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Rapid Method for Determination of Chloramphenicol Residues in Honey Using Gas Chromatography-Mass Spectrometry

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Chloramphenicol (CAP, 1-p-nitrophenyl-2-dichloroacetamido-1, 3-propanediol) is an effective broad-spectrum antibiotic against a wide range of gram-positive and gram-negative bacteria and rickettsia. Its antibiotic activity results from its interference with protein synthesis in invading microbes. CAP has been applied as veterinary drug to prevent and treat diseases in food producing animals such as bovine and poultry, and in aquiculture. This antibiotic has been also used in apiculture to protect honeybees from the American and European foulbroods, which are very dangerous diseases that attack honeybee broods and completely destroy the colony. There is evidence that CAP has a toxic effect on humans, causing serious health problems such as aplastic anaemia, and is considered as probably carcinogenic (Roybal 1998). Therefore, the European Union (EU) has banned the use of this drug in food-producing animals since 1994, although it is still used in some countries, particularly in Asia. Recently, the presence of traces of CAP in several foodstuffs from this area including honey, has been reported.

The classical extraction procedures developed for the determination of CAP usually involved the use of liquid-liquid extraction (Kessabi et al.1990; Akhtar et al. 1995; Verzegnassi et al. 2003). Solid-phase extraction (SPE) and matrix solid-phase dispersion (MSPD), simple and fast techniques, have been employed for sample preparation in CAP analysis more recently (Kubala-Drincic et al. 2003; Gikas et al. 2004). These extraction methods have been mainly applied to the determination of CAP in food-producing animals, particularly in tissues and fluids of cattle and poultry, as well as in fish and seafood. Numerous chromatographic methods have been reported for the determination of CAP in the aforementioned matrices. Gas chromatography with electron-capture detection (GC-ECD), after the derivatization of CAP extracts (Munns et al. 1994; Pfenning et al. 2000), and high-performance liquid chromatography (HPLC) with UV detection (Long et al. 1990) were initially employed. For confirmatory analysis, mass spectrometry (MS) coupled to GC (Epstein et al. 1994; Borner et al. 1995; Nagata & Oka 1996) or HPLC (Hormazabal & Yndestad 2001) has been reported. In the past few years, some analytical methods for the determination of CAP in honey have been developed, due to the application of this compound to bee colonies. However, only a few papers can be found in the available scientific literature, mainly based on liquid chromatography coupled to mass spectrometry (LC-MS) (Verzegnassi et al. 2003; Ortelli et al. 2004) whereas

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the use of GC with MS for the analysis of CAP residues in honey samples has been scarcely reported (Shen & Jiang 2005).

The aim of this work was to develop an analytical method based on solid-phase extraction and gas chromatography-mass spectrometry for the simultaneous quantification and confirmation of CAP at trace levels in honey samples. The developed method was applied to analyze some Spanish commercial honeys from different botanical origin.

MATERIALS AND METHODS

Chloramphenicol (CAP, 99 % purity) was obtained from Reidel-de Haën (Seelze, Germany). Meta-chloramphenicol (m-CAP), used as internal standard, was provided by USDA-FSIS Midwestern Lab (St. Louis, MO). Hexane, methanol, ethyl acetate and acetonitrile of residue analysis grade (Scharlab, Barcelona, Spain) were employed. A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to provide ultrapure water. The silylating reagent used, Sylon HTP (Supelco, Bellefonte, PA, USA), is a mixture of hexamethyldisilazane, chlorotrimethylsilane, and pyridine (3:1:9, v/v).

A stock solution (500 μg/mL) of CAP standard was prepared by dissolving 0.050 g in 100 mL of acetonitrile and stored at 4 °C. An intermediate standard solution was prepared by transferring 1 mL of the stock solution to a 100 mL volumetric flask and diluting to volume with acetonitrile to obtain a concentration of 5 μg/mL. Working solutions were prepared in water to fortify honey samples. The internal standard (m-CAP) was prepared in acetonitrile to make a 500 μg/mL solution.

Honey samples were analyzed to confirm the absence of CAP prior to spiking. Various Spanish commercial honeys were purchased, five unifloral (orange, lavender, thyme, eucalyptus, and rosemary) and two multifloral. Several citrus honeys were collected directly from the producers in Valencia. The honey samples were stored a 4 °C until analysis.

