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FOOD SAFETY IS AN IMPORTANT PUBLIC HEALTH ISSUE: CHLORAMPHENICOL RESIDUES DETERMINATION BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (LC-MS/MS) IN HONEY

Adela Krivohlavek[1], Irena Žuntar[2], Martina Ivešić[1], Ivana Mandić Andačić[1] & Sandra Šikić[1]

[1]Andrija Stampar Teaching Institute of Public Health, Zagreb, Croatia

[2]University of Zagreb, Faculty of Pharmacy and Biochemistry, Zagreb, Croatia

SUMMARY

Background: *Honey is used for nutritional, medicinal and industrial purposes and antibiotic residues may harm its quality and constitute a danger to human health. The broad spectrum antibiotic chloramphenicol (CAP) was used for curative purposes in veterinary medicine, but is now forbidden in European Union (EU) because of its many serious side effects (e.g. aplastic anaemia, grey syndrome, severe bone marrow depression and hypersensitivity).*

The aim of this study was to facilitate analyses of the quality and safety of Croatian honey distributed to whole European Union market; an assessment that has not previously been made.

Subjects and methods: *CAP in honey was qualifying and quantifying by validated liquid chromatography tandem mass spectrometry with negative electrospray ionisation method (LC-MS/MS). The target antibiotic was separated on chromatographic column Zorbax SB C18 (150 mm × 2.1 mm, 3.5 μm) with a gradient elution using acetonitrile - 0.1% formic acid mobile phase at a flow rate of 0.3 mL/min, with column temperature 35 oC for CAP and 5D-CAP as internal standard. Homogenised honey samples were diluted with acetate buffer solution and extracted on Oasis Hydrophilic-Lipophilic-Balanced (HLB) sorbents. The method was used to analyse 280 domestic honey samples collected throughout Croatia between 2005.–2013.*

Results: *Recoveries of the method for real (acacia, chestnut, linden and flower) honey samples were 102% with RSD 8.4%. The value CCα and CCβ were 0.09 and 0.12 μg/kg, respectively. Results showed only three subsequent positive detections (1.1%) of CAP in honey.*

Conlusions: *Analysed honey samples from Croatia showed good quality and safety what is the one of the main objective in consumer health policy in EU.*

Key words: *antibiotics – chloramphenicol - honey - liquid chromatography tandem mass spectrometry (LC-MS/MS)*

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INTRODUCTION

Honey, defined by European Commission, is the natural sweet substance produced by Apis mellifera bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (Council Directive 2001/110/EC). In the long human tradition it has been used not only as nutrition but also as a medicine. The belief that honey is nutrition, a drug and an ointment has continued to the present time. Currently, information on the use of honey for the treatment of many human diseases can be found in general magazines, beekeeping journals and natural products leaflets, suggesting a wide variety of unfounded properties. An alternative medicine branch, called apitherapy, has developed in recent years, offering treatments based on honey and other bee products for many diseases (Bogdanov et al. 2006). The same as any other natural food, honey can be contaminated by the environment, e.g. by heavy metals, pesticides, antibiotics etc. The use of antibiotics, as well as chloramphenicol (CAP), in apiculture has been known for decades and consequently, their residues can be found in honey (Bogdanov 2006, Reybroeck 2012).

Chloramphenicol is an antibiotic effective against a wide range of gram-negative and gram-positive bacteria in both humans and animals. Due to the resistance, toxicity and safety concerns, it is no longer a first-line agent for any infections in developed nations, with notable exception of topical treatment of bacterial conjunctivitis. The European Union banned CAP use in food-producing animals because of its many serious side effects (e.g. aplastic anaemia, grey syndrome, severe bone marrow depression and hypersensitivity) (Commission Regulation (EC) 1430/94). In Croatia, it was banned in 2003., but the minimum required performance level (MRPL) was set in 2005. at 0.3 μg/kg and now it is completely banned (Official Gazette of the Republic of Croatia 21/2011). Various analytical methods have been reported for determining CAP in honey (Ashwin et al. 2005, Ferguson et al. 2005, Forti et al. 2005, Huang et al. 2006, Pan et al. 2006, Ronning et al. 2006, Rodziewich & Zawadzka 2007, Scortichini et al. 2005, Shen & Jiang 2005, Turnipseed et al. 2002, Verzegnassi et al. 2003) or propolis (Bononi & Tateo 2008) and other biological materials like milk (Agui et al. 2002, Ashwin et al. 2005, Ferguson et al. 2005, Guy et

al. 2004, Huang et al. 2006, Nicholich et al. 2006, Pengov et al. 2005, Perez et al. 2002, Ronning et al. 2006), meat (Ashwin et al. 2005, Fergison et al. 2005, Scortichini et al. 2005, Shen & Jiang 2005, Rocha Siqueira et al. 2009, Ronning et al. 2006), eggs (Huang et al. 2006, Ronning et al. 2006) and sea food (Ferguson et al. 2005, Rocha Siqueira et al. 2009, Shen & Jiang 2005).

