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ORIGINAL ARTICLE

Simultaneous determination of prescription drugs, cocaine, aldicarb and metabolites in larvae from decomposed corpses by LC–MS–MS after solid–liquid extraction with low temperature partitioning

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Abstract The determination of toxic substances in insect larvae collected from corpses in an advanced state of putrefaction can help to elucidate the *causa mortis* in forensic cases. In this study, an analytical method for the simultaneous determination of six prescription drugs, cocaine and its metabolite benzoylecgonine, and aldicarb and its sulfone and sulfoxide metabolites in larvae was developed and validated. The method involved a solid–liquid extraction with low temperature partitioning, and determination by a liquid chromatography–mass spectrometry (LC–MS–MS) method. Significant matrix effects were observed for most analytes, indicating the need to use analytical curves in pre-extracted fortified matrices. The limits of quantification ranged from 1 to 40 ng/g, with precision between 2.83 and 16.9 %. The validated method was used to analyze 28 actual larval samples collected from corpses at the Forensic Medical Institute between 2009 and 2012 in the Federal District, Brazil. At least one substance was present in 11 samples. Benzoylecgonine and diazepam were found in four samples each, followed by cocaine (three samples), carbamazepine (two samples) and amitriptyline (one sample). The method proved to be simple, fast and of low cost, and can be used by forensic laboratories as a complementary tool to elucidate intoxication-related deaths where usual toxicological matrices are not available.

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Introduction

Poisoning patterns vary greatly throughout the world, with prescription drugs and pesticides being the chemicals most involved in fatal cases [1–5]. Worldwide, about 30 % of all suicide attempts involve pesticides, ranging from 4 % in Europe to over 50 % in the Western Pacific region [6]. Some poisoning cases, however, go unsolved, as the body is only found many days after death, and due to the advanced state of putrefaction, human tissues are not suitable for toxicological analyses. In such forensic cases, entomological samples have been used as an alternative to investigate *causa mortis* [7, 8]. Larvae are easily collected from a corpse, can be found for a long period after death, and are potentially easier to analyze due to fewer matrix effects [9]. Drug concentrations in these samples also appear to be more stable than in putrefied tissue [10]. However, interpretation of the quantitative results obtained in these studies is still under discussion, due to the wide inter-site and intra-site variations of drug concentrations, and the absence of a sound quantitative correlation between the concentrations found in larvae and in the corpse [11].

Benzodiazepines, antidepressants and anticonvulsants such as carbamazepine are among the drugs most involved in intoxication cases in Brazil [12, 13] and other countries [1, 3, 4, 14, 15]. Cocaine abuse and intoxication is a global problem, leading to many medical complications and death [16, 17]. Between 2006 and 2008, cocaine was present in 22 % of urine samples related to fatal events in the Federal District of Brazil, with the percentage almost doubling

from 2006 (14 %) to 2008 (26 %) [13]. Pesticides account for about 7 % of the cases of intoxication in Brazil, and are the main cause of fatal poisonings in the country [5]. These rates are higher in other countries including China [18] and Sri Lanka [19]. Most of the acute poisoning with pesticides in Brazil involves aldicarb, a *N*-methyl carbamate used illegally in the country as a rodenticide [13, 20, 21].

Many studies have been conducted over the last 30 years to investigate the presence of chemicals in larvae collected from decomposed corpses. The substances found include barbiturates [7], antidepressants and benzodiazepines [11], amphetamines [22], opiates [23], cocaine and its metabolite, benzoylecgonine [24, 25], and pesticides [26]. The techniques used to determine these chemicals include gas chromatography coupled to a nitrogen-phosphorus detector [25] or mass spectrometry (MS) [24], and liquid chromatography coupled to a UV detector [23], MS [11], or MS–MS [9, 27].

However, very few of the published studies have used validated methods, and to the best of our knowledge, no validated method for the simultaneous determination of substances from different chemical classes in larvae is available in the literature. Multiclass methods are important in a forensic laboratory due to the variety of substances potentially involved in poisoning cases, as well as the lack of information on the circumstances of the death in most cases.

The aim of the present study is to develop and fully validate a liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the determination of prescription drugs (amitriptyline, carbamazepine, bromazepam, clonazepam, diazepam and flunitrazepam), cocaine and its metabolite benzoylecgonine, and the pesticide aldicarb and its sulfone and sulfoxide metabolites, in necrophagous insect larvae. The method was applied to analyze larval samples collected from putrefied corpses at the Forensic Medical Institute of the Federal District, Brazil (IML, DF), between 2009 and 2012.

Materials and methods

Chemicals and reagents

High-performance liquid chromatography (HPLC)-grade methanol, acetonitrile (ACN) and ethyl acetate (AcOEt) were purchased from Merck (Darmstadt, Germany); glacial acetic acid and sodium chloride from J. T. Baker (Avantor Performance Materials, Center Valley, PA, USA); formic acid from Sigma–Aldrich (St. Louis, MO, USA); and ammonium formiate from Fluka (Buchs, Switzerland). Ultrapure water was obtained through a Milli-Q purification system (Millipore, Bedford, MA, USA).

