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Simultaneous determination of three residual barbiturates in pork using accelerated solvent extraction and gas chromatography–mass spectrometry

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Abstract

A new method was developed for the rapid extraction and unequivocal determination of barbital, amobarbital and phenobarbital residues in pork. The isolation of the analytes from pork samples was accomplished by utilizing an accelerated solvent extractor ASE 300. The procedure was automatically carried out in series for fat removing and extraction, respectively with *n*-hexane and acetonitrile pressurized constantly at 10.3 MPa for 30 min. After evaporation, the extracts were cleaned up on a C_{18} solid phase extraction (SPE) cartridge and the barbiturates were eluted with hexane–ethyl acetate (7:3), evaporated on a rotary evaporator and derivatized with CH_3I. The methylated barbiturates were separated on a HP-5MS capillary column and detected with a mass detector. Electron impact ion source (EI) operating in time program-selected ion monitoring mode (SIM) was used for identification and external standard method was used for quantification. Good linearity was obtained in the range from 0.5 μg/kg to 25 μg/kg. Average recoveries of the three barbiturates spiked in pork ranged from 84.0% to 103.0%, with relative standard deviations from 1.6% to 12%. The limit of detection (LOD) was 0.5 μg/kg for the three barbiturates (S/N ≥ 3). The quantification limit (LOQ) was 1 μg/kg for the three barbiturates (S/N ≥ 10).

Keywords: Barbiturates; Accelerated solvent extraction (ASE); Solid phase extraction; Methylation; Gas chromatography–mass spectrometry; Pork

1. Introduction

Barbital, amobarbital and phenobarbital, as the derivatives of barbituric acid (2,4,6-trihydroxypyrimidine, structures are shown in Fig. 1), are the main well-known sedative hypnotics used in clinic [1]. They have been used extensively in the past to reduce anxiety, reduce respiration, reduce blood pressure, reduce heart rate and reduce rapid eye movement (REM) sleep. Sedative barbiturate compounds distributed into all tissue and organs in vivo, even cross the placenta barrier. Since barbiturates and their metabolites accumulated in tissues and could lead to tolerance, dependence, excessive sedation and cause anesthesia, coma and even death, they have been prohibited to men and to acting as animal feed additive and chemical protection drugs in animal butchery at present. However, they could make animal (e.g. pig) sleepful and moveless, accelerate upgrowth, and decrease feed cost. They are still being misused as animal feed additive and chemical protection drugs in animal butchery and in horse races. It is necessary to monitor their residues to protect the consumer's health.

Gas chromatography–mass spectrometry (GC–MS) has been used many times for the identification and confirmation of barbiturates during the last few years. The representative reports were published for the analysis of barbiturates in biological fluids (e.g. urine and serum) [2–7]. As all the procedures described, it was necessary to concentrate the pertinent analytes and eliminate the interfering compounds from the matrix. Solid phase extraction (SPE) [2–4], solid phase micro-extraction (SPME) [5,6], or stir bar sorptive extraction (SBSE) [7] were the most frequently utilized measures. Adam and Reeves [8] have validated a confirmatory method with SPE–GC–MS for the identification of pentobarbital in dog feed, in considering that the feed composed of rendered products of some euthanized animal meat and bone meal. The limit of detection (LOD) was 0.7 μg/kg. Heller et al.

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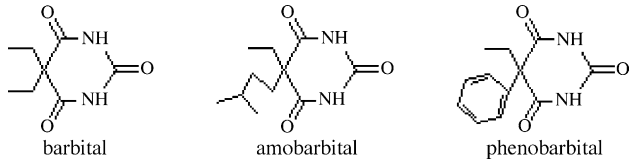

Fig. 1. Molecular structure of three barbiturates.