A 5 g amount of honey, heated at 50 °C in a water bath, was dissolved in 15 mL of water in a Sovirell tube and homogenized on a Vortex mixer (Selecta, Madrid, Spain) for 15 s until total dissolution. For the recovery assays, honey samples were fortified with a CAP standard in water. The aqueous solution was loaded into an 500 mg OASIS HLB (hydrophilic lipophilic balance) SPE cartridge (Waters, Milford, MA), placed on a multiport vacuum manifold (Supelco, Visiprep, Madrid, Spain), previously conditioned with 8 mL methanol and a 8 mL distilled water. The Sovirell tube was rinsed with 10 mL water-acetonitrile (9:1, v/v) that were transferred to the cartridge and then the Oasis cartridge was dried applying a gentle vacuum for 25 min. CAP was subsequently eluted with 10 mL acetonitrile and collected in 10 mL graduated tubes. The extract was transferred to a conical tube and 0.5 mL of the internal standard (m-CAP) at 0.4 μg/mL was added. Samples were evaporated in a Turbo Vap LV evaporator (Zymark Corporation, Hopkinton, MA) near dryness at 45 °C with a stream of nitrogen. The tubes were rinsed with ca. 1 mL of acetonitrile, which was evaporated to dryness to obtain residues for derivatization.

CAP and m-CAP were silylated by adding 100 µL of Sylon HTP, shaking on a Vortex mixer and allowing the reaction to take place at room temperature for 1.5 min. Excess of reagent was evaporated under a gentle stream of nitrogen. The residue was reconstituted in 1 mL of n-hexane and stirred on the Vortex mixer. A subsequent clean-up step was performed, particularly necessary for low residue levels. The extract was loaded in a glass column containing 1 g of Florisil (Fluka, Buchs, Switzerland). The column was washed with 3 mL of hexane and the analytes elution was carried out with 4 mL of ethyl acetate. The eluate was then concentrated to 1 mL and stored at 4 °C until analyzed by GC-MS.

GC-MS analysis was performed with an Agilent 6890 (Waldbronn, Germany) gas chromatograph equipped with an automatic split-splitless injector Model HP 7683, and a mass spectrometric detector (MSD) Model HP 5973N, which was equipped with an inert ion source. A fused silica capillary column (ZB-5MS), 5 % phenyl polysiloxane as nonpolar stationary phase (30 m x 0.25 mm I.D.) and 0.25 µm film thickness, supplied by Phenomenex (Torrance, CA), was employed. Operating conditions were as follows: injector port temperature 280 °C; helium as carrier gas at a flow-rate of 1.0 mL/min; pulsed splitless mode (pulsed pressure 310 kPa for 1.5 min). The column temperature was maintained at 150 °C, then programmed at 7 °C/min to 240 °C for 10 min; followed by a final ramp to 280 °C at a rate of 40 °C/min and held for 1 min. The total analysis time was 24.86 min and the equilibration time 2 min. A 2 µL volume was injected splitless, with the split valve closed for 1 min. Mass spectrometric detector (MSD) was operated in electron impact ionization mode with an ionizing energy of 70 eV, scanning from m/z 100 to 350 at 3.62 s per scan. The ion source temperature was 300 °C and the quadrupole temperature 150 °C. The electron multiplier voltage (EM voltage) was maintained 400 V above autotune and a solvent delay of 5 min was employed.

Table 1. Retention times (t_R), target (T) and qualifier ions (Q_1, Q_2, Q_3), and abundance ratios of qualifier ion/target ion (Q_1/T, Q_2/T, Q_3/T)[a]

	CAP-derivative	m-CAP-derivative
t_R (min)	16.65	15.87
T (m/z)	225	225
Q_1 (m/z)	194	194
Q_2 (m/z)	208	208
Q_3 (m/z)	227	227
Q_1/T (%)	61.8	31.5
Q_2/T (%)	178.2	106.4
Q_3/T (%)	43.6	37.2