Standards of carbamazepine (96 % purity) and amitriptyline (100 % purity) were donated by the Brazilian Pharmacopeia (Rio de Janeiro, Brazil). Standards of bromazepam (100 % purity), clonazepam (100 % purity), diazepam (99.7 % purity) and flunitrazepam (99.7 % purity) were kindly donated by Roche Pharmaceuticals (Anapolis, Brazil). Cocaine and benzoylecgonine were donated by the Federal Police (Brasília, Brazil). Standards of aldicarb (98 % purity), aldicarb sulfone (99 % purity) and aldicarb sulfoxide (99 % purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Larval materials

Method development and validation were performed using larvae produced in ground porcine offal. The offal was placed in a container that was closed with a grid, to protect it from wild animals while allowing for insect activities, and was left in a forested area at the Experimental Biology Station of the University of Brasilia. After colonization by necrophagous insects (about 1 week), active larvae at different developmental stages were collected from porcine offal and taken to the laboratory, where they were rinsed thoroughly with tap water, dried on absorbent filter paper and killed by freezing at −20 °C. Larvae collected from porcine material on three different occasions were pooled and homogenized in an industrial blender until a homogeneous pulp was obtained (blank sample).

Solid–liquid extraction with low temperature partitioning

The optimization of the solid–liquid extraction with low temperature partitioning (SLE–LTP) method was performed using 1 g portions of the larval blank sample fortified with known amounts of the analytes investigated in the study. Water (0.5 ml), and organic solvent (ACN or a mixture of ACN and AcOEt) were added to the homogenized samples; the mixture was sonicated for 5 min, centrifuged for 5 min at 3.500 rpm (2.383 × *g*), and frozen at −20 °C. Once frozen, the aqueous layer was separated from the organic phase, the latter of which was filtered and submitted to LC–MS–MS analysis. All experiments were performed in triplicate, with analyte concentrations ranging from 0.5 to 10 ng/ml.

Optimization of the relevant SLE–LTP parameters was performed for all analytes, except benzoylecgonine, which was included in the study only during method validation. Univariate experiments were used to evaluate the effect of solvent volume, addition of salt, freezing time, and manual agitation prior to sonication. Optimization of solvent composition and acidification of the extraction phase was performed using a 2^2 factorial design with a central point.

A factorial experiment is a widely used strategy to investigate individual effects of multiple factors that may interfere in an analytical procedure. In this study, we investigated extraction solvent composition and acidification. Solvent compositions that were tested were: 2 ml pure ACN; 1.625 ml ACN and 0.375 ml AcOEt; and 1.8 ml ACN and 0.2 ml AcOEt (central point). Extraction phase acidification conditions were: no acid; formic acid 1 %; and acetic acid 1 % (central point). A total of 15 tests were conducted, corresponding to the central point and four combinations of the other conditions, each performed in triplicate.

LC–MS–MS conditions

The equipment consisted of a Shimadzu LC system with a binary pump (LC-20AD), degasser (DGU-20A5), autosampler (SIL-20AC), column oven (CTO-20AC) and controller (CBM-20A) (Shimadzu, Kyoto, Japan). The LC system was coupled to a 4000 QTRAP triple-quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA) fitted with a Turbo Ion Spray interface in the positive electrospray ionization mode (ESI+). System operation and data acquisition were controlled by Analyst® (V 1.5.2) software (AB Sciex). Analytes were separated using a Phenomenex® Luna C18(2) column (150 × 2 mm, 5 µm) preceded by a C18 guard column (4 × 2.0 mm) (Phenomenex, Torrance, CA, USA), with an oven temperature at 40 °C, and a flow rate at 0.5 ml/min in gradient mode. Solvent A was water and solvent B was methanol; both contained 5 mM ammonium formiate. The mobile phase gradient started at 30 % B, held for 2 min, then increased to 50 % B in 1 min, to 57 % B in 7 min, to 65 % B in 1 min, to 70 % B in 5 min, was held for 4 min, then increased to 99 % B and was held for 4 min, and then returned to 30 % B in 1 min, resulting in a total run time of 25 min. The system was equilibrated at the initial condition for 10 min between consecutive runs.

The optimal conditions of the mass spectrometer ion source after automatic optimization by the software were: entrance potential 10 V, curtain gas 20 psi (138 kPa), ion source gas 1 and 2 at 45 and 50 psi (310 and 345 kPa), respectively, collision gas medium, ion spray voltage 4000 V, and an ion source temperature of 650 °C. Selective reaction monitoring (SRM) conditions for each analyte were found by direct infusion of standard solutions (0.2 µg/ml in methanol/water 50:50 containing 5 mM ammonium formiate). Declustering potential (DP), collision cell exit potential (CXP), and collision energy (CE) were optimized for the two most abundant transitions (quantifier and qualifier) for each analyte, as shown in Table 1.

Method validation

The optimized SLE–LTP procedure was submitted to a full validation process recommended for bioanalytical methods [28–30].

Selectivity was evaluated by analyzing the LC–MS–MS chromatographic profiles of a blank and of a fortified blank larval sample, checking for the elution of interferences at the same retention time as the analytes of interest.