Enzyme-linked immunosorbent assay methods (Ferguson et al. 2005, Scortichini et al. 2005, Shen & Jiang 2005, Rocha Siqueira et al. 2009) are very useful for preliminary analyses and screening purposes (mostly because of their easiness) but they can give false compliant results. Any subsequent results require confirmation by other suitable methods. A very sensitive method for determining chloramphenicol is gas chromatography, coupled with electron capture detector (GC-ECD) (Pengov et al. 2005, Shen & Jiang 2005), but this method requires a derivatization step and it is not a confirmative approach. There are some methods like voltametric (Agui et al. 2002), quick and easy capillary electrophoresis, liquid chromatography (Shen & Jiang 2005), with classical detectors (ultraviolet, UV), multi-diode detector (DAD) or fluorescent (FLD) detector which can achieve MRPL and also do not require derivatization for determination of CAP, but these are non-confirmative. For confirmative methods there are few choices. Specifically, GC-MS methods can provide definitive qualitative and quantitative results, but these require a derivatization step (Shen & Jiang 2005). The combination of LC-MS (Ashwin et al. 2005, Bogusz et al. 2004, Forti et al. 2005, Rocha Siqueira et al. 2009, Shen & Jiang 2005, Yibar et al. 2011) offers a rapid, simplified, specific and sensitive alternative to GC-MS methods and removes the need for derivatization reactions.

The present work describes a rapid method for determination and confirmation of CAP in honey, based on liquid chromatography with tandem mass spectrometry (LC-MS/MS) in electrospray negative ion mode. The method was validated according to Commission Decision 2002/657/EC (Commission Decision 2002/657/EC) and performed for the analysis of CAP in samples of Croatian domestic honey in the period from 2005. to 2013. in order to verify our hypothesis of its good quality.

SUBJECTS AND METHODS

Reagents

Analytical standard chloramphenicol (chemical purity 98.5%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany) and chloramphenicol D5 (100 μg/mL in acetonitrile; chemical purity ≥98%) used as an internal standard (IS) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). For chromatographic analyses and solid phase extraction (SPE), purification and concentration, organic solvents of high-performance liquid chromatography (HPLC) gradient grade methanol and acetonitrile were purchased from Baker (Deventer, Netherlands). An acetate buffer solution 0.01 mol/L was prepared by dissolving p.a. potassium acetate from Kemika (Zagreb, Croatia) and the pH adjusted to 6 via a pH-meter MPC 227, Mettler Toledo GmbH (Giessen, Germany). Samples were diluted with buffer solution and sonicated in ultrasound bath (Branson 1210, Branson Ultrasonics) (Danbury, USA). The 24-port vacuum manifold (Supelco) was used for solid-phase extractions. The honey samples were extracted using SPE cartridges Oasis HLB 6 mL/200 mg, 60 μm. Prior to analysis, all samples were passed through a 0.20 μm disposable filter (Millex-FG, Fluoropore PTFE, Millipore Corp., Sigma - Aldrich Chemie GmbH, Taufkirchen, Germany).

A CAP standard stock solution of 2.0 mg/mL was prepared by dissolving 20 mg CAP in 10 mL of acetonitrile and this solution was diluted in acetonitrile obtaining an intermediate standard solution of 3.0 μg/mL. A CAP working solution of 30 ng/mL was made by diluting a stock solution with acetonitrile. An internal standard of 5D-CAP was prepared by diluting 100 μL of 100 μg/mL stock solution in acetonitrile and then was adequately diluted until a working solution of 30 ng/mL was obtained. All standard solutions were kept at ~4 oC and protected from light for a year.

Equipment

Liquid chromatography analyses were performed on a ZORBAX SB C18 narrow bore column (150x2.1 mm i.d., 3.5 μm) (Agilent Technologies Deutschland GmbH Chemische Analysentechnik, Waldbronn, Germany) using an Finnigan Surveyor (Thermo Electron Corporation) series liquid chromatograph equipped with a binary pump and an autosampler. Data acquisition and quantification were conducted using Excalibur sofware. The column was thermostated at 35°C. Chromatographic separation was performed using gradient elution with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) starting with a ratio (80:20; v/v) then 0-4 min, 80% —> 25% A; 4-4.5 min at 25% A; 4.5-4.51 min 25% —>80% A; 4.51-6.5 min 80% A. The flow was set at 0.3 mL/min and the injection volume was 25 μL. Under these conditions, the retention time of CAP and 5D-CAP was observed at 3.15 min.

