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DETERMINATION OF CHLORAMPHENICOL RESIDUES IN HONEY BY MONOLITHIC COLUMN LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY AFTER USE OF QUECHERS CLEAN-UP

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SUMMARY

An HPLC method with a monolithic column and detection by mass spectrometry has been established for determination of chloramphenicol in honey samples previously cleaned by use of a modification of the QuEChERS procedure. Honey samples were dissolved in water containing sodium chloride and extracted with acetonitrile. Further sample clean-up was performed by simple reversed-solid-phase dispersion with primary–secondary amine (PSA) adsorbent. Chloramphenicol (CAP) residues at ppb concentrations were detected by liquid chromatography–mass spectrometry (LC–MS), with electrospray ionization, in negative-ion mode. The mobile phase was methanol–0.2% aqueous ammonium acetate solution, 45:55 (*v*/*v*). Under these conditions CAP is eluted after ~4 min and 8 min from a Merck RP-18e monolithic column and a conventional C_{18} column, respectively. Recovery at three fortification levels (0.2, 20, and 200 μg kg^{-1}) was in the range 78–93% with RSD from 3.7 to 3.9%. The coefficient of determination, R^2, was 0.9995 over a 0.1–100 μg L^{-1} linear range. Use of this method for determination of CAP in honey resulted in an LCL of 0.20 μg kg^{-1}. The method was validated on the basis of EU decision 2002/657. CCα and CCβ for the honey matrix were 0.002 and 0.006 μg kg^{-1}, so the method was fit for the purpose of monitoring commercial products or checking MRL compliance.

INTRODUCTION

Chloramphenicol (CAP) is a broad-spectrum antibiotic drug which has been widely used in farming and stock breeding and to treat bees

infected with bacterial diseases [1]. Because of many, serious side-effects (e.g. fatal aplastic anemia, grey syndrome, severe bone marrow depression) [2,3], use of CAP to treat food-producing animals was banned by the European Community in 1994 [4] and the European Community has established a 'zero tolerance residue limit' for CAP in food of animal origin, including honey [5].

A variety of methods have been established for determination of CAP residues in animal foodstuffs, e.g. liquid chromatography–mass spectrometry (LC–MS), enzyme-linked immuno-sorbent assay (ELISA), radio immuno-sorbent assay, gas chromatography with derivatization, and biosensor assay [6–9]. ELISA has been used for screening purposes; its sensitivity is poor, it requires large amounts of solvents, and it is time-consuming. Impens et al. [10] measured CAP in shrimps by use of GC–MS and LC–MS^n. Use of GC–MS needs complex pretreatment and derivatization. Ashwin et al. [11] reported that the detection limit of biosensor–LC–MS was 0.1 μg kg^{-1}, but the technique gave inconsistent results. So far, the most reliable method for determination of CAP residues is HPLC–MS^n. Vivekanandan et al. [12] determined CAP in honey by liquid–liquid extraction (LLE) and HPLC–MS^n. The minimum required performance limit (MRPL) for determination of CAP is 0.3 μg kg^{-1} in all food of animal origin [13].

Monolithic columns (a bed packed with porous particles or a straight rod of highly porous silica with a bimodal pore structure) have recently been introduced for use in HPLC [14–16]. This kind of column is more permeable and results in lower column pressure, because of its large pores, so greater hydrodynamic flow can be used to increase separation speed. The surface area required for chromatographic adsorption and desorption is obtained by creating mesopores on the surface of the monolith skeleton [17]. Thus, monolithic column can efficiently separate complex mixtures with high performance even at high flow rates. The performance of the method using monolithic and conventional reversed-phase columns has been compared.

The QuEChERS method, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe, was developed for preparation of vegetable and fruit samples for analysis by GC–MS [18] and is now widely used. In this technique reversed-dispersive-solid-phase extraction with a solid adsorbent is used to quickly remove interfering components. There are no reports of use of this technique for analysis of honey. In this study a modification of the QuEChERS method was used to clean honey samples.