[9] have used the same method to determine pentobarbital in dog feed, using isotope labeled pentobarbital as an internal standard. The limit of quantitation (LOQ) was 2 μg/kg. The derivatization method adopted in their study was based on a procedure described by Liu et al. [10]. A solution composed of 100 μL TMAH (25% in methanol)–DMSO (1:20, v/v) was added to the residue of purified samples and mixed before the addition of 25 μL iodomethane used as derivative reagents. The derivative was treated with 0.4 mL 0.1 mol/L HCl and back extracted to 2 mL of isooctane. Our research team has previously developed a simple derivatization method for simultaneous identifying of three barbiturates (barbital, pentobarbital and phenobarbital) in pork by SPE–GC–MS [11]. The derivative reagent was a mixture of 1 mL acetone, 20 μL iodomethane and 50 mg potassium carbonate. As the procedure did not need to add hydrochloric acid and to back extract the derivative into organic solution, it was a more simplified method. The identification of methylated barbiturates and the recovery showed that the method was effective. Heller [12] have identified the phenobarbital in dog feed by LC/MS/MS without derivatization. The limit of quantification, however, was 40 μg/kg, so the LC/MS method would not be recommended as a substitute for GC/MS in barbiturates determination because of poorer sensitivity.

Recently, automated accelerated solvent extraction (ASE), or pressurized liquid extraction (PLE), has attracted more and more attention in samples pretreatment for residues analysis. In a common ASE procedure, extractions were performed with conventional liquid solvents at elevated pressures (10.3–20.6 MPa) and moderate temperatures (50–200 °C). ASE could extract solid or semi-solid samples quickly and with much less solvent than conventional techniques. It has recently been reported for extraction of a variety of compounds, and a considerable number of applications have been reported in environmental, food, polymer, and pharmaceutical areas [12–17]. However, the method of using ASE to extract barbiturates residues in animal tissues has scarcely been reported. This article has presented a modification to our early experiments and ASE was used for extraction and defatting. The whole time for defatting and extraction has decreased to 35 min, and the follow up clean-up effects was proven to be more efficient. Good recoveries of 84.0–103.0% and lower LOD of 0.5 μg/kg showed that ASE was a good substitution for shaking.

2. Experimental

2.1. Chemicals

Barbital, amobarbital and phenobarbital standards (purity >99.0%) were provided by the courtesy of the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol, hexane, ethyl acetate, acetone were HPLC grade and purchased from Fluka Co. Ltd. (Switzerland). Analytical grade acetonitrile, iodomethane (CH_3I), sodium acetate (NaAc), di-potassium hydrogen phosphate (K_2HPO_4), anhydrous sodium sulfate and potassium carbonate (K_2CO_3) were obtained from Beijing Chemical Reagents Company (Beijing, China). Anhydrous sodium sulfate was baked for 6 h at 400 °C and kept in desiccator. Diatomaceous earth was obtained from Dionex (Salt Lake, USA). C_{18} solid-phase extraction cartridge (500 mg/3 mL tubes, No. 57012) was purchased from Supelco Co. Ltd. (Bellefonte, PA, USA).

2.2. Standard solutions

Three barbiturates stock standard solutions at 1.0 g/L were prepared in methanol separately and stored at 4 °C. Three barbiturates mixed standards 1 mg/L were obtained by transferring 0.1 mL of each stock solution into a 100 mL volumetric flask and diluting to volume with methanol. Series mixed working standard solutions were prepared by transferring appropriate volumes of 1 mg/L mixed standards into 100 mL volumetric flasks separately and diluting to mark with methanol.

A 0.1 mol/L K_2HPO_4 buffer (pH 7.4) was prepared by dissolving 14.1 g of K_2HPO_4 in 450 mL distilled water, adjusting pH to 7.4 with 1 mol/L phosphoric acid and diluting to 500 mL with distilled water. A 0.1 mol/L NaAc buffer (pH 7.0) was prepared by dissolving 13.6 g of sodium acetate trihydrate in about 800 mL distilled water, adjusting pH to 7.0 with 1 mol/L HCI and diluting to 1 L of distilled water. The washing solution of SPE was prepared daily by combining 2 mL of ethyl acetate with 38 mL of hexane. The SPE eluting solution was prepared daily by combining 12 mL of ethyl acetate with 28 mL of hexane.

2.3. Sample preparation

Blank pork samples were purchased from a supermarket in Beijing. Samples were removed skin, all the fat and lean meat were ground and homogenized in a blender. The blank samples had been proved to be free of the three barbiturates by the GC/MS method described by Zhao et al. [11]. Spiked samples were prepared in the way which an aliquot (5 g) of ground blank pork sample was spiked with appropriate amounts of mixed standards, homogenized 30 s in a vortex mixer (Yamato MT-5, Japan) and let sit for overnight at room temperature. The ultimate fortified concentrations in matrices are 0.5, 1, 2.5, 5, 10, and 25 μg/kg.

