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Determination of Chloramphenicol in Honey by Liquid Chromatography-Mass Spectrometry with Photodiode Array Detection

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Abstract. The aim of the present work was the development, validation and implementation in routine analysis of a technique that guarantees low detection limit for chloramphenicol in honey using liquid chromatography (LC)-mass spectrometry (MS) with photodiode array (PDA) detection. Sample preparation was carried out by liquid-liquid extraction of a honey solution in phosphoric buffer (PBS, pH=7.8). The determinations were performed using a Zorbax Eclipse XDB-C18 column and gradient mobile phase, using an electrospray ionization source (ESI) in negative ion mode. Recoveries were calculated at three concentration levels and higher values than 85% were obtained, with relative standard deviations less than 7.7%. The applicability of the present method was tested on 12 honey samples purchased from different beekeepers from Transylvania.

Keywords: Liquid chromatography-mass spectrometry, chloramphenicol, honey

INTRODUCTION

Drug residues may cause allergic or toxic reaction to consumers and promote occurrence of antibiotic-resistant bacteria (Kishida, 2007). Chloramphenicol (CAP) is a broad-spectrum antibiotic, active against aerobic and anaerobic microorganisms. The administration of CAP to humans, in relatively high doses has caused serious toxic effects such as agranulocytosis and aplastic anaemia (Chen *et al.*, 2008).

In apiculture, antibiotics are used for the prevention and treatment of American or European foulbrood, bacterial diseases which cause damage to beekeepers by massive colonies losses. Generally, antibiotics persist as contaminants especially in honey and royal jelly and endanger the consumers' health. The EU has established maximum residue limits (MRLs) for antibiotic residues in animal products by Regulation 2377/90 (EEC, 1990). List from Annex IV contains the pharmacologically active substances used in veterinary medicinal products for which a maximum residue limit cannot be established because residues of the substances concerned, at any limit, in foodstuff of animal origin constitute a hazard to the health of the consumer. CAP is included in this annex, its administration to food-producing animals being prohibited throughout the Community (EEC, 1990). Although it is known its toxicity, CAP is still used by beekeepers in some countries. In China, a very important honey exporter, CAP and streptomycin are preferred antibiotics in beekeeping and residues can therefore be found in honey (Ortelli *et al.*, 2004). In the case of CAP, EU has set a minimum required performance limit (MRPL) of $0.3\mu g \cdot kg^{-1}$. This fact does not mean that concentrations below this limit are permitted, but is an indication the method must be able to reach at least this level (Michaud, 2005).

In literature, different chromatographic techniques are described for determination of CAP at residual levels from honey (Chen *et al.*, 2008; Forti *et al.*, 2005; Hammel *et al.*, 2008; Lopez *et al.*, 2008; Ortelli *et al.*, 2004; Rønning *et al.*, 2006; Shen and Jiang, 2005; Sheridan *et al.*, 2008), royal jelly (Calvarese *et al.*, 2006; Jiang *et al.*, 2006) and propolis (Bononi and Tateo, 2008). According to Commission Decision 2002/657/EC, the confirmation of suspect positive samples must be performed by mass spectrometry together with the adequate chromatographic separation. Thus, chromatography coupled with MS techniques, such as GC/MS and LC/MS, have become the most reliable analytical techniques for the unambiguous confirmation of „zero-tolerance residue limit" substances in products of animal origin (Jiang *et al.*, 2006). LC/tandem MS is recognized as being the most performant method due to selectivity and sensitivity offered by the MS/MS detection.

The present work describes the improvements of analytical method for the sensitive determination of CAP in honey samples using LC-MS in the reverse-phase mode with aditional PDA detection, for obtaining low detection limit (LOD), below the value of MRPL required by the European legislation.

