrometric behaviour.

The LOQ determined according to the FDA guidelines for bioanalytical method validation [41,42] was found for dimethoate at 0.24 μg/ml plasma and 1.56 μg/ml urine. The corresponding values for omethoate were 0.49 and 0.78 μg/ml, respectively. LOQ and LOD are summarized in Table 1.

In general, no significant differences of quality criteria between dimethoate and omethoate or between both matrices were determined indicating no or negligible impacts of diluted matrix ingredients. However, the use of internal standards as stable isotope labelled analogs or structurally related compounds will

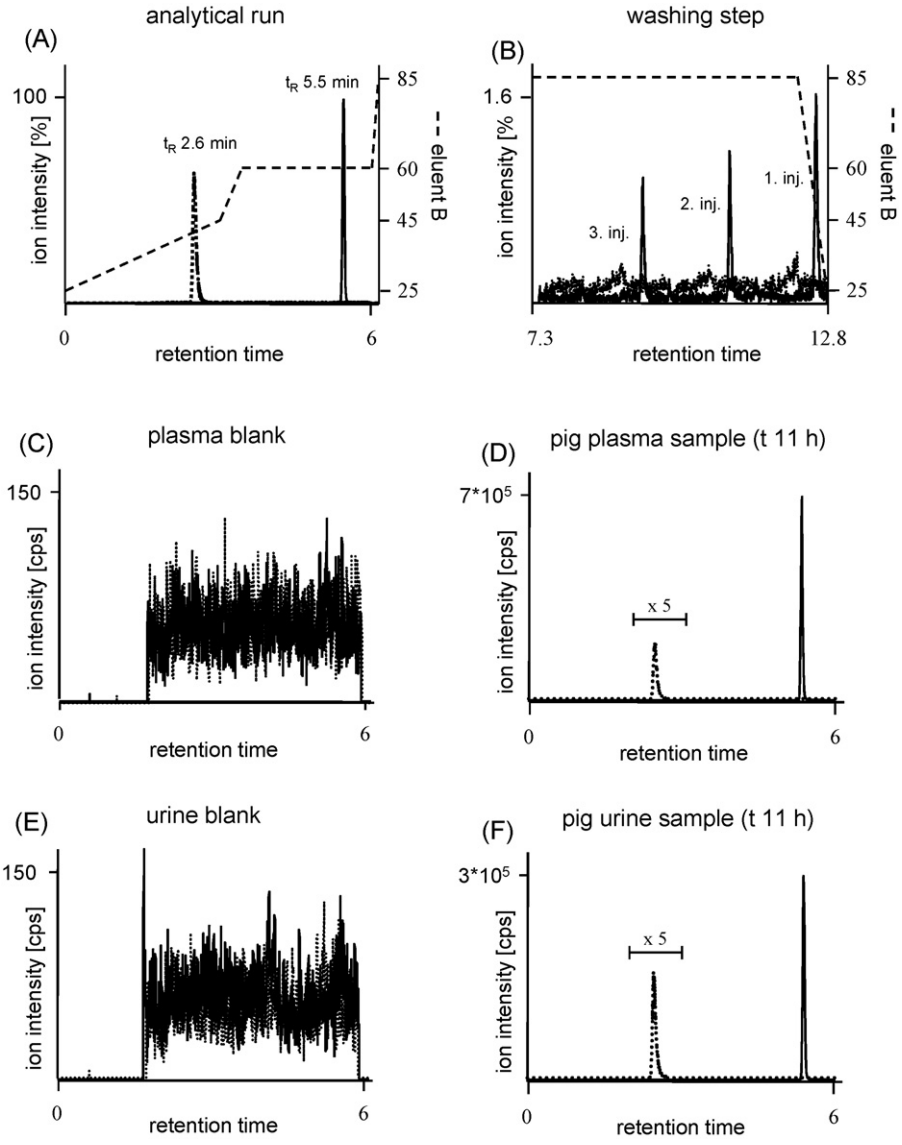

Fig. 2. LC–ESI-MS/MS for simultaneous analysis of dimethoate and omethoate. A: representative chromatogram of plasma standard E, 15.63 μg/ml (analytical run). B: representative chromatogram of neat solvent injected 3-times subsequent to analytical run (washing step). C–F: analytical runs of plasma blank (C), plasma sample from animal study at 11 h (D), urine blank (E), and urine sample from animal study at 11 h (F). Dotted line: omethoate (MRM, 214.2 → 183.1; +ESI, CE 17 V, DP 41 V, CXP 12 V), solid line: dimethoate (MRM, 230.2 → 199.1; +ESI, CE 15 V, DP 46 V, CXP 14 V). Samples were prepared by the standard protocol. Chromatography was performed in the gradient mode at 30 °C as indicated by the dashed line. Column: Atlantis T3 C18, 5 μm, 150 mm × 4.6 mm I.D.; solvent A: 0.1% (v/v) FA, solvent B: ACN/0.1% (v/v) FA 80:20 (v/v); flow: 1 ml/min; sample volume 100 μl; ionization spray voltage: 3000 V; curtain gas: 1.72×10^5 Pa (25 psi); heater gas (GS1): 4.83×10^5 Pa (70 psi); turbo ion spray gas (GS2): 4.14×10^5 Pa (60 psi); gas temperature (TEM): 700 °C; entrance potential (EP): 10 V, dwell time 50 ms; collision gas: nitrogen.

promise to improve quality characteristics and will thus be an option for future experiments.