Mass spectrometry analyses were performed on a Finnigan TSQ Quantum Ultra EMR triple stage quadrupole mass spectrometer (Thermo Electron Corporation) equipped with a heated-electrospray interface (HESI). The electrospray capillary temperature was 350°C and the capillary voltage was 4500 V. Nitrogen was used as a collision gas. MS detection was performed in negative mode using Multiple Reaction Monitoring (MRM). The monitored ion for CAP was m/z 321, and the product ions used for quantification were m/z 257, 194, and 152 and for 5D-CAP as internal standard monitored ion was m/z 326 and the product ions used for quantification were m/z 157 and 262. The scan time for each transition reaction was 500 ms with scan width 1.0 m/z. The MRM transition and their collision energies are shown in Table 1.

Table 1. Multiple reaction monitoring (MRM) transitions monitored for chloramphenicol (CAP) and internal standard 5D-CAP (IS) and their collision energies

Compound	Precursor ion m/z	Product ion m/z	Collision energy (eV)
CAP	321	152	20
CAP	321	194	19
CAP	321	257	12
5D-CAP (IS)	326	157	20
5D-CAP (IS)	326	262	12

Honey samples

Commercial domestic honey samples were randomly collected from all districts of Croatia during 2005.–2013. Honey samples were of different varieties, but mostly acacia (32%), flower (17%), chestnut (9%), linden (8%), honeydew (5%), sage (5%), lavender, fruit honey and honey with some substances added, including lemon, cherry, etc. Some of the samples were collected by sanitary inspection and others were analysed from distributors. Prior to analyses, all samples were stored in dark and dry places at ambient temperature (around 22°C) and in their original containers.

Honey sample preparation

The homogenized honey samples (5.0±0.01 g) were weighed in 200 mL beakers and fortified with 50 µL of working internal standard 5D-CAP and diluted with 10.0 mL acetate buffer. The samples were well mixed and 15 min sonicated at ultrasound bath and then purified and concentrated using HLB Oasis SPE cartridges. After preconditioning the cartridges by flushing 3 mL of methanol, 3 mL of water and 3 mL of acetate buffer, the whole sample was allowed to pass through the bed with suction. Purification was done by flushing 3 mL buffer and 6 mL of water.

Different extraction protocols were assayed using various eluting solvents, various volumes of solvent and different SPE columns (Krivohlavek et al. 2005). The best results were obtained with 2 mL of acetonitrile. Acetonitrile was evaporated until dry under a stream of nitrogen using a water bath at 35°C. The dry residue was redissolved in 0.5 mL mobile phase acetonitrile: water (20:80, v/v) and then filtered through a 0.20 µm disposable filter. Twenty-five µL was injected into LC-MS/MS.

Calibration curves at six concentrations levels were prepared by spiking blank honey samples with CAP at the following concentrations: 0.0 (blank samples), 0.10, 0.30, 0.50, 1.00 and 3.00 µg/kg. A fixed amount of an internal standard 5D-CAP was added to all the samples at concentration 0.30 µg/kg. The calibration curves were obtained relating to a ratio of CAP area/CAP-D5 area with CAP mass ratio in µg/kg. A calibration curve with standards was made every day.

RESULTS

For the purpose of the honey market control safety, especially domestic ones, according to banned chloramphenicol in food-producing animals in the European Union, the LC-MS/MS method was performed and validated.

A gradient LC-ESI/MS/MS method with an internal standard was developed to separate, quantify and confirm the presence of CAP in honey. A MRM procedure was applied. The three transitions were monitored m/z 321 —> m/z 257, m/z 321 —> m/z 194, m/z 321 —> m/z 152. According to Commission Decision 2002/657/EC for the confirmation of banned substances, a minimum of four identification points is required (Commission Decision 2002/657/EC). The four identification points can be obtained using LC-MS/MS with one precursor and two product ions. The presented research method detected 3 product ions and so the performance criteria for confirmation were fulfilled. Method validation was performed using both standard solution and spiked-honey samples. The method was validated according to the criteria of Commission Decision 2002/657/EC (Commission Decision 2002/657/EC). According to these criteria, validation included selectivity, linearity, precision (within-days and between-days), accuracy, decision limit (CCα), detection capability (CCß), robustness, sensitivity, stability and measurement uncertainty.