MATERIALS AND METHODS

Materials and reagents. Chloramphenicol standard was purchased from Sigma Aldrich (Steinheim, Germany). Stock solution at a concentration of $400 mg \cdot l^{-1}$ was prepared by dissolving the compound in methanol. Working standard solutions used for calibration and spiking were prepared by succesive dilutions of the stock solution with acetonitrile/water (25:75 v/v). HPLC grade methanol, ethyl acetate and analytical grade sodium sulfate anhydrous were obtained from Merck (Darmstadt, Germany). Formic acid 98-100% was purchased from Riedel-de Haen (Seelze, Germany) and acetonitrile HPLC grade was from Sigma Aldrich. Ultrapure water was generated using Ultra Clear Direct UV water purification system (SG Wasseraufbereitung, Germany). Phosphoric buffer solution (PBS, pH=7.8) was prepared from the mixture of $0.1 mol \cdot l^{-1}$ KH_2PO_4 and $0.1 mol \cdot l^{-1}$ Na_2HPO_4 solutions, Fluka (Steinheim, Germany).

Honey samples of different botanical origin (multiflower, acacia, sunflower, honeydew honey) were purchased directly from beekeepers. For validation of method, a free of CAP acacia sample was used and different levels of spiking were performed for recovery, LOD and LOQ (quantification limit) determinations.

Apparatus. HPLC analysis were performed on a Shimadzu 2010 EV series system (Kyoto, Japan), which included a DGV-20A5 degasser, a LC-20AD quaternary pump, SIL-20AC automatic injector with cooling capability of 70 vial tubes and a LCMS1 nitrogen generator (Claind, Italy). The LC system was equipped with two detectors: SPD-M 20A diode array and LCMS 2010 EV mass spectrometry. The instrument was operating in a negative ion mode. Other instrumental operational parameters are presented in Table 1. A Zorbax Eclipse XDB-C18 (2.1x150mm, 3.5μm) separation column was obtained from Agilent Technologies (CA, USA).

Sample preparation. Procedure is bases on the method described by Shen and Jiang (2005) with minor modifications. A sample of 10 g honey was weighed into a 50 ml polypropylene centrifuge tube and dissolved in 10ml PBS (pH=7.8). Then, 3g Na_2SO_4 and 10ml ethyl acetate were added. The mixture was mechanically shaken (Rotator-Mixer Multi RS-60, Biosan) for 15 min and centrifuged afterwards for 5 min at 5500rpm (Sigma 3K18 Laborzentrifugen, Germany). The organic phase was collected into a 50 ml round-bottomed flask and the extraction process was repeated with another 10 ml ethyl acetate. The organic

phases were mixed and evaporated to dryness under reduced pressure at 40°C using Heidolph VV rotary evaporator (Schwabach, Germany). The residue was dissolved in 1ml mixture of acetonitrile/water (25:75 v/v), vortexed for 50 seconds (Wizard Vortex, Velp Scientifica, Italy), filtered through a 0.2µm pore-size filter directly in the autosampler vial, and then injected into the HPLC system.

Tab. 1

LC-MS operating parameters

Parameter	Setting	
Mobile phase	0.1% formic acid in water (A) 0.1% formic acid in acetonitrile/water 90:10 v/v (B)	
Gradient	0-0.70 min	0%B
	0.70-0.75 min	0 -15%B
	0.75-5.0 min	15% B
	5.0-12.0 min	15 – 40% B
	120. – 14.0 min	40 – 60% B
	14.0-14.01 min	60 - 0% B
	Post time	5 min at 0% B
Flow-rate	0.3ml/min	
Column temperature	25°C	
Autosampler temperature	10°C	
Monitoring wavelength	278nm	
Injection volume	50µl	
Interface voltage	4.5KV	
Interface temperature	250°C	
Nebulizing gas flow	1.5l/min (N_2)	
Selected ion monitoring	m/z 321, 323 (CAP)	
Ionization mode polarity	ESI, negative ion	

Validation of the method. Validation of the method was performed according to criteria set by Commission Decision 2002/657/EC (EC, 2002). The specificity of the method was tested by analysing CAP free honey samples. There were no interfering peaks at the retention time (13.12 min) corresponding to the analyt. The chromatograms of a honey sample free of CAP and the spiked sample are presented in Fig. 1.

Seven standard solutions of CAP containing 0.3–25 $\mu g \cdot kg^{-1}$ were injected 7 consecutive times to test the repeatability of injection on the mass detector quadrupole instrument. By plotting the theoretical concentrations as a function of the peak area, a calibration curve was obtained. The same procedure was performed for PDA detection, with 5 to 50 $\mu g \cdot kg^{-1}$ concentrations of CAP. Each point of the calibration curves corresponded to the mean value obtained from 3 independent injections. Both MS and PDA showed a linear response for CAP in the specified concentrations range (r^2=0.999).