EXPERIMENTAL

Chemicals and Reagents

Chloramphenicol (CAP) standard (>99%) was obtained from Sigma, PSA (40 μm) was obtained from DIKMA, acetonitrile and methanol (chromatographically pure for HPLC) were obtained from Merck, and sodium chloride, magnesium sulfate, and ammonium acetate (AR) were domestic commercial products.

CAP Standard Solution

CAP (25 mg) was weighed into a 25-mL volumetric flask and dissolved in methanol to create a primary standard. The primary standard solution was further diluted with methanol to furnish working solutions with concentrations in the range 0.1 to 200 μg kg^{-1}.

Sample Preparation

A mixture of honey (5 g), water (5 mL), and acetonitrile (20 mL) was vortex mixed (QL vortex mixer; Jiangsu, China) for 30 s and placed in a KQ-50B (Jiangsu, China) ultrasonic bath for 2 min. Anhydrous sodium chloride (2 g) was added and the homogenate was vortex mixed, for 2 min, then returned to the ultrasonic bath for another 2 min. The mixture was centrifuged (TD-40B centrifuge; Anke, Shanghai, China) at 4000 rev min^{-1} for 5 min. After removal of the extract another 20 mL acetonitrile was added to the remaining sample and the process was repeated. The combined acetonitrile extracts were evaporated under vacuum and reconstituted in 1 mL acetonitrile. Further clean-up was performed by adding 25 mg PSA directly to the reconstituted extract, vortex mixing for 30 s, and centrifuging at 4000 rev min^{-1} for 1 min. A portion of the supernatant (500 μL) was transferred to a vial for analysis by LC.

On-Line High-Performance Liquid Chromatography-Mass Spectrometry

Analysis was performed with an Agilent (USA) 1100 LC–MS fitted with either a 100 mm × 4.6 mm RP-18e monolithic column (Merck USA) or a 4.6 mm × 250 mm, 5-μm particle, XDB conventional column (Agilent). For both columns the mobile phase was methanol–0.2% aqueous ammonium acetate, 45:55 (*v*/*v*), at a flow rate of 1 mL min^{-1}. The column temperature was 30°C, the injection volume 10 μL, and the detection wavelength 278 nm.

Electrospray ionization (ESI) in negative-ion mode was used for mass spectrometry. The cone potential was 25 V, the collision potential 115 V, the interface temperature 350°C, the drying gas flow 6.0 L min^{-1}, and the scan range (*m*/*z*) 100–450. In MASS mode MRM (multiple reaction monitoring) was performed with He as collision gas. The monitored ion was *m*/*z* 321 and the product ions used for quantification were *m*/*z* 257, 194, and 152.

RESULTS AND DISCUSSION

Extraction and Clean-Up

In the QuEChERS method, originally used for pesticide extraction, 10 mL acetonitrile is added to 10 g sample followed by addition of anhydrous magnesium sulfate to remove water and PSA to bind with organic acids, polar coloring, or glucides. The sample matrix was less disturbed when using PSA, which minimized problems with the LC column.

To evaluate possible adverse effects of PSA and anhydrous magnesium sulfate on determination of chloramphenicol, a series of experiments was conducted with different amounts of PSA and anhydrous $MgSO_4$ in 1-mL volumes of 100 ppb CAP standard solution. Similar clean-up and determination procedures were followed, as described in the experimental section. The results revealed that varying the amounts of PSA and anhydrous $MgSO_4$ had no effects on CAP peak area (Table I).