3.3.1.4. Ruggedness. The LC–MS/MS procedure was demonstrated to be rugged as obvious from slightly altered parameter settings (ionization spray voltage, HPLC flow, gas spray, and declustering potential) impairing neither precision nor accuracy for both analytes (data not shown).

3.3.2. Matrix effects in LC–ESI-MS/MS

3.3.2.1. Recovery and effects of dilution factors. It is well known that efficiency of electrospray ionization in pesticide analysis is affected by, e.g. (i) non-volatile compounds which prevent the analyte from liberation of the droplet to get into the gas-phase, (ii) compounds with high proton affinity, (iii) surface-active substances, (iv) ion-pairing reagents, and (v) salts and ionic compounds [43]. Therefore, it is mandatory to characterize such effects on the analytical performance. Several procedures were introduced in the literature allowing to determine this effect. A valuable approach was reported by Matuszewski analyzing the precision of the slope of calibration curves prepared from the matrix of 5 different lots of biofluids [44]. Slope precisions should not exceed a quite close 3–4% value to be applicable to clinical studies. However, these excellent data may only be achieved when using internal standards for quantification.

Alternatively, we chose a method as introduced earlier [34,35]. Ratios of the slopes of standard curves differing in their preparation procedure and matrix composition allow to determine effects on the ionization process and to calculate the recovery. Intensities of standards prepared in neat solvent are neither affected by matrix interferences nor by incomplete recovery thus typically causing maximum slopes. In contrast, standards prepared in supernatant (SN) will be influenced by matrix compounds (causing suppression or enhancement of ionization) but not by incomplete recovery. At least, standards prepared from plasma spiked prior to precipitation

Table 1
Characteristics of LC–ESI-MS/MS and FI-ESI-MS/MS for quantification of dimethoate and omethoate in porcine plasma and urine.

	LC–ESI-MS/MS		FI-ESI-MS/MS	
	Dimethoate	Omethoate	Dimethoate	Omethoate
Plasma (dilution 1:11,250)				
Linear range [μg/ml]	0.24–15.6 (l) 7.8–250 (u)	0.49–15.6 (l) 7.8–250 (u)	0.24–15.6 (l) 7.8–250 (u)	0.24–15.6 (l) 7.8–250 (u)
Absolute linear range [pg][a]	2.1–138.7 (l) 69.3–2222 (u)	4.2–138.7 (l) 69.3–2222 (u)	2.1–138.7 (l) 69.3–2222 (u)	2.1–138.7 (l) 69.3–2222 (u)
Regression coefficient (r^2)	≥0.998	≥0.998	≥0.999	≥0.999
Precision $(RSD)_{intra\text{-}day}$ [%]	2–7	4–8	3–8	4–9
$Accuracy_{intra\text{-}day}$ [%]	95–105	96–103	95–102	97–106
Precision $(RSD)_{inter\text{-}day}$ [%]	9–14	8–13	8- 11	6–10
$Accuracy_{inter\text{-}day}$ [%]	85–113	83–115	87–110	90–112
LOQ [μg/ml]	0.24	0.49	0.24	0.24
Absolute LOQ [pg][a]	2.1	4.2	2.1	2.1
LOD [μg/ml]	0.12	0.24	0.12	0.12
Absolute LOD [pg][a]	1.05	2.1	1.05	1.05
Urine (dilution 1:40,000)				
Linear range [μg/ml]	1.56–25 (l) 6.25–200 (u)	0.78–25 (l) 3.1–200 (u)	1.56–25 (l) 6.25–200 (u)	1.56–25 (l) 6.25–200 (u)
Absolute linear range [pg][a]	3.9–62.5 (l) 15.6–500 (u)	1.95–62.5 (l) 7.8–500 (u)	3.9–62.5 (l) 15.6–500 (u)	3.9–62.5 (l) 15.6–500 (u)
Regression coefficient (r^2)	≥0.999	≥0.999	≥0.998	≥0.998
Precision $(RSD)_{intra\text{-}day}$ [%]	1–4	2–4	1–6	2–11
$Accuracy_{intra\text{-}day}$ [%]	98–111	98–108	96–106	95–108
Precision $(RSD)_{inter\text{-}day}$ [%]	4–8	5–9	7–13	6–12
$Accuracy_{inter\text{-}day}$ [%]	92–112	91–112	86–109	88–114
LOQ [μg/ml]	1.56	0.78	1.56	1.56
Absolute LOQ [pg][a]	3.9	1.95	3.9	3.9
LOD [μg/ml]	0.78	0.39	0.78	0.78
Absolute LOD [pg][a]	1.95	0.98	1.95	1.95

[a] Absolute amount injected in a 100 μl volume; (l), lower concentration range; (u), upper concentration range; RSD and accuracy data (intra- and inter-day) represent the lowest and highest values determined from replicate measurement of 4 differently concentrated QC samples covering the lower, middle and higher calibration range for each analyte and both matrices as described.

(P) will reflect both the impact of matrix compounds on ionization as well as the loss of analyte due to precipitation.