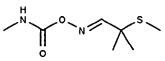
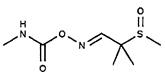
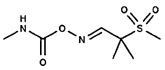
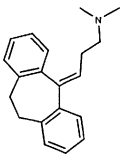
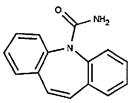
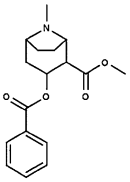
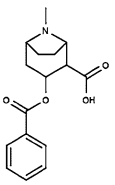
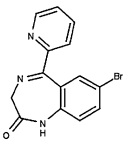
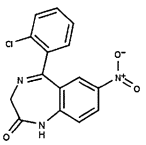
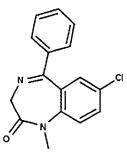
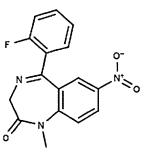
Matrix effect (in % of signal enhancement or suppression), extraction efficiency (in %), and linearity were evaluated for each compound at five concentration levels using six replicates at each level. The lowest fortification level for each compound was defined according to instrument sensitivity under optimized conditions in a fortified blank matrix (signal-to-noise ratio of 10). The fortification level ranges were: 0.2–20 ng/ml for carbamazepine, 0.4–40 ng/ml for flunitrazepam, cocaine, benzoylecgonine and aldicarb, 0.5–50 ng/ml for bromazepam and diazepam, 0.6–60 ng/ml for amitriptyline and clonazepam, 1.2–120 ng/ml for aldicarb sulfone, and 8.0–400 ng/ml for aldicarb sulfoxide.

Matrix effects (ME) were estimated by comparing the average instrument response (area) of a blank sample fortified with the analytes after the SLE–LTP procedure (set B) with the average response of the analytical standards in ACN (set A). Extraction efficiency was determined by comparing the average response of the blank sample fortified before the extraction procedure (set C) with the average response of set B.

Linearity was verified using set C. The ordinary least squares method was used to estimate the linear regression parameters, the Grubbs test was used to verify the presence of outliers, and the Cochran test to verify the homogeneity of variances. For the heterocedastic data, a regression was performed using the weighted least squares method; the weighting factor that produced the lowest sum of the relative errors, providing the most adequate approximation of variance, was chosen for the regression [31].

Repeatability was evaluated based on the analysis of blank samples fortified at three concentration levels (low, medium and high; $n = 6$ at each level), as performed by the same analyst on the same day. Intermediate precision was evaluated through the analysis of blank samples fortified at the low and medium concentration levels, by the same analyst on different days or by different analysts on different days. Accuracy (bias) was evaluated during the precision experiments, and is defined as the difference between the expected test results and an accepted reference value (in this work, the spiked concentration) [32].

Table 1 Investigated substances, chemical structures and selective reaction monitoring parameters for each analyte

Analyte	Structure	Parent ion	Molecular weight (Da)	DP (V)	Transition (*m/z*)		CE (V)	CXP (V)
Aldicarb		$[M + NH_4]^+$	190	31	q	208 → 116	11	8
					c	208 → 89	23	14
Aldicarb sulfoxide		$[M + H]^+$	206	51	q	207 → 132	11	10
					c	207 → 89	21	6
Aldicarb sulfone		$[M + H]^+$	222	66	q	223 → 86	21	6
					c	223 → 148	15	10
Amitriptyline		$[M + H]^+$	277	21	q	278 → 233	25	18
					c	278 → 91	39	6
Carbamazepine		$[M + H]^+$	236	41	q	237 → 194	29	14
					c	237 → 192	33	14
Cocaine		$[M + H]^+$	303	26	q	304 → 182	29	14
					c	304 → 82	45	4
Benzoylecgonine		$[M + H]^+$	289	66	q	290 → 168	27	12
					c	290 → 105	45	6
Bromazepam		$[M + H]^+$	316	86	q	317 → 182	45	12
					c	317 → 209	39	16
Clonazepam		$[M + H]^+$	315	76	q	316 → 270	37	22
					c	316 → 214	53	16
Diazepam		$[M + H]^+$	283	81	q	284 → 193	45	14
					c	284 → 222	39	16
Flunitrazepam		$[M + H]^+$	313	86	q	314 → 268	37	20
					c	314 → 239	49	18

q quantifier ion, *c* qualifier ion, *DP* declustering potential, *CE* collision energy, *CXP* collision cell exit potential

Actual larval samples

Larval samples were collected from corpses in advanced state of decomposition by the staff of the Forensic Medical Institute of the Federal District, Brazil (IML-DF) between 2009 and 2012. These samples were rinsed thoroughly with tap water, dried on filter paper, homogenized and stored at −20 °C until analysis. The identification of insect species

or of the developmental stage of the larvae was not performed in this study. Samples were analyzed according to the validated method in batches of up to ten samples. Three larval blank samples fortified at the intermediate level were included in each batch as an internal quality control. Quantification was performed against an analytical curve prepared daily with blank samples fortified before the extraction procedure (set C). Positive samples that were outside the linear range were either diluted with acetonitrile or concentrated under nitrogen to fit the analytical curve range, and reanalyzed by LC–MS–MS.