2.4. Extraction procedures

2.4.1. ASE extraction

Accelerated solvent extraction of pork sample was performed on a Dionex ASE 300 (Dionex, sunnvale, CA, USA). The ASE 300 was equipped with the Solvent Controller and AutoASE software. The Solvent Controller was an easy-to-use module that allowed automated solvent mixture and delivery from up to four solvents. AutoASE was a controller and reporting application software package. The ASE 300 was also equipped with an auto-

Table 1
The ASE 300 extraction conditions test

Test	ASE 300 conditions								Analyte recovery (%)		
	Temperature (°C)		Solvent composition		Static time (min)		Static cycles				
	Defatting	Extraction	Defatting	Extraction	Defatting	Extraction	Defatting	Extraction	Barbital	Amobarbital	Phenobarbital
1	125	125	Hexane	Acetonitrile	5	6	1	2	108.5	41.6	92.9
2	100	100	Hexane	Acetonitrile	5	5	1	1	77.8	39.9	66.4
3	100	100	Hexane	Acetonitrile	5	6	1	2	104.7	53.3	82.8
4	80	80	Hexane	Acetonitrile	5	6	1	2	102.5	68.8	89.5
5	50	50	Hexane	Acetonitrile	5	6	1	2	103.7	87.3	93.3
6	0[a]	100	Hexane	Acetonitrile	5	6	1	2	90.6	94.6	103.0
7	100	100	Hexane	Acetonitrile–actone (95:5, v/v)	5	6	1	2	120.8	47.5	84.0
8	80	80	Hexane	Acetonitrile–actone (85:15, v/v)	5	6	1	2	117.8	69.9	102.0
9	80	80	Hexane	Acetonitrile–dichloromethane (70:30, v/v)	5	6	1	2	127.6	70.9	105.8

[a]Test was performed at constant pressure (10.3 MPa) using 34 mL extraction cell. The Flush volume was 40% to defatting and 60% to extraction analytes. The spiked concentrations were 7.5 μg/kg; 0 means that extraction was carried out at room temperature.

Table 2
ASE 300 extraction conditions

	Defatting	Extraction
Solvent	Hexane	Acetrinile
Pressure	10.3 MPa	10.3 MPa
Temperature	0[a]	100 °C
Heat time	0[b]	5 min
Static time	5 min	6 min
Flush volume	40%	60%
Purge time	120 s	120 s
Static cycles	1	2

[a] 0 means that extraction temperature was room temperature.
[b] 0 means that the sample was not heated.

sampler carousel and a collection tray that allowed 12 separate samples to be extracted sequentially. The system pressure was 10.3 MPa. Thirty-four milliliter stainless steel extraction cells and 200 mL glass collecting vials were used.

An aliquot (5 g) of spiked sample or blank sample was put into a 100 mL mortar and well ground with 7.5 g of diatomaceous earth. The mixture was then loaded onto the 34 mL extraction cell which had a cellulose disk at the bottom, and the cell been capped and placed on the extractor. The extraction process was sequentially performed with hexane to remove the fat in sample and with acetonitrile to extract the analytes. Seven gram anhydrous sodium sulfate was added into the glass collecting vials to adsorb the co-extracted water. Operating conditions such as temperature, solvent composition and extracting time have been changed to get a set of optimized ASE extraction conditions as described in Table 1. The pressure of the solvent was kept constant at 10.3 MPa. The temperature '0' means the extraction at room temperature and the sample was not heated. The temperature '100' means the sample was heated to 100 °C in the preheated oven for 5 min. The hexane extractive solution was about 40 mL. The acetonitrile extractive solution was about 65 mL. The optimized extraction conditions are described in Table 2. As the sample amount been treated in the conventional shaking method was 2 g, an aliquot of the extracted solution was taken as follows: extract was diluted to 100 mL with acetonitrile and 40 mL of the solution was pipette into a 100-mL pear-shaped flask with ground-glass stopper. The extract was then evaporated in a rotary evaporator (100 rpm, 35 ± 5 °C) (Yamato R-134, Japan) till an oily dark residue remains. The residue was re-dissolved in buffer solutions as described by Adam and Heller et al. [8,9] which composed of 5 mL 0.1 mol/L K_2HPO_4 (pH 7.4) and 2 mL 0.1 mol/L NaAc (pH 7.0) and was shaken on a vortex mixer for 30 s.