Table I

Effect of PSA and anhydrous $MgSO_4$ on recovery of chloramphenicol

Mass PSA (mg)	Peak area	$MgSO_4$ (mg)	Peak area
0	5.273	0	5.273
5	5.267	50	5.268
15	5.266	100	5.264
25	5.266	150	5.262

Because honey contains a small amount of water (e.g. 5 mL of an aqueous sample containing 2 g NaCl is already saturated) addition of anhydrous $MgSO_4$ could create colloids which could hinder vortex mixing and the extraction steps. No anhydrous $MgSO_4$ was therefore added to the sample in this study. Accordingly, the optimized QuEChERS conditions

used in this study were: addition of 2 × 20 mL acetonitrile to 5 g sample; addition of PSA to the acetonitrile extractant; and no addition of anhydrous $MgSO_4$. In this optimized procedure, clean-up by PSA was performed after evaporation of the acetonitrile phase to increase enrichment of the analyte and the limit of quantification.

Chromatography and Mass Spectrometry

The sample was analyzed by a mass spectrometry coupled with high-performance liquid chromatography equipped with a conventional column or a monolithic column. Column temperature was maintained at 30°C. Sample (1 mg L^{-1}, 10 µL) was injected at a flow rate of 1 mL min^{-1} with methanol–0.2% aqueous ammonium acetate, 45:55 (*v*/*v*), as mobile phase. Electrospray ionization (ESI) was used in negative-ion mode and the mass spectrometer was operated in multiple reaction monitoring (MRM) mode. A typical mass spectrum is shown in Fig. 1.

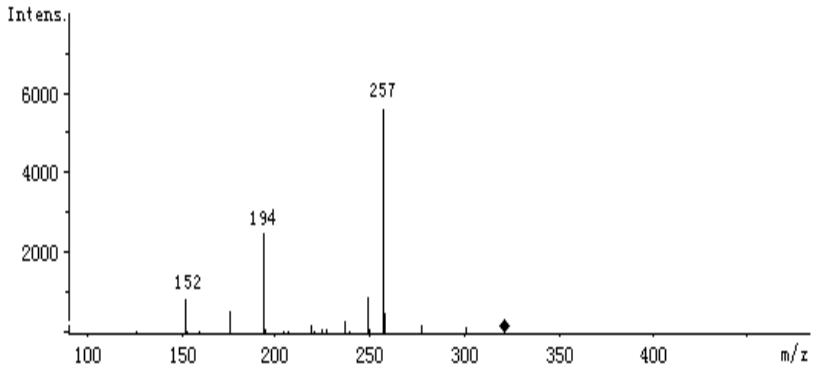

Fig. 1

Full scan ESI mass spectrum of CAP residue from honey (MS^2 potential 1.85 V)

Testing the Performance of the Monolithic Column

Monolithic columns should enable separation of complex mixtures with high performance even at a high flow rate, because monolithic materials with large through pores are more permeable. They have, however, seldom been used for analysis of drug residues. To compare the performance of monolithic and conventional columns, a series of experiments were performed under constant instrumental conditions. The total-ion and extracted ion-chromatograms (TIC and EIC, respectively) obtained by use of conventional and monolithic columns are shown in Figs. 2 and 3.

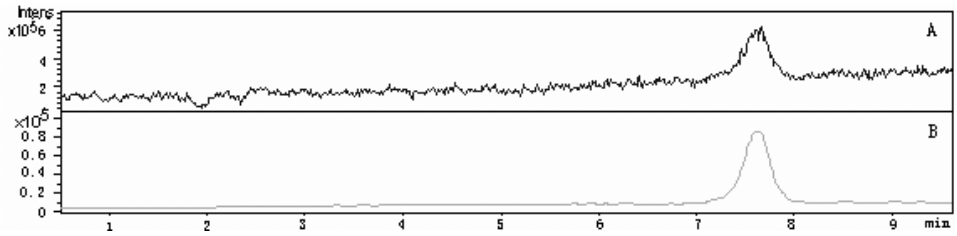

Fig. 2

TIC (A) and EIC (B) obtained from chloramphenicol by use of a conventional column (4.6 mm i.d. × 250 mm, 5 µm particle XDB; Agilent). The injection volume was 10 µL, the concentration of CAP 100 µg L^{-1}, the mobile phase methanol–0.2% aqueous ammonium acetate solution, 45:55 (*v*/*v*), and the flow-rate 1 mL min^{-1}. The ions monitored were *m*/*z* 321, 257, and 194