Results and discussion

Method optimization

The univariate experiments showed few statistically significant effects on analyte recovery. Increasing the ACN volume from 2 to 4 ml significantly increased recovery only for clonazepam (+17 %; $P = 0.005$), and 2 ml was chosen to minimize solvent consumption. The addition of salt (0.2 g sodium chloride) to the extraction solvent significantly decreased the recovery of aldicarb (−13 %; $P = 0.026$), and was not included in the subsequent experiments.

The 2^2 factorial design experiments showed that acidification of the extraction solvent significantly decreased the recovery of carbamazepine by about 6 %, and although not significant, negatively affected the recovery of all other substances, except cocaine. The effect of solvent composition was only significant for aldicarb, resulting in a 9 % decrease in recovery when pure ACN was used. Although also not significant, higher recoveries were found for the other analytes, and for simplicity, pure ACN was chosen as the extraction solvent in the SLE–LTP procedure.

The optimal freezing time was evaluated after freezing the extracted sample for 4, 6, 8, 10, 14 and 16 h. The average recoveries were not significantly different at any freezing time, with a tendency of higher recoveries after 6 or 10 h for most of the analytes. The optimal freezing time was set at 6 h in order to allow analysis in a single day. Manual agitation for 1 min prior to sonication resulted in a lower fluctuation around the average recovery (lower relative standard deviation, RSD) for all analytes except cocaine, and a significant increase in the diazepam recovery (+8.2 %; $P = 0.012$). Although this step decreased carbamazepine recovery (−8.6 %; $P = 0.012$), manual agitation was found to be beneficial for the overall performance of the method.

Hence, the optimized SLE–LTP procedure for the extraction of the 11 analytes from authentic larval samples is as follow: 1 g of homogenized larval sample or less, depending on the availability, was weighted in a 15-ml falcon tube; 0.5 ml of water and 2 ml ACN were added; and the tube was manually agitated for 1 min and sonicated for 5 min. The tube containing the sample extract was centrifuged for 5 min at 3500 rpm, frozen at −20 °C for 6 h, and the organic layer filtered through a 0.45 µm syringe filter. The organic extract that was obtained was directly analyzed by LC–MS–MS.

The extraction procedure optimized in this work used a smaller sample size than those used in the application of SLE–LTP on other matrices [33–35]. The solvent-to-water proportion was similar to that used to determine veterinary drugs in meat [33]. This miniaturization proved to be adequate for entomotoxicological analysis in cases where the availability of larval material is limited.

The use of ACN, a solvent with intermediate polarity, enabled the extraction of a vast array of substances, complying with the purpose of a multi-class method. A significant correlation was found between the analyte octanol–water partition coefficient (K_{OW}) and recovery using ACN extraction ($r = 0.60$, $P = 0.035$), with lipophilic compounds showing better recoveries. A higher correlation was found between water solubility and recovery ($r = -0.76$, $P = 0.006$), with compounds of lower polarity showing better recovery. Aldicarb and cocaine presented high recoveries, despite their high water solubility.

Method validation

Whenever possible, the same biological matrix as that in the intended samples should be used for validation purposes [36], and some guidelines require a minimum of six independent sources of the same matrix to evaluate matrix effects [37]. However, unlike other biological matrices, such as blood and urine, larvae from decomposed corpse are of limited availability and a larval homogenate produced in different batches of ground porcine offal was used in this study to validate the method. Furthermore, multiple matrices may not be necessary for hyphenated mass spectrometry-based methods [36]. Ideally, isotope-labeled internal standards should be used to compensate for extraction losses and matrix effects [37]. These standards are expensive and are not always available in a forensic laboratory for routine analysis, and although method validation and analyses using external standards are more time consuming and more prompt to bias, the process is more feasible for most forensic laboratories.

In this study, the comparison of the chromatograms of blank and fortified blank samples did not show interfering peaks eluting at the same retention times for any of the 11 analytes evaluated (Fig. 1), confirming that the chromatographic conditions used ensured satisfactory selectivity of the method.