2.4.2. Shaking

An aliquot (2 g) of spiked ground pork sample or blank sample was put into a 50 mL plastic centrifuge tube, followed by addition of 2.5 g anhydrous sodium sulfate and 30 mL acetonitrile. The mixture was mixed well. The centrifuge tube was placed onto a shaker and shaked for 2 h and centrifuged at 4000 rpm for 5 min. The top extractive solution was decanted into a 100 mL separatory funnel with ground glass stopper and then defatted with two aliquots of 20 mL hexane. The hexane

layer was discarded. The remaining extractive solutions were evaporated to nearly dryness and re-dissolved in 5 mL buffer solution composed of 0.1 mol/L K_2HPO_4 (pH 7.4) and 2 mL 0.1 mol/L NaAc (pH 7.0).

2.5. C_{18} SPE clean-up

A C_{18} SPE cartridge was pre-washed with 5 mL methanol and 5 mL 0.1 mol/L NaAc buffer (pH 7.0). A flow rate of 1–2 drops per second was maintained by adjusting vacuum and made sure the SPE bed not to be dry. The re-dissolved aqueous extracts were transferred directly to the SPE cartridge. When the surface of the liquid reached the bed of the SPE, the cartridge was washed with 5 mL 0.1 mol/L NaAc buffer (pH 7.0). After that the cartridge was dried under vacuum and washed again with 5 mL hexane–ethyl acetate (95:5). The barbiturates residue was eluted with 5 mL hexane–ethyl acetate (7:3) into a graduated 10 mL centrifuge tube with ground-glass stopper.

2.6. CH_3I methylation

The derivatization was based on a method described previously by our group [11]. The SPE cartridge eluate collected in centrifuge tubes were evaporated to dryness under a gentle stream of nitrogen on an evaporator (CNM MST-1, China) at room temperature. One milliliter acetone, 20 μL CH_3I and 50 mg K_2CO_3 were added to the residues and were shaken on a Vortex mixer for 30 s. The tube was capped and placed into the muffle furnace, where the reaction took place for 2.5 h at 52 °C. After that the tube was taken out and cooled to room temperature and centrifuged at 4000 rpm for 2 min. The acetone layer was decanted to a 10 mL ground glass capped centrifuge tube. The organic solvent was evaporated to dryness at room temperature under a stream of nitrogen. The residue was reconstituted in 1 mL ethyl acetate, vortexed for 30 s and transferred into a GC screw cap vial. The ultimate solutions were ready for GC–MS determination.

2.7. GC–MS determination

Analysis was performed with Agilent 6890 gas chromatograph equipped with an Agilent 5973 inert MSD, a split/splitless injector, a series 7683 automatic sampler and the GC–MS Chemstation Data System (Agilent, Palo Alto USA). The GC column was a HP-5MS capillary column, 30 m in length, 0.25 mm i.d., and 0.25 μm in film thickness (Agilent, Palo Alto, USA).

Table 3
Three barbiturates monitoring ions and retention time

	Retention time (min)	Monitoring ions
Barbital	7.05	126, 169[a], 183, 184
Amobarbital	8.69	169[a], 170, 184, 226
Phenobarbital	10.83	175, 232[a], 245, 260

[a] *Note*: This ion was quantification ion.

GC oven temperature was initially kept at 70 °C for 1 min, raised at a rate of 20 °C/min to 195 °C and kept for 1 min, then raised at a rate of 20 °C/min to 250 °C, kept for 1 min. The whole procedure was held for 12 min. The injector temperature was 250 °C and 1 μL solution was injected splitlessly. Helium was used as carrier gas (purity >99.999%). the flow rate was set at 1.1 mL/min constantly.

The mass selective detector was operated in electron ionization (EI) mode with 70 eV of electron energy. The ion source temperature was 230 °C. The GC–MS interface temperature was 280 °C. Quadrupole module temperature was 150 °C. Full scan mode was chosen with a wide range from *m/z* 50 to *m/z* 450. Solvent delay was set for 5.5 min. Based on scanning chromatograms of three barbiturates standard, four monitored ions were selected for each barbiturate for confirmation and a quantified ion was selected for each analyte, including *m/z* 169 for barbital and amobarbital, and *m/z* 232 for phenobarbital. The monitoring ions and the retention times are described in Table 3. The main fragmentation patterns of their derivatives are shown in Fig. 2. Monitoring ions and the retention times were obtained after triplicate injection of working standard solutions (0.5 μg/L and 1 μg/L) with different concentrations in fullscan mode.