As expected and illustrated in Fig. 3A, the LC–ESI-MS/MS standard curve of dimethoate in neat solvent (black circle) belongs to the steepest curves. Comparison to congruent standard curves in supernatant of plasma (SN1:9, SN1:90, SN1:900, and SN1:9000) indicated that ion suppression was smaller than 3% being in the range of precision. Furthermore, comparison of supernatant curves (SN1:90, SN1:900, and SN1:9000) with standard curves generated from plasma (P1:90; P1:900; and P1:9000) revealed a recovery rate of 94 ± 3%. To perform these measurements it had been necessary to spike plasma standards with higher concentrations of the pesticide (≥30 ng/ml) thus allowing high dilution (1:90–1:9000) to meet the linear range of the method. Interestingly, when spiking with smaller concentrations a lower recovery of 83 ± 3% was found as calculated from the smaller slope of the shallower standard curve P1:9 (Fig. 3A, black squares). This reproducible effect might be due to interactions with plasma proteins [45–47] potentially causing co-precipitation of the pesticide. However, this phenomenon was of insignificant relevance for the highly concentrated *in vivo* samples and their corresponding calibration standards thus not deteriorating quantitative analysis.

Similar results of quantitative recovery (98 ± 2%) and no ion suppression (≤2%) were obtained for omethoate as demonstrated by identical slopes of congruent standard curves generated from neat solvent, spiked supernatant (SN1:90–SN1:9000) and spiked plasma (P1:90–1:9000) (Fig. 3B).

A very interesting difference to dimethoate was observed for standards containing the highest concentrations of matrix-compounds as derived from a 1:9 dilution of plasma (P1:9, black diamond) and of supernatant (SN1:9, white triangle) (Fig. 3B). Slopes of corresponding linear standard curves were nearly identical but were much steeper (factor 2.3) than that of neat solvent (black circle). These facts proved a rather seldom effect of ionization enhancement (increase in signal intensity) caused by unidentified plasma matrix ingredients and were reproducibly found in additionally tested individual specimens to a comparable extent. However, these interactions could be eliminated by a higher dilution of the sample (≥1:90) thereby reducing signal intensity to the unaffected level of neat solvent (Fig. 3B).

Comparable phenomena of ion suppression or enhancement and incomplete recovery were absent for both pesticides in urine samples due to the less complex matrix and the lack of separating sample preparation steps. Related standard curves were nearly identical (±4%) independent of the dilution used (1:100–1:80,000, data not shown).

3.3.2.2. Matrix effects in individual specimens. To elaborate whether plasma or urine matrices obtained from individual pigs exert different effects on ionization biofluids from six study animals were tested. Plasma and urine were taken from pigs prior to the administration of any drug or poison before starting the animal study.

LC–ESI-MS/MS analyses of pesticide-spiked specimens ($n = 3$) showed that no significant differences in the remaining intensity were found between the six plasma (Fig. 4A, light grey bars) and six urine matrices (Fig. 4B, light grey bars).

Mean and SD of remaining relative intensity for plasma #1–6 was 99.4 ± 2.6% for dimethoate and 101.5 ± 3.0% for omethoate. In urine samples #1–6 the corresponding values were found at 99.5 ± 2.0% for dimethoate and at 100.8 ± 2.1 for omethoate. In addition, these data demonstrate that no significant ion suppression or enhancement occurred as obvious from comparison to the