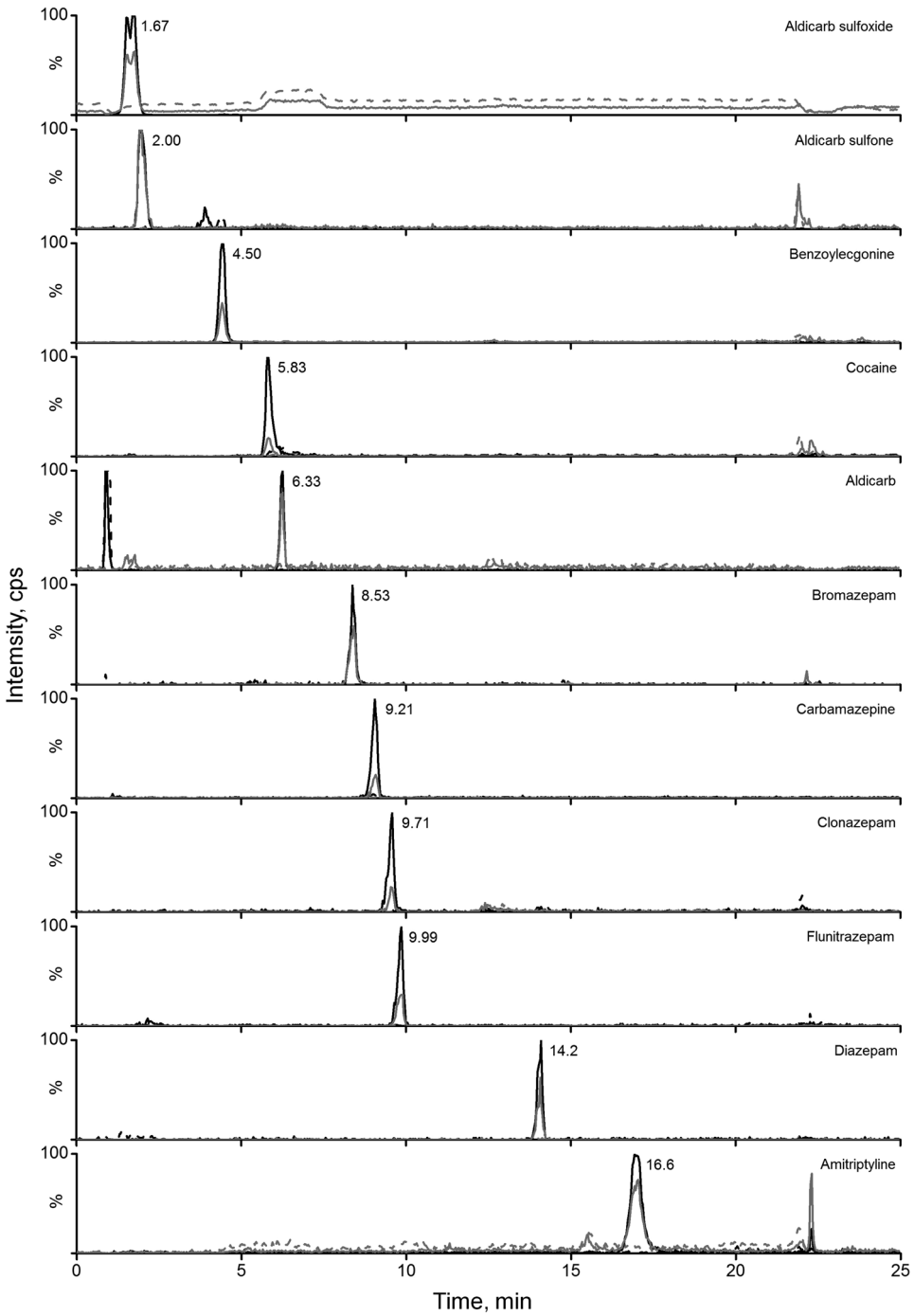


Fig. 1 Selective reaction monitoring chromatograms of a post-extraction fortified blank matrix (*black*: quantifier ion; *gray*: qualifier ion) and blank matrix (*dotted lines*); *cps* counts per second

Matrix effects occur when molecules coeluting with the compound of interest alter (mainly suppress) the ionization efficiency of the electrospray interface [38]. Figure 2 show the average matrix effect for the eleven analytes in larvae samples fortified at five concentration levels. No significant matrix effect was found at any level for benzoylecgonine and carbamazepine. With the exception of bromazepam, signal suppression was found for all the other compounds in at least one level, with the highest suppression observed for clonazepam (−33.5 %). Signal enhancement for bromazepam decreased with higher concentrations, and disappeared at 50 ng/ml ml, the highest level. Zancanaro et al. [39], however, found inversed matrix effects for bromazepam and clonazepam in oral fluid at 5 ng/ml ml (about 20 % suppression and enhancement, respectively). Although Bonfiglio et al. [40] found a higher signal suppression for caffeine compared to two less polar compounds, we did not find a significant correlation between