[a] Q/T (%) are the results of abundance values of the qualifier ion (Q_1, Q_2, Q_3) divided by the abundance of the target ion (T) × 100

Analysis was performed with selected ion monitoring (SIM) using one target and three qualifier ions. The target and qualifier abundances were determined by the injection of a silylated CAP standard under the same chromatographic conditions using full-scan with the mass/charge ratio ranging from 90 to 400 m/z. Quantification was based on the peak area ratio of the target ion divided by the peak area of the internal standard in the calibration standard versus those found in samples. Table 1 lists the retention time, the target and qualifier ions and the qualifier to target abundance ratios of CAP and the internal standard m-CAP. The SIM program used to determine and confirm CAP in honey has one acquisition window at *m/z* 194, 208, 225, and 227 with an ion dwell time of 100 ms and a scan rate of 2.86 cycles/s. CAP was confirmed by its retention time, the identification of target and qualifier ions and the determination of qualifier to target ratios. Retention time has to be within ± 0.3 min of the expected time and qualifier-to-target ratios have to be within a 20 % range for positive confirmation.

RESULTS AND DISCUSSION

CAP was determined by gas chromatography-mass spectrometry with selected ion monitoring (GC-MS-SIM) after obtaining its trimethylsilyl derivative with Sylon HTP. The molecular ion of the disilylated trimethylsilyl chloramphenicol was not observed and, therefore, the analysis was carried out with the main fragment ions *m/z* 194, 208, 225, and 227. The internal standard, m-CAP, presented the same fragment ions but different relative abundances than CAP.

The linearity of the method was assayed by analyzing standard solutions, previously derivatized, in the range of 1- 200 μg/L containing 200 μg/L of the internal standard, m-CAP. As a certain response increase was observed when fortified samples were injected (matrix effect), blank extracts, previously analyzed to confirm the absence of residues, were spiked with the appropriate amounts of CAP and m-CAP and used as standards. The MS response for CAP was linear in the concentration range studied with a determination coefficient of 0.999. The results of the linear regression analysis were: slope: 4.0 and y-intercept: $-4 \cdot 10^{-2}$.

The repeatability of the chromatographic method was determined by performing the analysis of a honey sample spiked at 50 μg/L. The sample was injected 10 times with an automatic injector and the relative standard deviation (RSD) values obtained for the retention time and peak area were 0.005 % and 0.2 %, respectively. The repeatability of the whole analytical method was also determined by replicate analysis of a fortified sample during different days. The repeatability of the method, expressed as relative standard deviation (RSD), was lower than 10 % for CAP.

CAP was extracted from honey samples by SPE using acetonitrile as elution solvent. Previously, the cartridge was rinsed with 10 mL of a water-acetonitrile (9:1, v/v) solution to remove interfering materials. In a first approach, water was used to wash the cartridge, however some compounds, such as pigments and other honey components, were retained in the sorbent and interfered in the CAP determination at low levels. Therefore, a small proportion of acetonitrile (10%) was added in the washing solvent and cleaner eluents were obtained without reduced recovery of the

target analyte. Nevertheless, after the derivatization of the extracts, a further clean-up step using a Florisil column was necessary to allow the determination of CAP at very low levels.

Table 2. Chloramphenicol recoveries[a] from honey samples

Fortification level (μg/kg)	Orange	Rosemary	Multifloral
100	88.2 ± 3.7	97.9 ± 6.2	91.7 ± 5.8
50	86.4 ± 4.0	89.1 ± 6.3	98.0 ± 2.2
10	86.2 ± 3.2	85.7 ± 2.8	93.7 ± 7.8
5	89.5 ± 3.8	83.6 ± 2.6	103.7 ± 2.7
1	96.5 ± 4.1	103.5 ± 8.6	91.8 ± 7.7
0.2	93.5 ± 4.9	94.8 ± 4.1	93.6 ± 7.3

[a] Recovery, % ± RSD, % (n = 5)

Table 2 shows the CAP recovery results obtained. The honey was fortified at 100, 50, 10, 5, 1 and 0.2 μg/kg before extraction by adding 0.5 mL of the appropriate working standard solution. Five sample replicates spiked at each fortification level were carried out. Prior to the derivatization step, m-CAP was added as internal standard. The use of the internal standard decreased the variability of results normally found when a derivatization step is necessary. The recovery obtained ranged from 83 to 104 %. The precision of the method, expressed as the relative standard deviation (RSD) of analyte recoveries, was good, lower than 9 %. The obtained values are similar to the recoveries reported by other authors for the analysis of CAP in honey (Gikas et al. 2004; Ortelli et al. 2004).