2.8. Calibration, recovery and precision

Calibration and quantitation results were determined and analyzed by using the GC–MS Chemstation Data System. Calibration curves were obtained from GC–MS analysis of extracts of blank and spiked pork samples. The spiked concentrations were 0.5, 1, 2.5, 5, 10, and 25 μg/kg. Triplicated analysis was taken for every concentration. Peak areas of spiked samples were measured and corrected with the blank control samples and plotted against the spiked concentration to generate the calibration curves. The calibration curves and the correlation coefficients are described in Table 4.

For the assessment of the recovery and precision of the PLE–SPE–GC–MS method, six replications of blank pork sample and spiked sample of 2.5, 5 or 10 μg/kg were extracted and

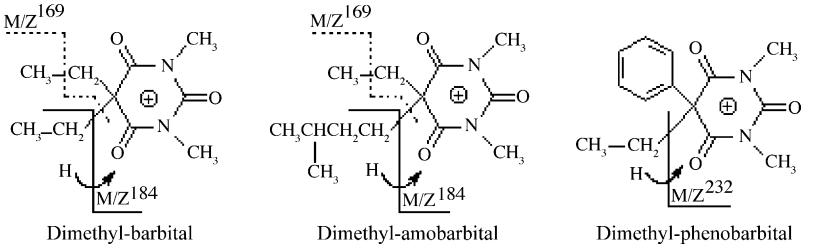

Fig. 2. Fragmentation patterns of main ions for the three dimethyl-derivatives.

Table 4
Linear equations and regression coefficients of the calibration curves ($n = 3$)

Analyte	Linear equations	Regression coefficients
Barbital	A = 133.74C + 114.25	0.9993
Amobarbital	A = 1368.1C + 737.83	0.9998
Phenobarbital	A = 247.84C + 145.3	0.9994

A: peak area, C: spiked concentrations.

Table 5
Recoveries of three barbiturates in pork ($n = 6$)

Analyte	Spike (μg/kg)	Recovery (%)	CV (%)
Barbital	2.5	100.4	11
	5	90.6	7.3
	10	89.0	12
Amobarbital	2.5	84.0	1.6
	5	94.6	4.7
	10	90.6	7.6
Phenobarbital	2.5	101.4	5.0
	5	103.0	6.1
	10	89.7	9.0

analyzed according to the above procedures. External standard method was used for the quantitation. A signal-to-noise ratio of 10:1 was set for the three barbiturates derivatives quantitation and signal-to-noise ratio of 3:1 for their identification. The results are described in Table 5.

3. Results and discussion

3.1. ASE Optimization

Three barbiturates were considered to be slightly polar and thermo-stable chemicals. So they could be extracted by ASE 300. Our ASE procedure included two steps: extraction of interfering matters with hexane, and then extraction of the drugs with acetonitrile. The influential factors on extraction procedure included temperature, pressure, solvent composition, static time and the number of static cycles. The ASE 300 worked at constant pressure (10.3 MPa). Table 1 describes the extraction conditions of three barbiturates by considering various ASE 300 parameters.

The extraction temperature was 50, 80, 100, and 125 °C. The ASE 300 extraction temperature greatly affected the barbiturates' recoveries and the extraction behavior of three drugs from the pork matrix. The effect of extraction temperature on the extraction efficiency of ASE is presented in Fig. 3. The results indicated that the recovery of amobarbital and phenobarbital decreased with the temperature's elevation, while the recovery of barbital was almost unaffected. This result contravened the usual conclusion that the ASE extraction recovery would increase with the temperature's elevation [13–17]. We only reduced the defatting step temperature to "0" that means in defatting procedure, the sample was not heated and extracted at room temperature when the other extraction conditions remained the same. As a result, the recovery of three barbiturates was all above 90%. We considered that the causes of the barbiturates structure could