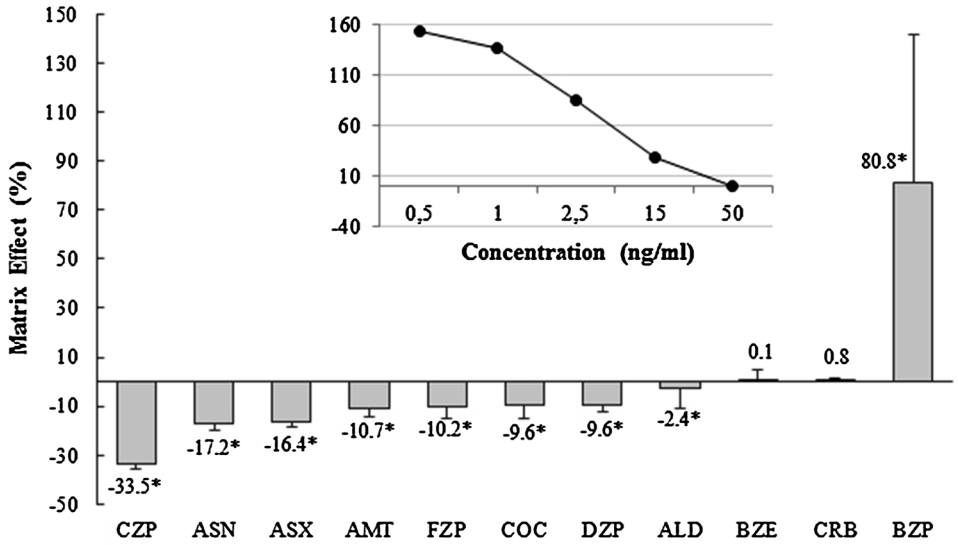


Fig. 2 Average matrix effects (%) for the five fortification levels. *Asterisks* indicate significant matrix effects in at least one level tested (each level being the mean of 6 samples). The insert shows the bromazepam matrix effects at different concentrations. *CZP* clonazepam, *ASN* aldicarb sulfone, *ASX* aldicarb sulfoxide, *AMT* amitriptyline, *FZP* flunitrazepam, *COC* cocaine, *DZP* diazepam, *ALD* aldicarb, *BZE* benzoylecgonine, *CRB* carbamazepine, *BZP* bromazepam

Table 2 Extraction efficiencies (%)[a] after solid–liquid extraction with low temperature partitioning and analysis by LC–MS–MS for the five levels evaluated

Analyte (range, ng/ml)	Extraction efficiency ± uncertainty (%) per fortification level[b]				
	1X	2X	5X	30X	100X
Aldicarb (0.4–40)	95 ± 26	118 ± 18	93 ± 11	68.8 ± 6.7	78.4 ± 5.5
Ald. sulfoxide (8–400)[c]	40.8 ± 3.5	51.6 ± 6.2	50.7 ± 6.7	48.5 ± 5.0	52.1 ± 7.7
Ald. sulfone (1.2–120)	57.9 ± 6.2	73.8 ± 6.8	68.3 ± 7.2	60.7 ± 4.9	68.8 ± 6.1
Amitriptyline (0.6–60)	65 ± 12	89 ± 14	68.7 ± 9.6	62.6 ± 5.9	66.3 ± 6.9
Carbamazepine (0.2–20)	71 ± 12	75.9 ± 9.6	71.1 ± 2.6	62.2 ± 2.9	68.1 ± 4.0
Cocaine (0.4–40)	54.2 ± 4.4	65.4 ± 5.5	63.0 ± 4.8	58.1 ± 4.0	62.7 ± 5.6
Benzoylecgonine (0.4–40)	29.5 ± 3.3	36.9 ± 6.1	36.4 ± 3.1	38.8 ± 3.7	39.1 ± 6.0
Bromazepam (0.5–50)	43.3 ± 7.8	66 ± 13	69.4 ± 7.7	60.3 ± 6.1	68.5 ± 5.7
Clonazepam (0.6–60)	56 ± 12	73 ± 11	67.1 ± 9.0	66.3 ± 9.1	66.1 ± 5.4
Diazepam (0.5–50)	59.6 ± 8.0	80.1 ± 8.8	75.7 ± 6.8	66.3 ± 5.3	74.0 ± 6.2
Flunitrazepam (0.4–40)	63.7 ± 6.2	86.6 ± 7.7	81.2 ± 6.2	68.5 ± 5.1	78.3 ± 4.1

[a] Extraction efficiency (EE) is the ratio between the average absolute peak areas of sets C and B. The uncertainty is given by $EE \times \sqrt{\frac{\Delta C}{C} + \frac{\Delta B}{B}}$, where $\frac{\Delta C}{C}$ and $\frac{\Delta B}{B}$ are the relative standard deviations of sets C and B, respectively; set B refers to blank samples fortified after the SLE-LTP procedure, and set C to blank samples fortified before extraction

[b] X is the lowest fortification level

[c] For aldicarb sulfoxide, the levels are 1X, 2X, 5X, 15X and 50X

the average absolute matrix effect and the polarity of the analytes investigated in this study ($r = 0.11$, $P = 0.745$).

Table 2 show the mean extraction efficiencies of the SLE–LTP procedure for the analytes at the five fortification levels. Aldicarb had the highest efficiency at all levels, with means ranging from 69 to 118 %. The LC–MS–MS was less sensitive for aldicarb sulfoxide (fortification levels from 8 to 400 ng/ml), with extraction efficiency between 40 and 50 %. Among the benzodiazepines, the efficiency was lower for bromazepam (43.3–69.4 %) and higher for flunitrazepam (63.7–86.6 %). The efficiency for cocaine ranged between 54 and 65 %, but was lower than 40 % for its metabolite benzoylecgonine. The uncertainty of the mean extraction efficiency was below 20 % for all

compounds at all concentrations tested, with the exception of aldicarb at the lowest level (0.4 ng/ml; 26 %).