The sensitivity of the method was estimated by calculating the limit of detection (LOD) and limit of quantification (LOQ) for CAP standard solutions. These two parameters were calculated separately for both detectors (MS and PDA) by the system software (LCMS Solutions). For mass detector the level of concentration injected for LOD and LOQ determination was 0.3$\mu g \cdot kg^{-1}$ and 5$\mu g \cdot kg^{-1}$ was the concentration level for diode array detector (Tab. 2).

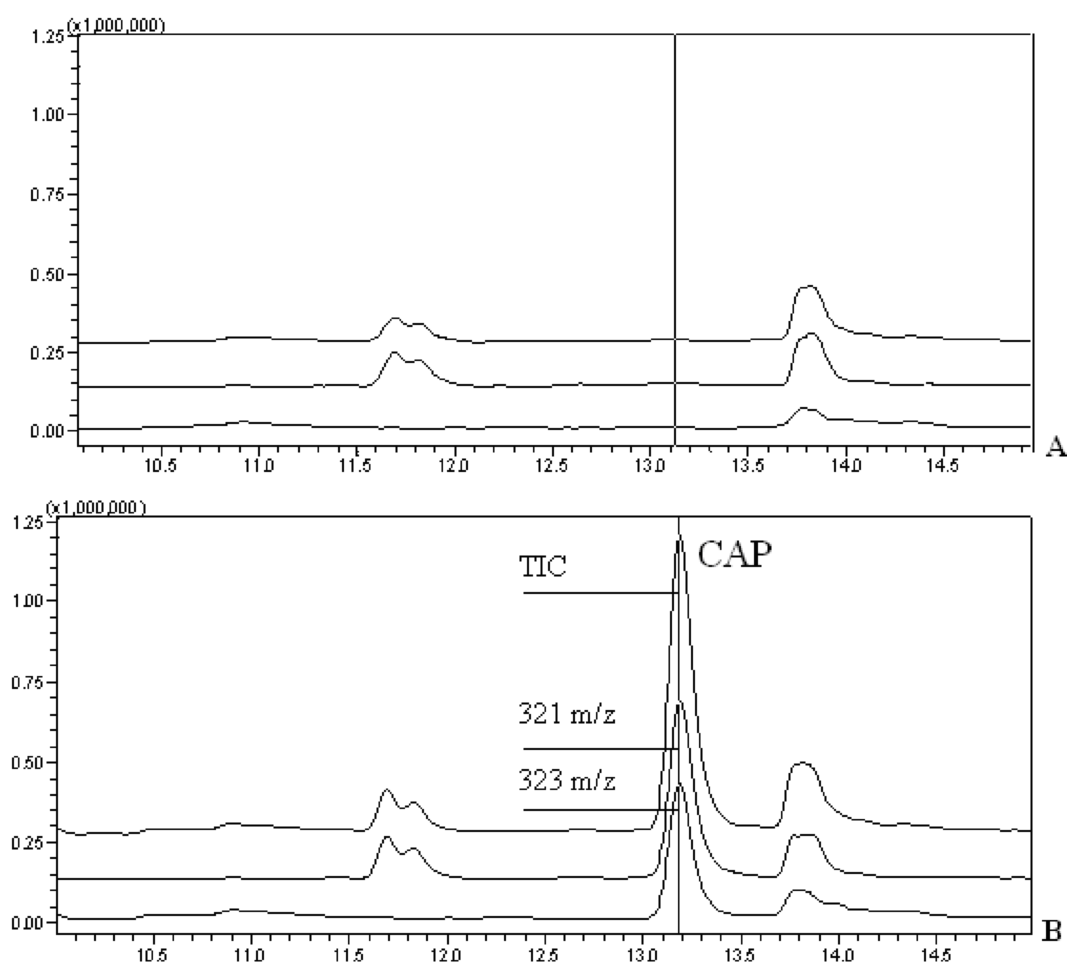

Fig. 1. Chromatograms obtained for blank honey sample (A) and spiked honey sample (B) with 5µg/kg CAP (TIC = Total ion current)

For recovery studies, each 6 independent spiked CAP free samples at 3 different concentrations (0.6; 2 and 5 µg·kg^{-1}) were analysed. The recovery was calculated by comparing the extracted quantity of CAP registered in the chromatogram with the spiking amounts. Precision was calculated by measuring relative standard deviations (RSD %). The results obtained are summarized in Tab. 3.