The selectivity of the method was checked by the preparation and analysis of blank and spiked honey samples from different origins (acacia, chestnut, linden and flower) to verify the absence of potential interfering compounds in honey. No interference was observed around CAP retention times in honey samples. Figure 1 show MRM chromatograms of a blank honey sample, same blank honey sample with the addition of 0.30 µg/kg CAP and appropriate standard solution, respectively.

The linearity response was studied using seven working standards injected three times, covering the entire working range of 0.5–50 ng/mL containing a fix amount of 5D-CAP (3.0 ng/mL). Chloramphenicol standard solution/internal standard peak area ratio was calculated versus chloramphenicol amount in ng/mL. Calibration curve was built using blank honey samples with the addition 0.00–3.00 µg/kg CAP as shown in Figure 2. For both curves, the linear correlation coefficient was greater than 0.99.

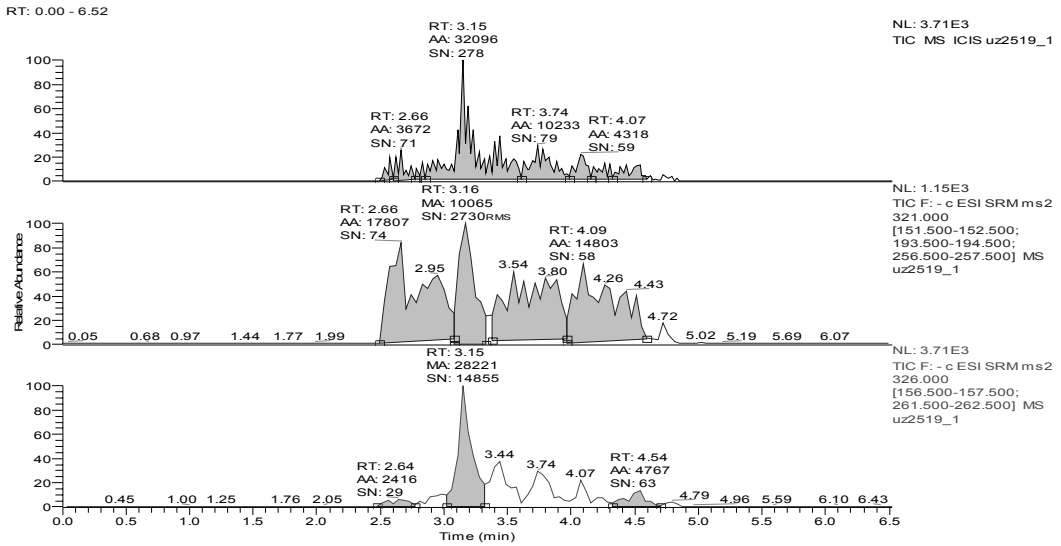

Fig. 1a. Liquid chromatography tandem mass spectrometry (LC-MS/MS) chromatogram of blank acacia honey extract

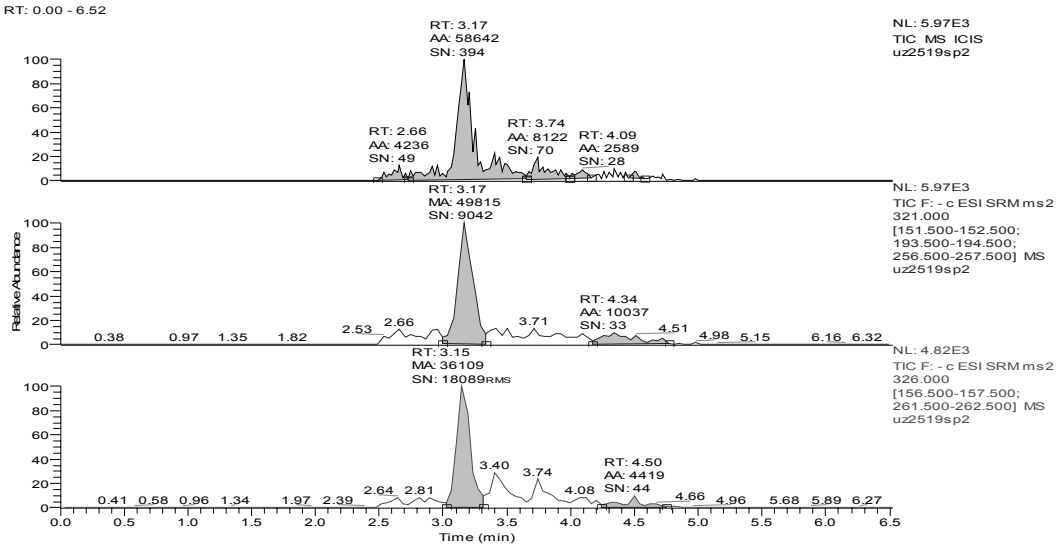

Fig. 1b. Liquid chromatography tandem mass spectrometry (LC-MS/MS) chromatogram of blank acacia honey sample with the addition of 0.30 µg/kg chloramphenicol (CAP)