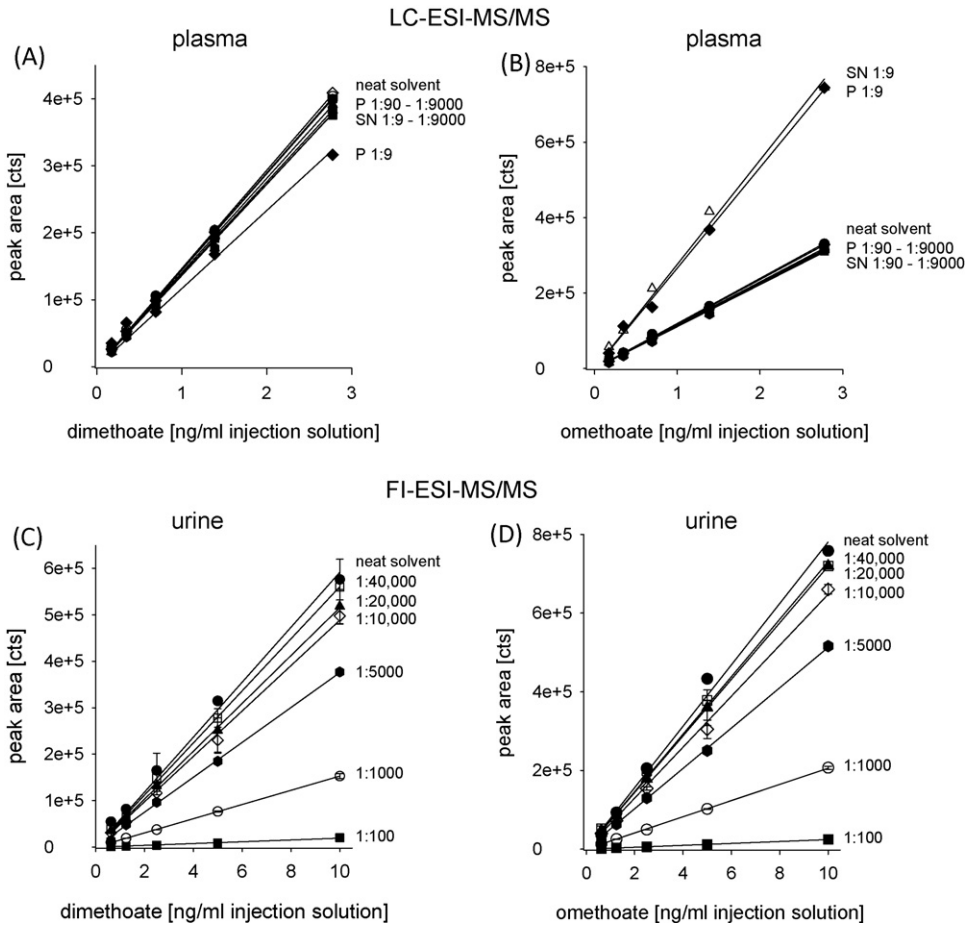

Fig. 3. Recovery and matrix effects on dimethoate and omethoate analysis by LC–ESI-MS/MS and FI-ESI-MS/MS. A: detection of dimethoate in plasma standards by LC–ESI-MS/MS. B: detection of omethoate in plasma standards by LC–ESI-MS/MS. Standard curves were generated in different solvents to determine recovery after plasma precipitation and matrix effects on ionization. P, plasma spiked prior to precipitation and dilution; SN, supernatant of blank plasma spiked prior to dilution; black circle, neat solvent; white square, supernatant 1:9000; black triangle, plasma 1:9000; white diamond, supernatant 1:900; black hexagon, plasma 1:900; white circle, supernatant 1:90; black square, plasma 1:90; white triangle, supernatant 1:9; black diamond, plasma 1:9. C: detection of dimethoate in urine standards by FI-ESI-MS/MS. D: detection of omethoate in urine standards by FI-ESI-MS/MS. Standard curves were generated in differently diluted urine. Black circle, neat solvent; white square, dilution 1:40,000; black triangle, dilution 1:20,000; white diamond, dilution 1:10,000; black hexagon, dilution 1:5000; white circle, dilution 1:1000; black square, dilution 1:100. For reasons of simplicity we do not show the curves obtained for dilution 1:160,000 and 1:80,000 as well as dimethoate or omethoate alone in neat solvent. Mentioned curvatures run nearly identical to the one of neat solvent (black circle) thus documenting no suppressing effects of urine matrix. All measurements by both methods were done in duplicate.

reference samples. Furthermore, no different effects on dimethoate (Fig. 4, blank bars) and omethoate (Fig. 4, hatched bars) were found.

These data revealed that the high dilution factors of the samples (11,250 for plasma and 40,000 for urine) appeared to be appropriate to eliminate individual differences of the matrix composition with respect to their potential effect on ionization.

3.3.2.3. Matrix effects in the presence of antidotes. During the therapy of dimethoate poisoning animals were treated with atropine and 2-PAM which in principle may influence the ionization of both analyte pesticides. Quantification of both therapeutics in body fluids from animals subjected to the study revealed that typical maximum concentrations of atropine were approximately 30 ng/ml in plasma and 1 μg/ml in urine whereas for 2-PAM 8 μg/ml in plasma and about 500 μg/ml in urine were found. Antidotes were measured by methods based on those published recently [48,49].

To elaborate corresponding matrix effects blank plasma and urine was spiked with both pesticides as well as with both antidotes (separately and in combination in two different concentrations) in study-relevant concentrations. Resulting peak areas were compared to reference samples (simply neat solvent). Fig. 4 summarizes the results obtained from plasma (Fig. 4A, light grey bars) and urine (Fig. 4B, light grey bars) indicating the absence of any suppressing effect. The lack of deteriorating influence will be due to chromatographic separation of analyte and antidotes. However, drug retention times were not obvious from the method used and not determined otherwise.

These data pointed out that the chromatographic method is suitable for pesticide quantification in samples from the animal study not being affected by apparent antidote concentrations.

Taken together the presented LC–ESI-MS/MS procedure is highly suitable for selective analysis of samples from dimethoate-poisoned animals providing an appropriate robust linear range of satisfying precision and accuracy. Merely the quite long run times were of slight disadvantage. Therefore, the high dilution factors which reduced potential deteriorating interferences led to the assumption that quantification of dimethoate and omethoate might also be possible without any chromatographic separation just by direct flow-injection. Consequently, we developed and validated an additional ESI-MS/MS-based procedure with an enormously reduced analytical run time. However, renunciation of the chromatographic step provokes coeluting matrix components which potentially might influence analyte ionization and provide interfering isobaric compounds which might also exhibit isobaric ion pairs in MS/MS detection. These limitations were pointed out in detail by Martens-Lobenhoffer et al. underlining the drawbacks of FI analysis of endogenous concentrations of arginine and its