Tab. 2

Method sensitivity

Detector	Concentration level (µg·kg^{-1}) (n=6)	$\overline{C}$ ±SD (µg·kg^{-1})	RSD (%)	LOD (µg·kg^{-1})	LOQ (µg·kg^{-1})
Mass spectrometry	0.3	0.4±0.027	5.49	0.13	0.27
Diode Array	5	4.29±0.471	9.98	4.75	6.94

Tab. 3

Mean recoveries and precision of the method (n=6) at 3 concentration levels

Fortification level ($\mu g \cdot kg^{-1}$)	Average found ($\mu g \cdot kg^{-1}$)	Recovery (%)	RSD (%)
0.6	0.48	85	3.8
2	1.68	87	5.6
5	4.35	91	7.7

RESULTS AND DISCUSSION

This study presents a suitable method for the extraction, detection and quantification of CAP in honey by LC-MS using ESI in negative ion mode. By comparing the other chromatographic methods based on the use of conventional detectors with the separation techniques coupled to very selective MS detectors systems, Bogialli and Di Corcia (2009) state that the latter methods, beside supplying precious information about the identity of a specific compound, offers the additional advantage that older laborious and time-consuming sample treatment procedures can be greatly simplified. The ESI source is suitable for analysis because of the polar nature of CAP. Literature (Forti *et al.*, 2005; Lopez *et al.*, 2008; Rønning *et al.*, 2006; Shen and Jiang, 2005; Sheridan *et al.*, 2008) shows that this analyt has a much better MS response in negative ionisation mode. Only one study (Hammel *et al.*, 2008) monitored the CAP in positive mode. Under their working conditions, molecule of CAP appeared as protonated species with o loss of water $[M+H-H_2O]^+$.

After scan measurement, in SIM the targeted mass numbers were selectively detected. The ions at m/z 321 and 323 represent the analyte of interest with the chlorine isotopes $^{35}Cl_2$, $^{35}Cl^{37}Cl$ respectively. The most abundant ion is the one with two ^{35}Cl-atoms ($^{35}Cl_2[M-H]^-$). For identification and confirmation of CAP in a sample is necessary to:

- both m/z 321 and 323 ions give signal at the same retention time with the analyte in the standard solution. Tolerance value obtained must be as low as possible (we obtained a tolerance below ±1%).
- 321 and 323 ion intensity ratio must have the same value, both in sample and in standard solution. If their ratio has different values, is an indication that there are interferences in the respective ion. Maximum permitted tolerance for relative ion intensities must be ±10%, if the relative intensity is higher than 50%. In our study, we obtain a relative intensity for standard solutions 64.5% and for sample solutions 65.7%.

Quantification involves the comparison of the intensity of the signal generated by the analyte determined in sample, with that obtained from standards containing known amounts of that analyt. In order to detect traces of CAP in bee products it is necessary to develop sensitive analytical methods with low detection limits. In the case of LC coupled with PDA detector, LOD and LOQ obtained were much higher than when using MS detection. For samples contaminated with CAP at higher levels of concentration than $7 \mu g \cdot kg^{-1}$, PDA detection is an extra confirmatory tool, together with MS detection, supplying UV-VIS absorption spectra for the analyt.

The sample preparation procedure is simple, based of liquid/liquid extraction with ethyl acetate and few steps for minimizing the loss of analyt before analysis. The use of solid-phase extraction (SPE) cartridges for sample preparation was investigates, but some interferences close to the retention time of CAP were registered, towards no interferences

when using liquid/liquid extraction. Because of the limited steps in sample preparation, recoveries were satisfactory and reproducible.

The gradient profile was established after several mixture proportions and time intervals runs. Our goal was to obtain a symmetrical peak shape for CAP. The number of theoretical plates (N=49816) indicate the high efficiency of the used separation column.