The limit of detection (LOD) of the proposed method, determined by considering a value 3 times the background noise at the retention time of CAP obtained for blank samples, was 0.05 μg/kg. This low LOD could be obtained due to the low background noise given by the new inert ion source of the MSD and the good selectivity achieved with the sample preparation technique. The limit of quantitation (LOQ), considered as the lowest concentration of analyte quantitatively determined, was 0.2 μg/kg. This value is lower than the minimum required performance limits for CAP in honey and other foodstuffs from animal origin (0.3 μg/kg) established by the European Community (European Community 2003). The LOD achieved is similar but somewhat lower than those recently published (Ortelli et al. 2004; Shen & Jiang 2005), which are around 0.1 μg/kg. Figure 1 shows the chromatograms of a blank rosemary honey sample and a rosemary honey sample fortified at the LOQ (0.2 μg/kg) together with a chromatogram of a standard showing a fragmentation pattern equal to that obtained in the honey sample.

The present method was applied to the analysis of twelve Spanish honey samples, some unifloral (orange, eucalyptus, lavender, rosemary, and thyme) and two multifloral and no detectable CAP residues were found in these samples.

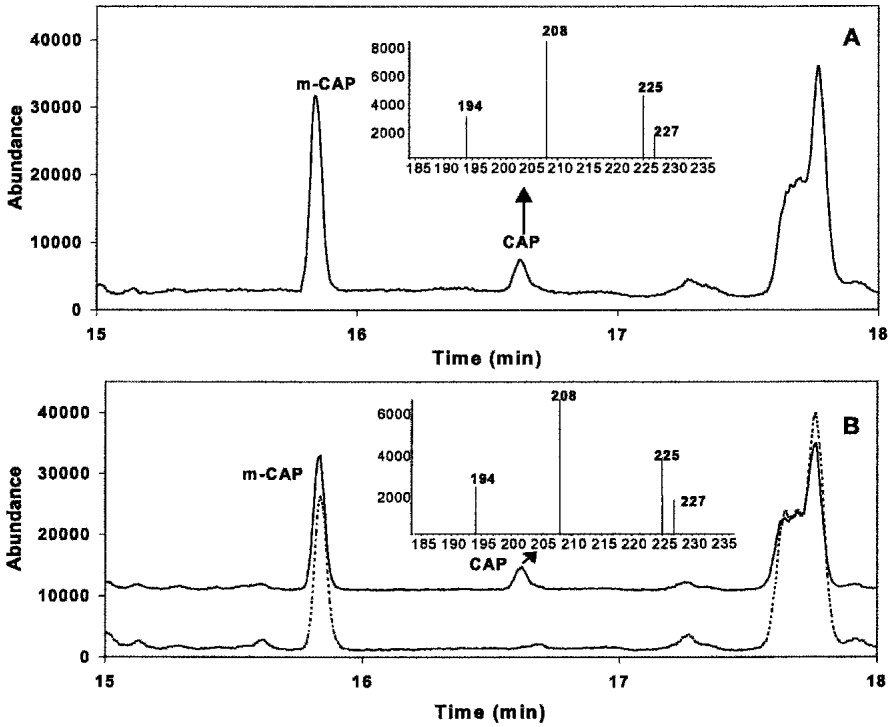

Figure 1. GC-MS-SIM chromatograms. A, a standard at 0.2 μg/kg with the mass spectrum of CAP showing the selected ions; B, a blank rosemary honey sample (dotted line) and a rosemary honey sample fortified at 0.2 μg/kg together with the mass spectrum of CAP showing the selected ions.

The developed analytical method, based on solid-phase extraction, allows the simultaneous quantification and confirmation of CAP residues in honey. This method is a simple and rapid procedure able to determine CAP with good reproducibility and very low detection and quantification limits.

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