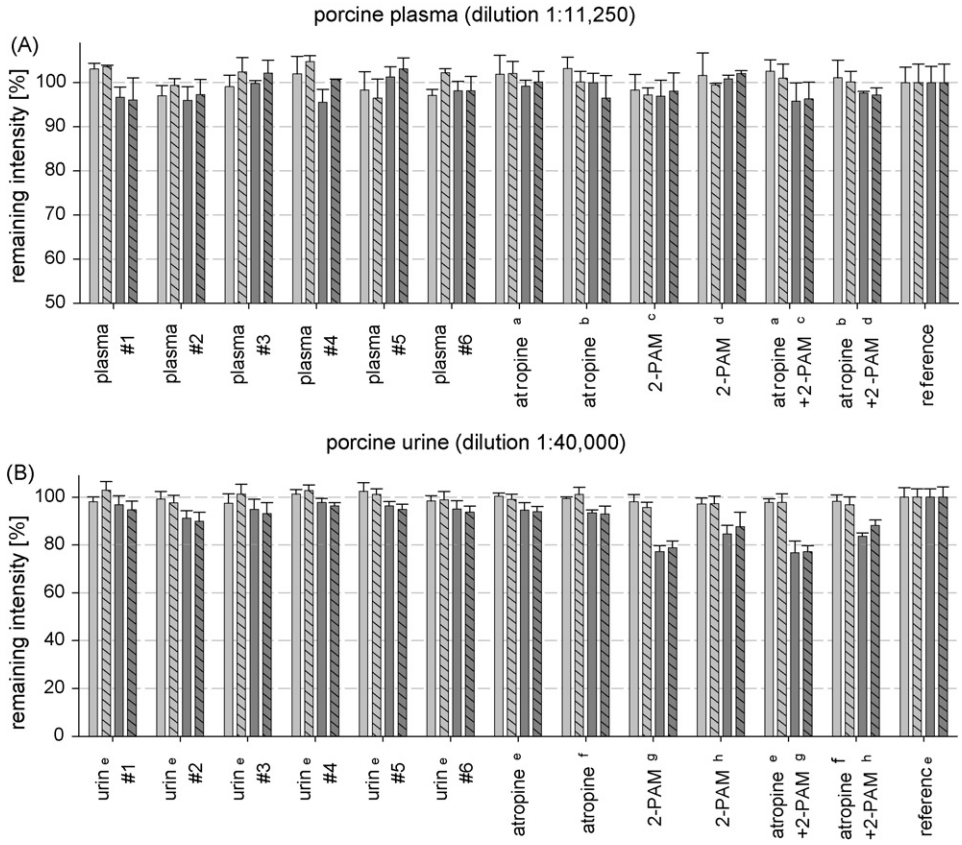

Fig. 4. Matrix effects caused by individual specimens and antidotes. A: porcine blank plasma of six individuals (plasma #1–6) was spiked with dimethoate (blank bars) and omethoate (hatched bars) at 16.7 μg/ml and analyzed by LC–ESI-MS/MS (light grey bars) as well as FI-ESI-MS/MS (dark grey bars) to be compared to peak areas from references. In addition, corresponding analyses were done for one lot of plasma spiked with both pesticides (5 μg/ml each) as well as with atropine and 2-PAM in two different concentrations each alone and in combination as indicated: a, 41.7 ng/ml; b, 20.8 ng/ml; c, 41.7 μg/ml; and d, 8.3 μg/ml. B: porcine blank urine of six individuals (urine #1–6) was spiked with dimethoate and omethoate at 20.8 μg/ml and analyzed by LC–ESI-MS/MS as well as FI-ESI-MS/MS to be compared to peak areas from references. In addition, corresponding analyses were done for one lot of urine spiked with both pesticides (41.6 μg/ml each) as well as with atropine and 2-PAM in two different concentrations each alone and in combination as indicated: e, 1.7 μg/ml; f, 0.85 μg/ml; g, 833 μg/ml; and h, 417 μg/ml. Symbols and colours are assigned as described above. All analyses were performed in triplicate each and measured in duplicate.

mono- and dimethylated analogs in plasma and urine [50]. Therefore, selectivity in specimens from different individuals had to be tested for potential interferences.

3.4. Analysis by FI-ESI-MS/MS

Pesticide quantification by direct injection appeared to be applicable very well to the plasma and urine specimens (Fig. 5).

Whereas the total run time for one LC–MS/MS analysis (analytical run plus washing step) was found to be about 12.8 min (Fig. 2A and B), the FI-MS/MS design required only 2 min: 1 min operational time (including flushing steps, drawing up the syringe of the autosampler, filling the sample loop and switching the injection valve) and 1 min for analysis (Fig. 5A). Therefore, the FI-ESI-MS/MS procedure was as suitable as its chromatographic variant but allowed a valuable six-fold speed-up of sample throughput. The elution time for both analytes was found to be 0.096 ± 0.002 min (detected at peak maximum) and the averaged full-width at half maximum (FWHM) was determined to be 0.135 ± 0.002 min. Therefore, peaks from FI analysis are at least twice as broad as those from LC analysis (FWHM 0.075 ± 0.002 min for omethoate and 0.046 ± 0.002 min for dimethoate). However, this fact did not impair the applicability of the FI method to the animal study.

3.4.1. Characteristics of FI-ESI-MS/MS

The flow-injection analysis was subjected to the same validation experiments as described for the chromatographic procedure. Simultaneous selective detection was reproducible and stable resulting in very similar characteristics of quality criteria as determined by LC–MS/MS (Table 1).