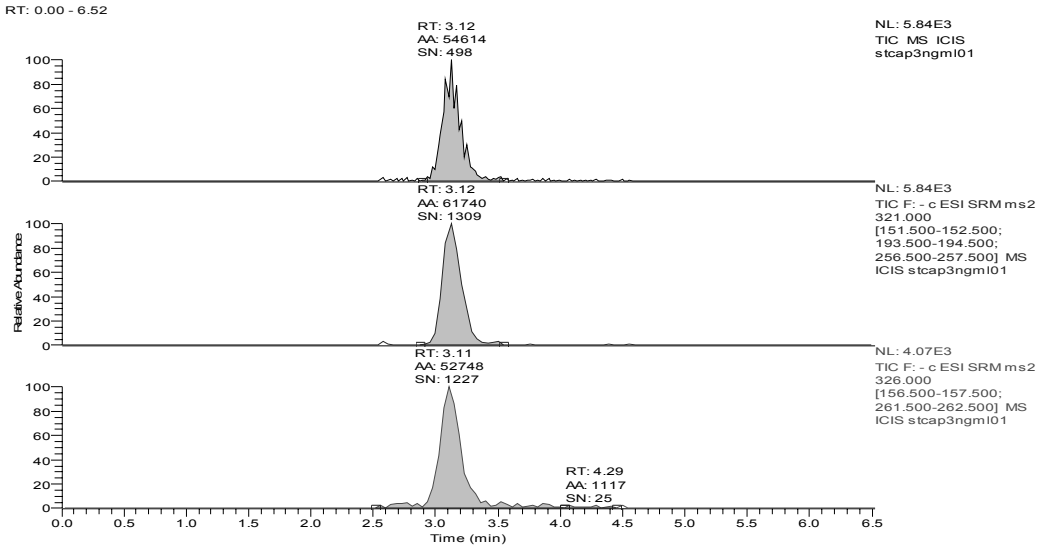

Fig. 1c. Liquid chromatography tandem mass spectrometry (LC-MS/MS) chromatogram in standard solution (3.0 ng/mL)

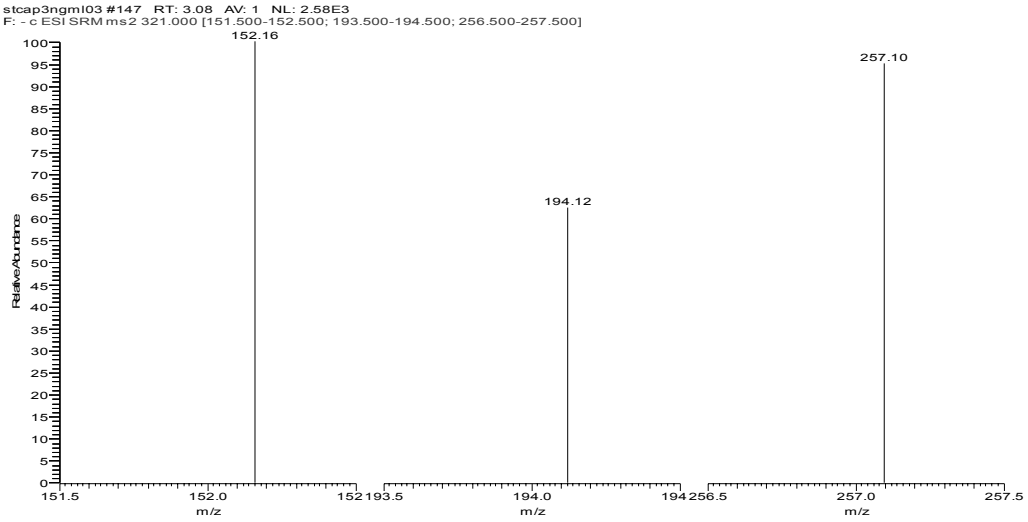

Fig. 1d. Mass spectrometry spectra of three multiple reaction monitoring (MRM) transitions monitored for CAP in standard solution