In summary, a significant matrix effect was found for nine of the 11 analytes in at least one fortification level, and extraction efficiency was below 70 % for most analytes. These results justified the use of an analytical curve fortified before extraction (pre-fortified in-matrix analytical curve) for the quantification of analytes in real samples. This procedure compensates for both matrix effects and bias due to losses during sample extraction.

The adjusted least-square pre-fortified-in-matrix analytical curve was heteroscedastic ($C_{calculated} > C_{critical;5;6}$) for all analytes. The best weighting factors found were $1/x$ for aldicarb sulfoxide and flunitrazepam, $1/x^2$ for aldicarb sulfone, amitriptyline, benzoylecgonine, clonazepam and cocaine, and $1/y^2$ for aldicarb, bromazepam, carbamazepine and diazepam. All correlation coefficients were significant ($T_{rw} > T_{critical,\ 28}$) and higher than 0.98.

Precision was evaluated in terms of the repeatability (same analyst, same day) and the intermediate precision (same or different analyst on different days) experiments. Repeatability was acceptable for all analytes (RSD < 20 %), ranging from 3.6 % (for aldicarb sulfone) to 18.2 % (for carbamazepine) at the lowest concentration level, and from 2.8 % (carbamazepine) to 15.0 % (benzoylecgonine) at the other two fortification levels (Table 3). Intermediate precision ranged from 9.4 % (cocaine) to 30.0 % (carbamazepine) at the lowest level, and from 8.7 % (cocaine) to 16.3 % (aldicarb sulfoxide) at the intermediate level. Trueness (in % recovery) was within the range of 80–120 % for most analytes at all levels in both precision experiments, with the exception of carbamazepine and clonazepam at the lowest level and amitriptyline at two levels.

The limit of quantification (LOQ) of the method, defined as the lowest level in which repeatability was within the acceptable range, was set at the lowest fortification level for all analytes, ranging from 1 (carbamazepine) to 40 (aldicarb sulfoxide) ng/g in the larvae (Table 3). These values are within the same range as LOQs reported for different analytes in larvae [9, 27, 41–44], and are below the concentrations usually found in larvae collected in intoxication cases, rendering the method applicable for forensic investigations.

Analysis of actual larval samples

In this study, twenty-eight larval samples (from 27 cases) collected by the IML-DF between 2009 and 2012 were analyzed using the optimized and validated method. Sample mass varied from 0.30 to 16.8 g. Regarding their origin, five samples were not clearly identified. Among the 22 identified samples, 17 were obtained from male corpses and five from females. Precise age information was available for 17 cases, and ranged between 13 and 53 years of age (mean of 32.6). The cause of death was not determined for 12 of the identified cases. The reported causes were traumatism ($n = 4$), stabbing ($n = 3$), asphyxia ($n = 2$) and cardiopathy ($n = 1$). Toxicological analyses of tissues or fluids were not performed for any of these cases during necropsy.

Of the 28 analyzed samples, 11 (39.3 %) were positive for at least one of the investigated compounds, with two samples containing more than one compound (Table 4).

Table 3 Limit of quantification (LOQ) and trueness (RSD) in %, obtained from repeatability ($n = 6$) and intermediate precision ($n = 13$) experiments in fortified blank matrices

Analyte	LOQ (ng/g)[a]	Repeatability			Intermediate precision	
		LOQ (% RSD)	5 × LOQ (% RSD)	100 × LOQ[b] (% RSD)	LOQ (% RSD)	5 × LOQ (% RSD)
Aldicarb	2.0	88.1 (9.05)	104 (7.47)	102 (4.55)	93.6 (9.73)	95.7 (11.4)
Aldicarb sulfoxide	40.0	98.7 (8.13)	95.5 (7.24)	107 (12.0)	91.4 (10.0)	104 (16.3)
Aldicarb sulfone	6.0	111 (3.59)	102 (6.28)	99.4 (6.68)	102 (13.7)	97.9 (9.70)
Amitriptyline	3.0	122 (14.8)	100 (10.5)	98.9 (3.74)	122 (12.4)	102 (10.5)
Carbamazepine	1.0	127 (18.2)	105 (2.83)	100 (5.36)	101 (30.0)	97.8 (10.3)
Cocaine	2.0	100 (4.81)	89.1 (5.20)	100 (8.90)	107 (9.42)	89.9 (8.74)
Benzoylecgonine	2.0	107 (9.61)	87.7 (7.27)	98.0 (15.0)	113 (10.8)	91.7 (13.4)
Bromazepam	2.5	100 (15.4)	108 (6.80)	99.7 (8.69)	103 (14.3)	98.5 (14.8)
Clonazepam	3.0	126 (16.9)	107 (7.80)	93.0 (7.34)	117 (15.7)	94.5 (15.4)
Diazepam	2.5	110 (11.4)	102 (8.40)	101 (7.12)	106 (14.2)	94.4 (12.1)
Flunitrazepam	2.0	110 (6.51)	101 (4.81)	99.1 (4.89)	106 (13.5)	90.6 (12.1)