3.4.1.1. Linear range, LOQ and LOD. The calibration range was again subdivided into an upper (u) and a lower (l) range identical to the LC approach allowing optimum accuracy for samples of interest. The LOQ was sufficient and appropriate for sample analysis being found at 0.24 μg/ml in plasma and 1.56 μg/ml urine for both pesticides. The LOQ for plasma might be due to the initial precipitation step discarding interfering proteins. Data are given in Table 1.

3.4.1.2. Precision (RSD) and accuracy (intra- and inter-day). As depicted in Table 1 intra- and inter-day precision (RSD) was between 1% and 11% and accuracy was found to range from 94% to 106%. No significant differences between both pesticides appeared.

3.4.1.3. Selectivity. Investigation of six individual lots of plasma and urine did not show any interfering signals neither for dimethoate nor for omethoate indicating an appropriate selectivity for the diluted samples. Representative elution profiles are illustrated in Fig. 5B and D.

3.4.1.4. Ruggedness. Furthermore, investigation of ruggedness (influence of altered spray voltage, eluent flow, gas spray, and declustering potential) proved the method to be stable and rugged (results not shown).

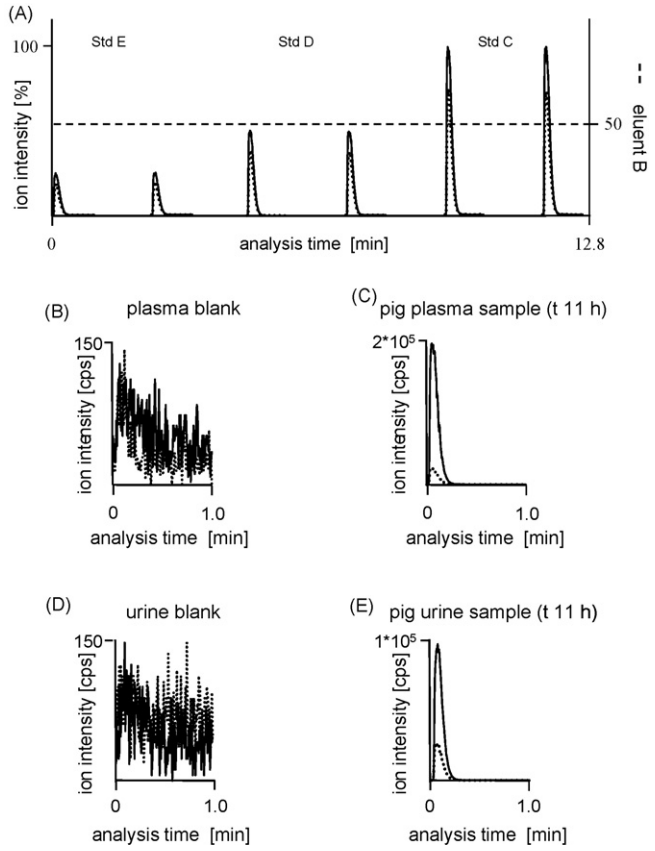

Fig. 5. FI-ESI-MS/MS for simultaneous analysis of dimethoate and omethoate. A: MRM traces of plasma standards C, D and E (62.5, 31.25, and 15.63 μg/ml, diluted 1:11,250) injected in duplicate each; dotted line: omethoate (MRM, 214.2 → 183.1; +ESI, CE 17 V, DP 41 V, CXP 12 V); solid line: dimethoate (MRM, 230.2 → 199.1; +ESI, CE 15 V, DP 46 V, CXP 14 V) B–E: MRM traces of plasma blank (B), plasma sample from animal study at 11 h (C), urine blank (D), and urine sample from animal study at 11 h (E). FI was performed at 30 °C with solvent A–solvent B (50:50, v/v) as indicated by the dashed line. Solvent A: 0.1% (v/v) FA, solvent B: ACN/0.1% (v/v) FA 80:20 (v/v); flow: 1 ml/min; sample volume 100 μl; ionization spray voltage: 3000 V; curtain gas: 1.72×10^5 Pa (25 psi); heater gas (GS1): 4.83×10^5 Pa (70 psi); turbo ion spray gas (GS2): 4.14×10^5 Pa (60 psi); gas temperature (TEM): 700 °C; entrance potential (EP): 10 V, dwell time 200 ms; collision gas: nitrogen.

3.4.2. Matrix effects in FI-ESI-MS/MS

In flow-injection analysis the lack of a sample separation step provokes coelution of the analyte and matrix ingredients, thus strong effects on ionization are to be expected. Therefore, we characterized different dilution factors on the signal intensities of dimethoate and omethoate in urine. This body fluid contains, e.g. large amounts of urea, creatinine, diet-dependent polar metabolites, and inorganic salts potentially deteriorating analyte ionization [50].

3.4.2.1. Matrix effects of dilution factors. Peak areas of both pesticides obtained from urine samples steadily decreased with smaller dilution factors (lower slope of standard curve) indicating strong suppression due to higher matrix concentrations as depicted for dimethoate (Fig. 3C) and omethoate (Fig. 3D). Only very slight ion suppression was observed when diluting urine by a factor of at least 40,000 (white squares) resulting in 96% and 90% remaining intensity for dimethoate and omethoate, respectively. Higher dilutions tested (1:80,000 and 1:160,000) resulted in standard curves being congruent to the neat solvent (data not shown for reasons of simplicity). Nevertheless, suppressed standard curves were of good linearity within the measured concentration range thus being applicable for quantification. In addition, dimethoate and omethoate did not affect ionization among each other (data not shown for reasons of simplicity). These data depict the meaningful relevance of matrix ingredients affecting validity and reliability of analyte quantification.