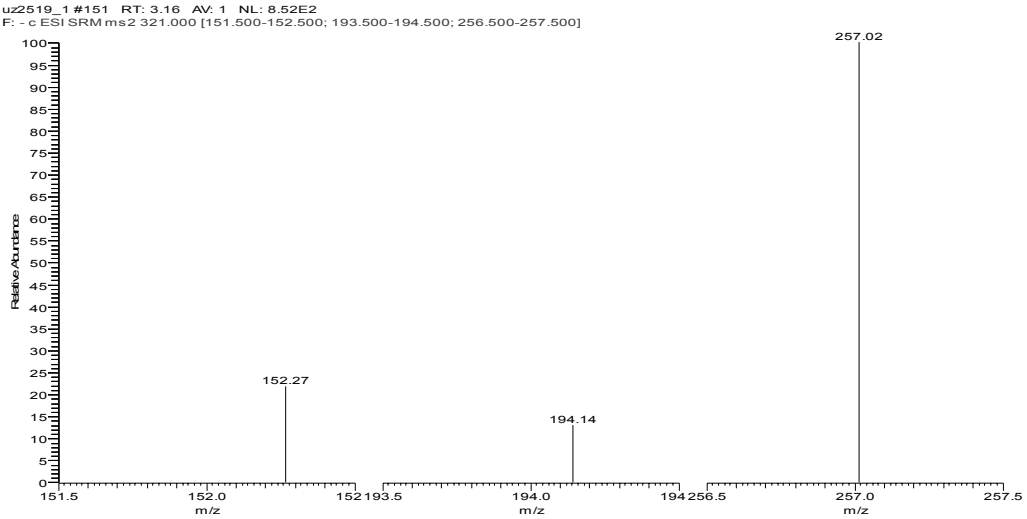

Fig. 1e. Mass spectrometry spectra of three multiple reaction monitoring (MRM) transitions monitored for CAP in blank sample

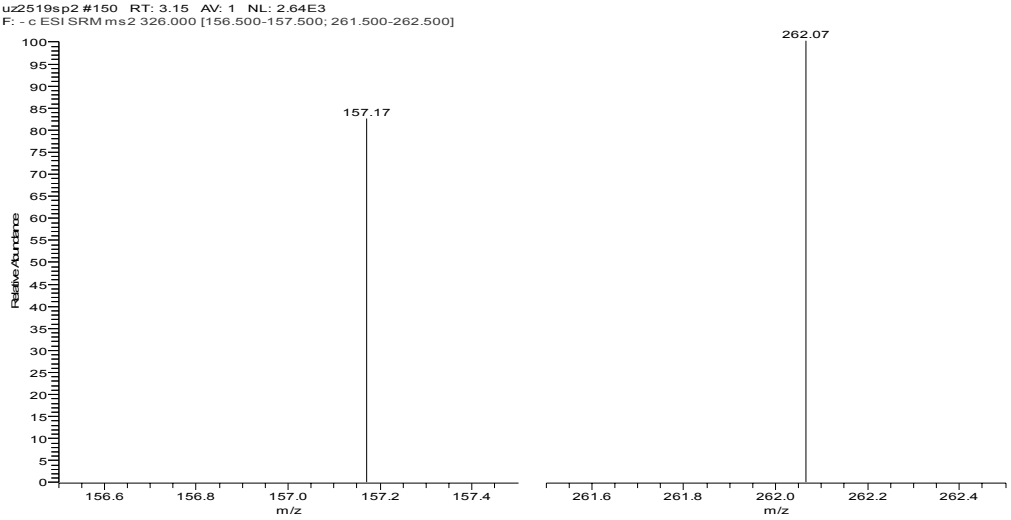

Fig. 1f. Mass spectrometry spectra of two MRM transitions monitored for internal standard 5D-CAP

Figure 1. Liquid chromatography tandem mass spectrometry (LC-MS/MS) chromatograms of blank acacia honey extract (a), blank acacia honey sample with the addition of 0.30 μg/kg chloramphenicol (CAP) (b) and appropriate standard solution (3.0 ng/mL) (c) with three multiple reaction monitoring (MRM) transitions monitored for CAP in standard solution (d) and in blank sample (e) and two MRM transitions monitored for internal standard 5D-CAP (f)