RSD relative standard deviation

[a] In the larvae

[b] For aldicarb sulfoxide, the level was 50 × LOQ

Table 4 Analyte concentrations found in actual larval samples

Case	Description	Concentration (ng/g)				
		BZE	COC	CRB	DZP	AMT
A	Male, 19 yrs, death by stabbing	0.84	1.00	<LOQ	3.39	nd
B	No identification	nd	nd	nd	4.43	nd
C	Female, undetermined age and cause of death	177	392	nd	nd	nd
D	Male, undetermined age and cause of death	4.36	nd	nd	nd	nd
E	No identification	nd	nd	nd	5.89	nd
F	No identification	2.44	nd	nd	nd	nd
G	Male, 51 yrs, undetermined cause of death	nd	nd	2.21; 503	nd	nd
H	Female, 47 yrs, undetermined cause of death	nd	4.63	nd	nd	nd
I	No identification	nd	nd	nd	6.70	nd
J	Male, undetermined age and cause of death	nd	nd	nd	nd	16.3

BZE benzoylecgonine, *COC* cocaine, *CRB* carbamazepine, *DZP* diazepam, *AMT* amitriptyline, *nd* not detected

The sample from case A (19-year-old male), showed the presence of diazepam, cocaine and its metabolite, benzoylecgonine, and traces of carbamazepine (<LOQ). Four samples (14 %, Cases A, B, E and I) were positive for diazepam (3.39–6.70 ng/g), the only benzodiazepine found in the study. Benzodiazepines were found in 4.6 % of the urine samples of postmortem cases investigated by the IML-DF from 2006 to 2008 [13]. The two samples collected from case G (51-year-old male) were positive for carbamazepine, with concentrations differing by two orders of magnitude. Most likely, the two samples were collected from different sites of the corpse, a procedure that is recommended in forensic investigation [9, 11, 45]. Concentration variability could also be due to different physiological states of the larvae collected in different locations [9]. To the best of our knowledge, this is the first report worldwide of diazepam and carbamazepine in entomological samples collected from corpses.

Five larval samples contained cocaine and/or benzoylecgonine. Cocaine half-life in humans is route and dose-dependent, but generally lies below 5 h [46]. Information on metabolite concentration or the parent drug-metabolite ratio may help to determine the time between exposure and death. However, drug accumulation in insects is unpredictable, making quantitative extrapolations unreliable [11]. Cocaine alone was found in Case H, and it was found together with benzoylecgonine in Cases A (COC/BZE = 1.2) and C (COC/BZE = 2.2). The presence of cocaine (alone or at a higher concentration than its metabolite) in these cases indicates that cocaine exposure may have occurred within a short interval before death, while in Cases D and F, the presence of the metabolite only reflects a more remote exposure to the drug. Furthermore, possible cocaine metabolism by the larvae or via nonenzymatic hydrolysis could also affect the ratio between the parent compound and its metabolite. Using a gas chromatographic method, Nolte et al. [25] detected both cocaine and benzoylecgonine in larval and muscle samples from the corpse of a drug user.

The sample from Case J showed amitriptyline at a concentration of 16.3 ng/g. Amitriptyline had been previously quantified by Miller et al. [47] in fly puparia, beetle exuvia and fecal material in a case of death by multiple-drug intoxication (3.400–5.400 ng/g), and by Tracqui et al. [11] in fly larvae (133 ng/g).

Aldicarb, found in an illegal rodenticide known as "chumbinho" in Brazil, has been reported as one of the main substances involved in lethal cases of intoxication in Brazil [13, 20, 21], mostly in suicide cases. This pesticide was detected in the gastric contents of 13.3 % of cases investigated by the IML-DF from 2006 to 2008 [13]. Our study, however, detected neither aldicarb nor its sulfoxide/sulfone metabolites in any of the larval samples analyzed. Gunatilake and Goff [26] found malathion in fly larvae (2.05 ng/g) collected from the remains of a corpse of one poisoning case that was suspected to involve the insecticide. In this study, malathion was also present in the gastric contents and body fat.

The internal quality control (QC) samples ($n = 12$) analyzed along with the different batches of authentic samples showed satisfactory accuracy and precision, ensuring the quality of the extraction procedure during routine analysis. Accuracy of the method estimated from the QC samples varied from 96 % for bromazepam to 114 % for carbamazepine, and precision ranged between 5 % for flunitrazepam and 15 % for carbamazepine.

Conclusions

We developed and satisfactorily validated an SLE–LTP method for the simultaneous determination of 11 substances from different chemical classes in larval samples by LC–MS–MS. The extraction procedure was shown to be simple, easily performed, time effective, with a low solvent consumption, and suitable for the analysis of several samples in a single batch. Currently, very limited information is available on method development and validation for the determination of xenobiotics in larval samples, and

to the best of our knowledge, this is the first fully validated method for the simultaneous determination of multiclass substances in such a matrix. The method was used to analyze 28 actual samples collected from corpses for which no previous toxicological investigation had been conducted.

In conclusion, the analytical method developed herein can be easily implemented in a forensic laboratory, and may provide an additional tool during the investigation of forensic cases when tissues of putrefied corpses are not available. Although the results obtained are quantitative, they only indicate that an exposure to the detected substance has occurred before death, and no direct inference about the cause of death can be made.

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