A similar behaviour of peak areas was observed for prepared plasma samples analyzed by FI-MS/MS. Strong suppression (39% remaining intensity) was found for both pesticides when injecting less diluted samples (1:100) whereas already a dilution of 1:1000 yielded in 89% remaining intensity. No suppression effects were found for both analytes when diluting at least by a factor 5000. Therefore, plasma samples prepared by the standard procedure (dilution 1:11,250) were free from effects of endogenous plasma derived compounds making the method suitable for analysis (data not shown).

3.4.2.2. Matrix effects in individual specimens. As illustrated in Fig. 4 no significant differences between six individual lots of body fluid (plasma, Fig. 4A and urine Fig. 4B) were observed for the detected pesticide peaks (dark grey bars, blank bars: dimethoate, hatched bars: omethoate). The mean and RSD of remaining intensity of all six lots compared to the reference was found to be in plasma $97.9 \pm 2.3\%$ for dimethoate and $99.6 \pm 2.8\%$ for omethoate. For urine the following data were calculated: $95.4 \pm 2.3\%$ for dimethoate and $93.8 \pm 2.2\%$ for omethoate. As obvious from these data no relevant suppression was observed for plasma samples whereas slight suppression was found for urine. These results were in accordance to the systematic investigations of diluted samples revealing no suppression for plasma and slight suppression for urine dilutions. However, as no lot-dependent deviation were determined the FI-ESI-MS/MS method also appeared to be suitable for pesticide quantification in urine. The high reproducibility from lot to lot indicating sample homogeneity might be favoured by the origin of the pig body fluids that were taken from laboratory animals of similar weight and age held under controlled diet and environmental conditions.

3.4.2.3. Matrix effects in the presence of antidotes. In contrast to the chromatographic method not any to slight (plasma) or moderate suppression effects (urine) were observed in pesticide detection in the presence of antidotes (Fig. 4). In plasma neither the lower nor the higher concentration of atropine nor the lower 2-PAM concentration resulted in suppression (Fig. 4A, dark grey bars). Combination of these antidotes did also not affect the measurement. Merely the presence of 2-PAM in the higher concentration (41.7 μg/ml plasma, approximately 5-times as high as present in most study samples) influenced the ionization process. Nevertheless, suppression was only slight and within the deviation of the method thus not deteriorating the applicability to the animal study.

In urine suppression effects were much more obvious instead. As discussed before the detection of dimethoate and omethoate is per se slightly suppressed by endogenous ingredients (Fig. 4B). The presence of atropine in both concentrations did not change the relative remaining intensity. In contrast, in the presence of 2-PAM (single antidote or in combination with atropine) moderate signal reduction by 16% (higher concentration, 833 μg/ml) or by 10% (lower concentration, 417 μg/ml) for both pesticides was achieved. Precision was not impaired. In principle, these suppressing effects limit the use of the FI method for urine analysis in the animal study with respect to an optimum accuracy. However, with respect to the concentration-time profiles measured in several study animals a 10%-concentration shift would not be of critical importance to evaluate pesticide behaviour.

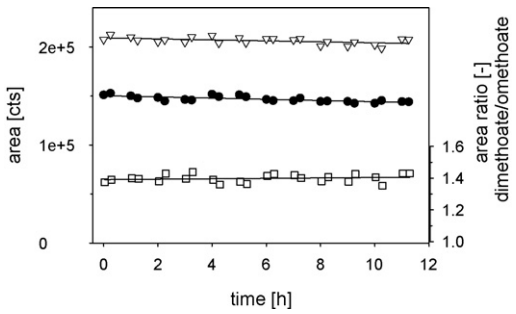

Fig. 6. Stability of dimethoate and omethoate in prepared plasma samples in autosampler. Medium plasma QC (40 μg/ml) was prepared according to the standard protocol modified to obtain a larger volume of the last dilution step separated into 12 HPLC sample vials. These aliquots were left in the autosampler at room temperature and every hour one vial was analyzed in duplicate by LC–ESI-MS/MS. Triangles: dimethoate; circles, omethoate; squares, peak area ratio dimethoate/omethoate; data points are from single measurement. Constant peak areas for dimethoate and omethoate as well as the constant peak area ratio demonstrate analytes stability over the 12 h test period. Identical results were obtained for the less concentrated QC (5 μg/ml) not shown here.

3.5. Stability of pesticides

3.5.1. Thaw-and-freeze cycles

Following that procedure dimethoate and omethoate did not show any degradation in plasma or urine.

Plasma and urine samples were stable at −80 °C for at least 6 months.

3.5.2. Stability in the autosampler

Both pesticides remained stable in both concentrations during storage at room temperature in the autosampler as deduced from constant peak areas and constant peak area ratios (dimethoate/omethoate). Exemplarily data are shown for the medium QC (40 μg/ml plasma) in Fig. 6. Therefore, pesticide concentrations will not change during the time needed for the analysis of a set of samples.