In order to verify the practical applicability of the improved method, a screening of 12 samples of honey with different botanical origin was performed. The sample preparation followed the same steps like in method validation, under the same working conditions. Only one of the samples was found positive for CAP, the detected concentration being 1.4 $\mu g \cdot kg^{-1}$.

CONCLUSIONS

Because of the well known CAP toxicological effects on humans, a great importance must be followed to protect the safety of the consumer.

In order to monitor the zero tolerance level of CAP, sensitive and accurate analytical methods are needed. Method presented in this study is able to identify and quantify the residues of CAP in honey at a concentration below the European MRPL. The good validation results obtained show that the proposed method can be applied in routine analysis.

In conclusion, the LC-MS procedure described is suitable for routine monitoring of CAP in honey, without the use of more costly LC-MS/MS systems.

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REFERENCES

1. Bogialli, S. and A. Di Corcia (2009). Recent applications of liquid chromatography-mass spectrometry to residue analysis of antimicrobials in food of animal origin. Anal. Bioanal. Chem. 395:947-966.
2. Bononi, M. and F. Tateo (2008). Liquid chromatography/tandem mass spectrometry analysis of chloramphenicol in propolis extracts available on the Italian market. J. Food Compos. Anal. 21:84-89.
3. Calvarese, S., A.F. Forti, G. Scortichini and G. Diletti (2006). Chloramphenicol in royal jelly: analytical aspects and occurrence in Italian imports. Apidologie 37:1-6.
4. Chen, H., J. Ying, H. Chen, J. Huang and L. Liao (2008). LC Determination of Chloramphenicol in Honey Using Dispersive Liquid-Liquid Microextraction. Chromatographia. 68:629-634.
5. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. L221:8-36.
6. Council Regulation (EEC) (1990) No. 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. L224:1-135.
7. Forti, A.F., G. Campana, A. Simonella, M. Multari and G. Scortichini (2005). Determination of chloramphenicol in honey by liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta 529:257-263.
8. Hammel, Y.-A., R. Mohamed, E. Gremaud, M-H. LeBreton and P.A. Guy (2008). Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1177:58-76.

9. Jiang, Y., J-Z. Xu, C-Y. Shen, T. Ding, H-L. Chen, B. Wu and W-J. Shen (2006). Determination of Chloramphenicol in Royal Jelly by liquid Chromatography/Tandem Mass Spectrometry. J. AOAC Int. 89(5):1432-1436.

10. Kishida, K. (2007). Quantitation and confirmation of six sulphonamides in meat by liquid chromatography-mass spectrometry with photodiode array detection. Food Control 18:301-305.

11. Lopez, M.I., J.S. Pettis, I.B. Smith and P-S. Chu (2008). Multiclass Determination and Confirmation of Antibiotic Residues in Honey Using LC-MS/MS. J. Agric. Food. Chem. 56:1553-1559.

12. Michaud, V. (2005). Antibiotic Residues in Honey- The FEEDM View. Apimondia Journal Apiacta 40:52-54.

13. Ortelli, D., P. Edder and C. Corvi (2004). Analysis of Chloramphenicol Residues in Honey by Liquid Chromatography-Tandem Mass Spectrometry. Chromatographia 59:61-64.

14. Rønning, H.T., K. Einarsen and T.N. Asp (2006). Determination of chloramphenicol residues in meat, seafood, egg, honey, milk, plasma and urine with liquid chromatography- tandem mass spectrometry, and the validation of the method based on 2002/657/EC. J. Chromatogr. A 1118: 226-233.

15. Shen, H-Y. and H-L. Jiang (2005). Screening, determination and confirmation of chloramphenicol in seafood, meat and honey using ELISA, HPLC-UVD, GC-ECD, GC-MS-EI-SIM and GCMS-NCI-SIM methods. Anal. Chim. Acta. 535:33-41.

16. Sheridan, R., B. Policastro, S. Thomas and D. Rice (2008). Analysis and Occurrence of 14 sulfonamide Antibacterials and Chloramphenicol in Honey by Solid-Phase Extraction Followed by LC/MS/MS Analysis. J. Agric. Food. Chem. 56:3509-3516.