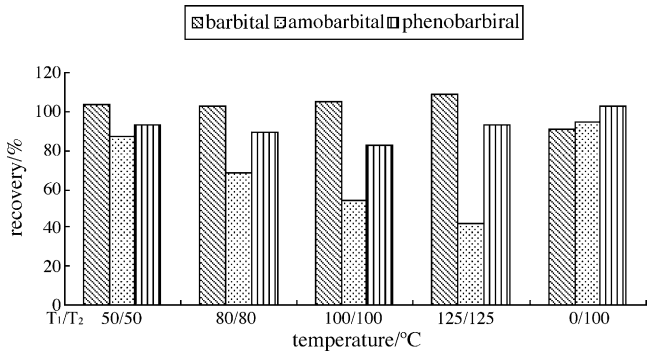

Fig. 3. The effect of extraction temperature on the extraction efficiency of ASE (T_1, the temperature for hexane extraction; T_2, the temperature for acetonitrile extraction).

attribute to the test results. Amobarbital molecule had a group of $–CH_2–CH_2–CH–(CH_3)_2$ which was the long carbon chains. The dissolvability of amobarbital in hexane would increase with the elevated temperature in the defatting step. It could lead to higher amobarbital recovery extracted with hexane and lower the recovery extracted with acetonitrile. The same but relatively slight effect existed in phenobarbital due to a phenyl group in its molecular structure. Barbital, however, showed no variation on recovery due to the short carbon chain and two ethyl groups in its molecular structure.

Table 1 also shows the increase of static time and the numbers of static cycles of acetonitrile extraction step could cause the increase of recoveries, but did not obviously change the recovery of amobarbital. Two different solvent compositions were studied. One mixture was acetonitrile–acetone (95:5, 85:15) and the other was acetonitrile–dichloromethane (70:30). The result indicated that the recovery of amobarbital did not obviously increase; the recovery of phenobarbital obviously increased and reached 100%; the recovery of barbital exceeded 120%, there was a result of extracted impurity.

Based on the test results, we have selected the optimized ASE extraction as follows: using hexane through one static cycle of 5 min at room temperature and then using acetonitrile to exhaustively extract three barbiturates by two static cycles of 6 min at 100 °C (see Table 2). The entire procedure was completely automated and required about 30 min.

3.2. Comparison between ASE and shaking

Comparison of ASE and shaking extraction technique is shown in Table 6. ASE extraction was done within about 30 min (including the time of defatting and extraction), while shaking extraction required about 3 h. ASE was conducted automatically, using less hexane solvent with higher sensitivity. In addition, the defatting step was prior to the extraction of target drugs in ASE, whereas, in the shaking the steps were reverse. The shaking method used acetonitrile to extract target drugs, after that, liquid–liquid partition was used to eliminate the fat. The cleaning-up efficiency of ASE was better than that of shaking. The TIC chromatograms of sample solutions from shaking and from ASE are presented in Fig. 4. It was obvious that the interfer-

Table 6
Comparison between ASE and shaking extraction technique

Parameters	ASE (5 g sample)	Shaking extraction (2 g sample)
Extraction solvent	Acetonitrile	Acetonitrile
Extraction time	15 min	2 h
Acetonitrile consumption	65 mL	30 mL
Defatting method	ASE	LLE
Defatting solvent	Hexane	Hexane
Defatting time	10 min	50 min
Hexane consumption	40 mL	40 mL
Automatic/manua	Automatic	Manual
LOD	0.5 μg/kg	1 μg/kg
LOQ	1 μg/kg	2.5 μg/kg
Average recoveries (spiked 5 μg/kg)	90.6 (7.3), 94.6 (4.7), 103.0 (6.1)	64.9 (11), 107.8 (11), 109.8 (5.4)
Capital investment	High	Low

ence peaks in ASE extracted sample chromatogram were fewer than those in shaking extracted sample chromatogram and the base line signal fell from 1000 to 500.

3.3. Analytical data

Specificity of the ASE–SPE–GC–MS method was proved by processing and analysing pork blank control samples (Fig. 4B). No interference was noticed around the retention times of the analytes. Under the adopted chromatographic conditions, the separation was acceptable.

The calibration curves using fortified samples provided correlation coefficients (r^2) higher than 0.999 for three analytes in the whole range of tested concentrations (0.5–25 μg/kg) (Table 4).