In addition, Fig. 6 demonstrates the signal stability over 12 h in HPLC analysis thus underlining the reliability the method. Due to the extensive dilution of plasma and urine prior to measurement it appeared reasonable that the stability of prepared samples is determined by the 80:20-mix and not by the original matrix. Therefore, only prepared plasma samples were analyzed.

3.6. Correlation of LC–ESI-MS/MS and FI-ESI-MS/MS: application to animal study

To underline the validity and usefulness of both ESI-MS/MS methods for porcine plasma and urine analysis we measured relevant samples derived from the animal study and correlated the resulting concentrations. As demonstrated in Fig. 7 concentrations of dimethoate (triangles) and omethoate (circles) measured by LC–MS/MS (white symbols) and by FI-MS/MS (black symbols) were identical within the standard deviations independent from the matrix analyzed, plasma (Fig. 7A) or urine (Fig. 7B). Exemplarily, the chromatograms of a plasma and urine sample taken 11 h after poisoning are given in Fig. 2 D and F and the corresponding data from FI analyses are shown in Fig. 5C and E. These data illustrate that under present conditions simple flow-injection analysis proved to be an adequate, valuable and advantageous alternative to the LC–ESI-MS/MS design allowing to sextuple sample throughput while keeping good quality and robust performance.

The dimethoate solution was applied to the pig by gavage to provoke poisoning via the gastrointestinal tract representing typical situations of intentional or accidental poisoning with pesticides.

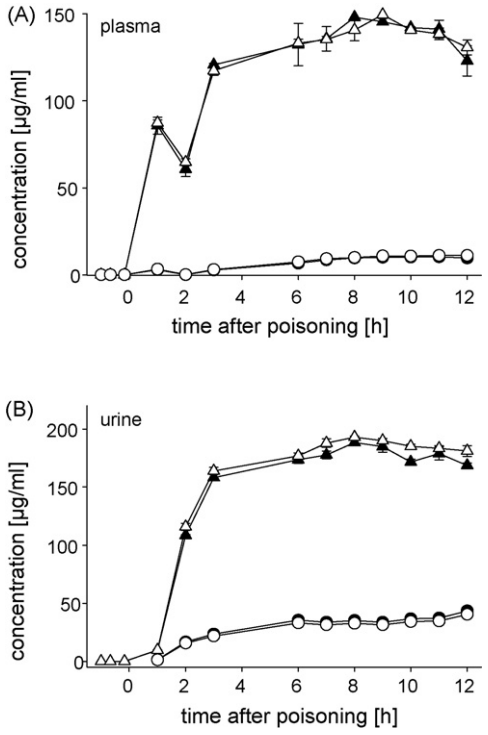

Fig. 7. Quantification of dimethoate and omethoate by LC–ESI-MS/MS and FI-ESI-MS/MS in plasma and urine samples from minipigs poisoned with dimethoate. (A) Concentration-time profile obtained from plasma samples. (B) Concentration-time profile obtained from urine samples. White symbols, LC–ESI-MS/MS; black symbols, FI-ESI-MS/MS; triangles, dimethoate; circles, omethoate. Göttingen minipig was poisoned with dimethoate (p.o., 22.5 ml of EC 40, 400 g/l) and subjected to therapy as described in Section 2.7. Samples were analyzed in duplicate by both ESI-MS/MS methods according the standard protocols. Data are given as means ± SD. Maximum antidote concentrations were approximately 8 μg/ml plasma and 300 μg/ml urine for 2-PAM and 20 ng/ml and 1 μg/ml for atropine, respectively.

Dimethoate was readily absorbed and distributed systemically via the circulation causing high plasma concentrations of about 130 μg/ml 10 h after poisoning (Fig. 7A). While passing the liver dimethoate was desulfurated to its oxon-derivative (omethoate) thus initiating the deliberation of this metabolite into blood. Therefore, omethoate was also detected in plasma exhibiting rising concentrations reaching about 10 μg/ml after 12 h (Fig. 7A).

Both pesticides were excreted via kidney thus causing rising concentrations in urine being significantly higher than in plasma (Fig. 7B).

A closer discussion of clinical symptoms, therapeutic intervention and toxicokinetic parameters is beyond the focus of this article and will thus not be presented in more detail.

As obvious from the concentration-time profiles (Fig. 7A and B) both presented ESI-MS/MS techniques were highly suitable for reliable quantification of pesticides in plasma as well as in urine allowing to monitor the toxicants' kinetics.

4. Conclusions

The chromatographic procedure presented herein allowed the quantification of dimethoate and its metabolite omethoate in the middle nano-molar range. However, quite high concentrations of both pesticides provoked by gastrointestinal poisoning of minipigs required extensive sample dilution prior to measurement thereby allowing us to analyze concentrations in the higher micro-molar range. High dilution also reduced the concentration of matrix-derived components thus minimizing resulting effects on ionization of analytes. Consequently, quantification of analytes was

possible by direct flow-injection thereby reducing analytical cycle time and enhancing sample throughput drastically. This instrumental set-up might also be of important benefit when used as a generic method for future studies including additional pesticides. However, optimization of these methods will be possible by inclusion of at least one internal standard helping to improve precision and accuracy.

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