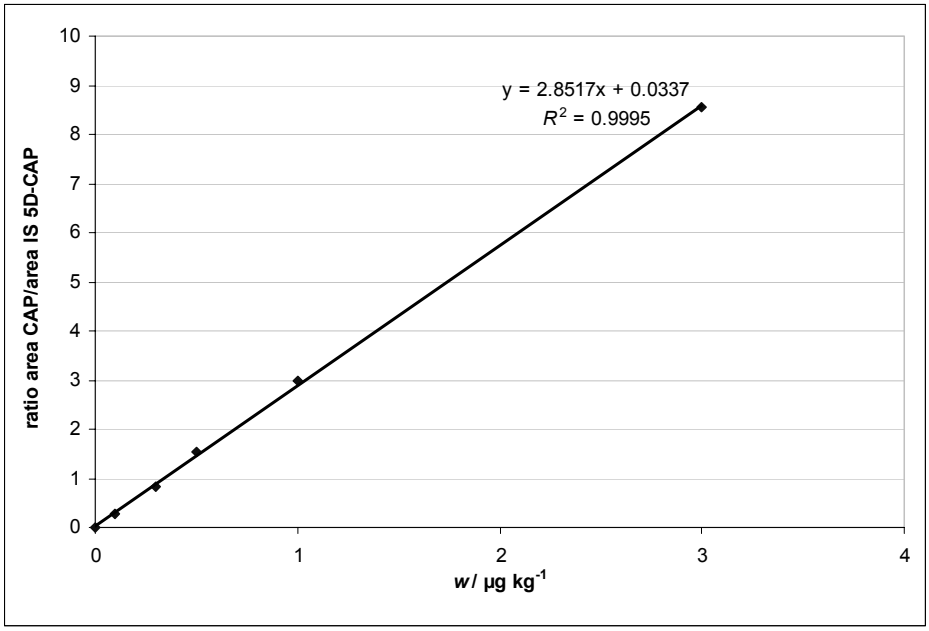

Figure 2. Linearity of calibration curve using blank honey samples with the addition of 0.00–3.00 µg kg^{-1} chloramphenicol (CAP) and with 0.30 µg/kg of internal standard 5D-CAP added at all six concentration levels

Table 2. Precision and accuracy for chloramphenicol (CAP) determination in spiked acacia honey samples

Precision	Spiked acacia honey samples		
Fortification levels (µg/kg)	0.10	0.30	0.50
Average (µg/kg) (n=9)	0.11	0.29	0.43
Within-day precision, RSD (%)	6.90	5.70	6.90
Recovery (%)	113	98	87
Between-day precision, RSD, (%), (n=3x9)		9.00	

DISCUSSION

Precision (within-day) and accuracy (recovery) were calculated from the analysis of blank honey spiked at three levels: one at MPRL and two around the MPRL (0.10, 0.30, and 0.50 µg/kg, respectively) of CAP. Nine replicates were obtained for each concentration. Precisions (within-day) were found that satisfied the three levels studied and RSD values were 5.7–6.9%. The recovery (trueness) was calculated by comparing the measured concentration to the spiked concentrations. The average recovery was in the range of 87–113% for all levels. Precision (between-day) was calculated in spiked samples at 0.30 µg/kg on three different days (3×9). The relative standard deviation was 9%. The precision and accuracy are presented in Table 2.

The stability of standard solutions was also investigated. Working standard solutions of CAP (25 pg/µL) were prepared on the same day from the stock solution (kept in dark and around 4°C) prepared over a year and then analysed. The relative standard deviation was 11%. The stability of the working standard solutions and prepared samples were also investigated. Standard solutions and prepared samples with standard added at 0.30 µg/kg were tested every second day for a ten-day period and the results showed that RSD values were 9.7 and 11.4% respectively. For a test of robustness, the matrix effect was assessed. Nine replicates of different types of honey (acacia, chestnut, linden and flower) were spiked at a limit of quantification of 0.30 µg/kg and analysed (Table 3).

The revised criteria also introduce the decision limit (CCα) and detection capability (CCß) to replace the limit detection and quantification, respectively. In accordance with the Commission Decision 2002/657/EC, more than 20 representative blank samples with internal standard added were analysed to determine CCα and CCß. The values of CCα and CCß were 0.09 and 0.12 µg/kg, thus below the MRPL set at 0.3 µg/kg by the EU amending Decision 2002/657/EC (Commission Decision 2002/657/EC).

The validated method, LC-MS/MS was used for routine analysis of CAP in Croatian honey samples. It is relatively fast, but some literature data showed that using molecularly imprinted polymers (MIPs) or magnetic MIPs (MMIP) may overcome multistep pretreatment, time and labour work of purification and extraction of complex matrix as it is honey, prior LC-MS/MS analysis (Boyd et al. 2007, Chen & Bin 2013).