The overall repeatability was determined by calculating the relative standard deviation (RSD) for repeated measurements

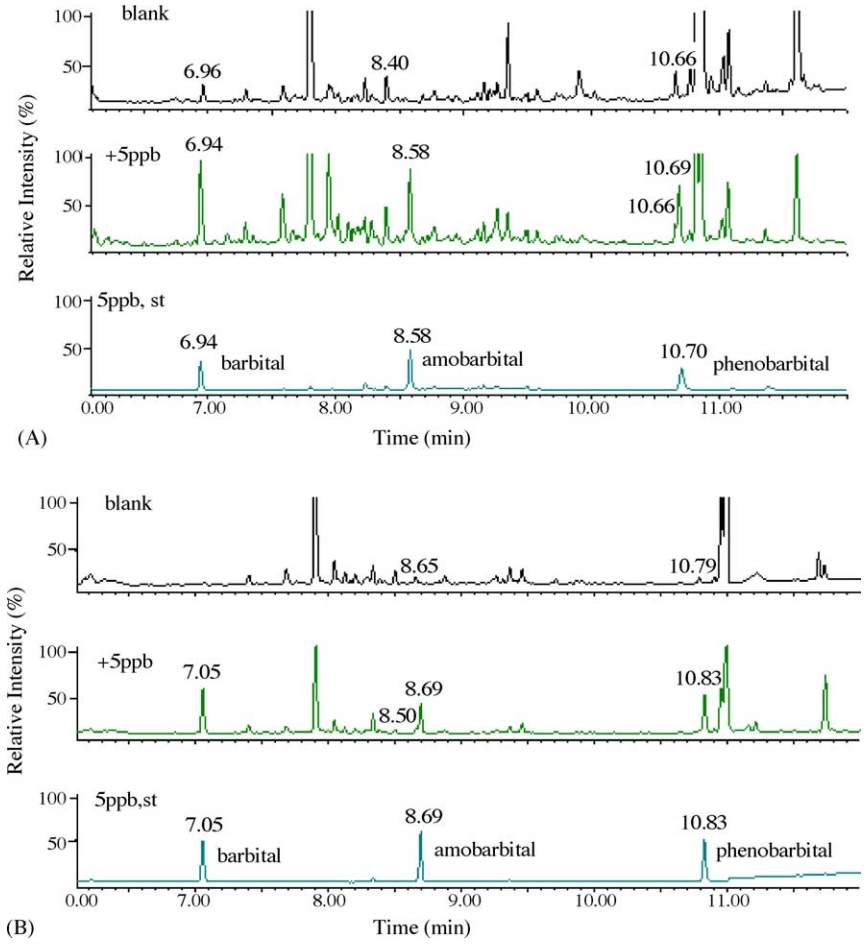

Fig. 4. Chromatograms of ASE 300 extraction and shaking extraction (2 g pork samples). (A) The chromatograms of extracts from shaking. Concerning the chromatographic conditions and the derivatization conditions, see [11]; (B) the chromatograms of extracts with ASE 300. The chromatographic conditions and the derivatization conditions are described in Section 2 of the text.

and ranged from 1.6% to 12% (Table 5). The recovery of the analytes was calculated by comparing the peak areas of fortified samples with those of the corresponding standards. Recovery values ranged from 84% to 103% (Table 5).

The limit of quantification (LOQ) of each barbiturate residue in pork was 1 μg/kg (signal-to-noise ratio of at least 10:1). It was the lowest concentration for which acceptable accuracy and precision were obtained. LOD reduced to 0.5 μg/kg (signal-to-noise ratio of at least 3:1). The LOQ and LOD were lower than the result of our previous test [11], thus proving the ASE–SPE–GC–MS method suitable for confirmatory purposes.

4. Conclusion

The major subject of this study was to investigate the suitability and convenience of ASE for the extraction of some barbiturates from pork samples. The result demonstrated that this technique offered many advantages over extraction methods currently used in the extraction of three barbiturates. The extraction procedure was rapid, simple and highly automated. This method made defatting and extraction run continually. The combination of ASE extraction step with C_{18} SPE cleaning-up step could provide better cleaning-up results.

The ASE–SPE–GC–MS method was proved to be highly sensitive for the determination of the analytes in pork. The developed ASE–SPE–GC–MS method provided an unequivocal identification and accurate quantification of three barbiturates.

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