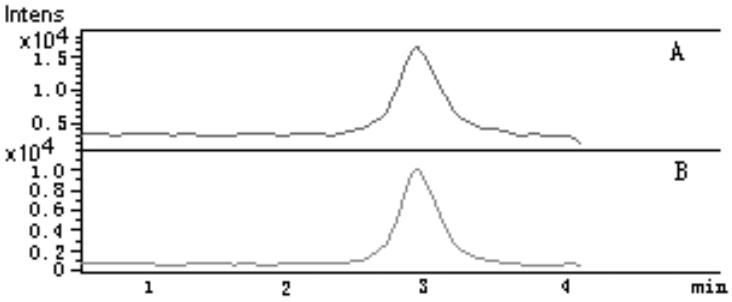

Fig. 3

TIC (A) and EIC (B) obtained from chloramphenicol by use of a 100 mm × 4.6 mm i.d. monolithic column (Merck RP-18e). The injection volume was 10 µL, the concentration of CAP 100 µg L^{-1}, the mobile phase methanol–0.2% aqueous ammonium acetate solution, 45:55 (*v*/*v*), and the flow-rate 1 mL min^{-1}. The ions monitored were *m*/*z* 321, 257, and 194

When the same mobile phase was used under the same experimental conditions chloramphenicol was eluted from the conventional column in 8 min and from the monolithic column in 4 min. Although analysis time was reduced by use of the monolithic column, peak shape and signal-to-noise ratio (*S*/*N*) were significantly improved, resulting in a lower LOQ. These results reveal the applicability of the method for rapid and efficient analysis of CAP residues in honey.

Linearity and Recovery

Linearity was evaluated by analysis of CAP solutions containing 0.2, 2, 20, 50, 100, and 200 μg kg^{-1} and a calibration plot was constructed by plotting peak area against concentration. Regression analysis revealed a linear relationship between response and concentration with a correlation coefficient of 0.9995. The regression equation was $y = 58.753x + 34867$.

Blank honey samples were fortified at 0.2, 20, and 200 μg kg^{-1} with CAP standard and subjected to the complete analytical procedure to determine the recovery and accuracy of the method (Table II). Precision was evaluated by assaying the same group of spiked samples on three different days. The relative standard deviations (RSD) ranged from 3.2–3.9% and recovery ranged from 78–92%. The precision (%, RSD) between days ranged from 4.8 to 8.9% for CAP concentrations of 0.2 to 200 μg kg^{-1} ($n = 5$).

Table II

Recovery of chloramphenicol from honey

Fortification (μg kg^{-1})	Recovery (%)			Mean recovery (%)	RSD (%)
	I	II	III		
0.2	78	85	86	83	3.7
20	92	88	85	88	3.9
200	89	85	93	89	3.2

The limit of reporting level (LCL) for the method, established as the lowest fortified level for which recovery and precision were reasonnable, was 0.2 μg kg^{-1}. The method decision limit (CCα) and the detection capability (CCβ) [19] were evaluated in accordance with the guidelines in 2002/657/EC. The values of CCα and CCβ obtained from the within-laboratory standard deviation were 0.002 and 0.006 μg kg^{-1}, respectively. These are both well below the MRPL of 0.3 μg kg^{-1}.

CONCLUSION

A new LC–MS method is reported for determination of CAP in honey at an LCL of 0.20 μg kg^{-1}. It relies on dispersive solid-phase extraction (modified QuEChERS) for clean-up of the honey sample, and HPLC on a monolithic column, with ESI–MS^2 detection, for analysis of the cleaned extract. The accuracy (recovery) and precision (RSD) are well within the range generally regarded as acceptable.

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