Table 3. Matrix effect for chloramphenicol (CAP) determination in different honey samples (acacia, chestnut, linden and flower) spiked at 0.30 μg/kg

Type of honey	Acacia (n=9)	Chestnut (n=9)	Linden (n=9)	Flower (n=9)	All (n=36)
Average recovery (%)	99	94	100	113	102
Standard deviation (%)	5.7	2.4	6.1	4.0	8.5
Coefficient of variation (%)	5.8	2.6	6.1	3.5	8.4

CAP residues were analysed in 280 samples but only detected above the CCα in three samples using this procedure. One sample was acacia honey from 2005. and the other from 2006. and the third was from 2008. with 1.6 μg/kg, 1.8 μg/kg and 0.54 μg/kg, respectively. Since 2008., no honey samples with chloramphenicol were detected above CCα (Table 4) showing good quality of domestic honey. To the best of our knowledge, this is the first time that such investigation has been done on the Croatian market.

Table 4. Analysed honey samples collected randomly from all Croatian districts present at Croatian market in period between 2005-2013

Year	Total number of analysed honey samples	Number of non compliant honey samples	% of non compliant honey samples
2005	9	1	11.1
2006	51	1	2.0
2007	16	0	0
2008	10	1	1.00
2009	37	0	0
2010	47	0	0
2011	20	0	0
2012	31	0	0
2013	59	0	0
2005-2013	280	3	1.1

It is known that the presence of xenobiotics, antibiotic residues in honey may harm its quality and constitute a danger to human health. Safety of food and feed is the one of the main objective in consumer health policy, and CAP is completely banned in food producing animals within EU due to its toxicity in humans (Commission Regulation (EC) 1430/94), but not in some countries outside of Europe (for example Asia). Therefore, it was necessary to develop a sensitive and rapid method as it is presented in our study to control and monitor CAP residues in honey on European market even a decreased trend was noted also by European Rapid Alert System for Food and Feed (RASFF) (European Rapid Alert System for Food and Feed - Reports and Publications).

Furthermore, honey consumption is very high in developed countries, where domestic production does not always meet the market demand. In the EU, which is both a major honey importer and producer, the annual consumption per capita varies from medium (0.3-0.4 kg) in Italy, France, Great Britain, Denmark and Portugal to high (1-1.8 kg) in Germany, Austria, Switzerland, Portugal, Hungary and Greece, while in countries such as the USA, Canada and Australia the average per capita consumption is 0.6-0.8 kg/year. The major honey exporting countries, China and Argentina, have small annual consumption rates of 0.1-0.2 kg per capita (Bogdanov et al 2008). According to the data of Statistical Yearbook 2013 of the Republic of Croatia annual average of honey per household member was 1.1 kg in 2011. and 1.2 kg in 2010. (Statistical Yearbook of the Republic of Croatia 2013). The consumption grew from 0.3 kg to approximately 1 kg in the period of 10 years (Statistical Yearbook of the Republic of Croatia 2003, Statistical Yearbook of the Republic of Croatia 2013). Additionally, it was showed that honey has a variety of positive nutrition and health effects, if consumed at higher doses of 50 to 80 g per intake (Bogdanov et al. 2008). But, health benefits of popular food could be diminished or even become disadvantage if it contains CAP residues. It is not only because of harm effect of CAP as a well known bone marrow depressant, but also because of possible interactions of CAP and some prescribed conventional drugs (Baxter K & Preston CL 2008). CAP is also a known enzyme inhibitor and could grow up level of many drugs by reducing their metabolism and thus caused toxicity. Some well documented and established interactions of clinical importance were between CAP and tolbutamide, phenytoin, iron compounds and vitamin B12. So, the result of interaction could be acute hypoglycaemia, phenytoin toxicity and opposes the treatment of anaemias with iron or B12, respectively, depending of the dose of the two (Baxter K & Preston CL 2008).

Since, consumption of honey and various honey products constantly grows, safety of products and consumer's health becomes of the biggest importance, and therefore routine control of the market by validated and confirmative method of forbidden CAP residue is necessity.

CONCLUSIONS

Chloramphenicol residue analysis of various honey samples (acacia 32%, chestnut 9%, linden 8%, flower 17% others 34%) from Croatia in the year period of 2005. to 2013. showed very good quality. Only three of 280 (1.1%) honey samples were non compliant having chloramphenicol above CCα. All non compliant samples were acacia ones. Presented LC-MS/MS method

for determining chloramphenicol in various honey samples is fast, robust and confirmative. The sample preparation is simple and has good precision and recoveries. Thus is appropriate for routine honey analyses of CAP residue. The validation results are in accordance with the performance method criteria of the European Commission Decision 2002/657/EC.

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Conflict of interest : None to declare.

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Correspondence:

Adela Krivohlavek, PhD
Andrija Stampar Teaching Institute of Public Health
Mirogojska cesta 16, HR-10000 Zagreb, Croatia
E-mail: adela.krivohlavek@stampar.hr