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Rapid determination of chloramphenicol and its glucuronide in food products by liquid chromatography–electrospray negative ionization tandem mass spectrometry

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Abstract

Chloramphenicol (CAP) is subjected to monitoring in food products, with a minimum required performance level set at 0.3 ng/g. CAP was isolated from chicken meat and seafood by very simple solvent extraction procedure. For honey, a fast SPE procedure was applied. CAP-D5 was used as internal standard. HPLC separation was done on RP18 123 mm × 3 mm column in acetonitrile–ammonium formate 10 mM, pH 3.0 (40:60) at flow rate of 0.3 ml/min. A TSQ Quantum instrument with ESI source has been used in negative ionization mode. A MRM procedure has been applied and following transitions were monitored: *m/z* 321 > 152 (quantifier), 321 > 194, 321 > 257 (qualifiers), 326 > 157 (IS). CAP peak was eluted at around 5 min; the total run time was 7 min. LOD was around 0.1 ng/g meat or 0.05 ng/g honey. Matrix effects were studied for all materials used, involving injection of blank extracts with post-column infusion of CAP, as well as checking the influence of the co-injected blank extracts on the signal intensity of CAP. No influence of matrix on the results of CAP determination were observed. The method allows analyzing up to 30 duplicate samples per day, including all calibration standards. Additionally, the method for determination of CAP glucuronide (CAP-G) was established, using urine from rats that were given this drug as a source of the metabolite. Full validation of the metabolite was not possible, due to the unavailability of reference standard.

Keywords: Chloramphenicol; Chloramphenicol glucuronide

1. Introduction

Chloramphenicol (CAP) is a potent, broad-spectrum antibiotic drug. Its use is associated with many toxic effects in humans and therefore is limited only to life-threatening situations. The principal toxic effect is the development of usually fatal bone marrow depression (aplastic anemia) in susceptible individuals. The onset of this effect is not dose dependent [1]. The use of CAP in food producing animals, particularly in aquaculture, is prohibited in Europe and US. However, this drug is still used in Asiatic countries [2]. This may be concluded from the incidence of aplastic anemia in the population involved. The incidence of aplastic anemia in Europe is 0.2 cases per 100,000 inhabitants. In contrary, in South Asiatic countries, which are the greatest producers of seafood, aplastic anemia is much more frequent; in China it reached two cases per 100,000, and in Thailand 3.7 cases per 100,000 [3]. A need of permanent control of food samples, particularly originating from Asiatic countries, is therefore obvious. Since there is no "safe level" or "tolerance level" of CAP in food, any detectable amount of this drug is reportable. Recently, European Commission established minimum required performance limit (MRPL) for CAP detection in food products at 0.3 μg/kg [4].

The methods applied for CAP may be divided into two groups: preliminary screening done by immunoassay [5–7] and confirmatory methods, based on gas chromatography–mass spectrometry [7,8], or liquid chromatography–mass spectrometry.

The use of liquid chromatography–negative ionization electrospray tandem mass spectrometry (LC–ESI–MS–MS) was proven as most selective and sensitive method for CAP determination in seafood and meat matrices. Several LC–MS

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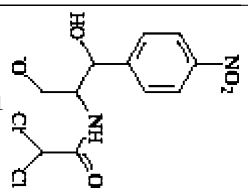
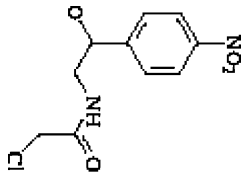
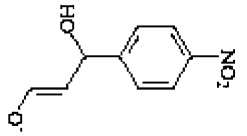
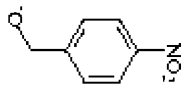
Table 1
MRM transitions monitored for CAP, internal standard (CAP-D5) and CAP-G

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Collision energy (eV)
CAP	321 	257 	12
		194 	10
		152 	20
CAP-D5	326	157	20
CAP-G	497	321	12
		152	20

methods have been published recently, with reported detection limit ranging from 0.02 to 1 ng/g. Most often, triple quadrupole instruments were applied [9–16]. Also, ion trap mass spectrometers [17] and atmospheric pressure photoionization instrument [18] have been used. From the existing literature is obvious that all authors applied quite elaborate and sometimes extremely tedious [15–17] sample preparation procedures.

The purpose of this study was to develop a rapid and selective confirmatory method for determination of CAP

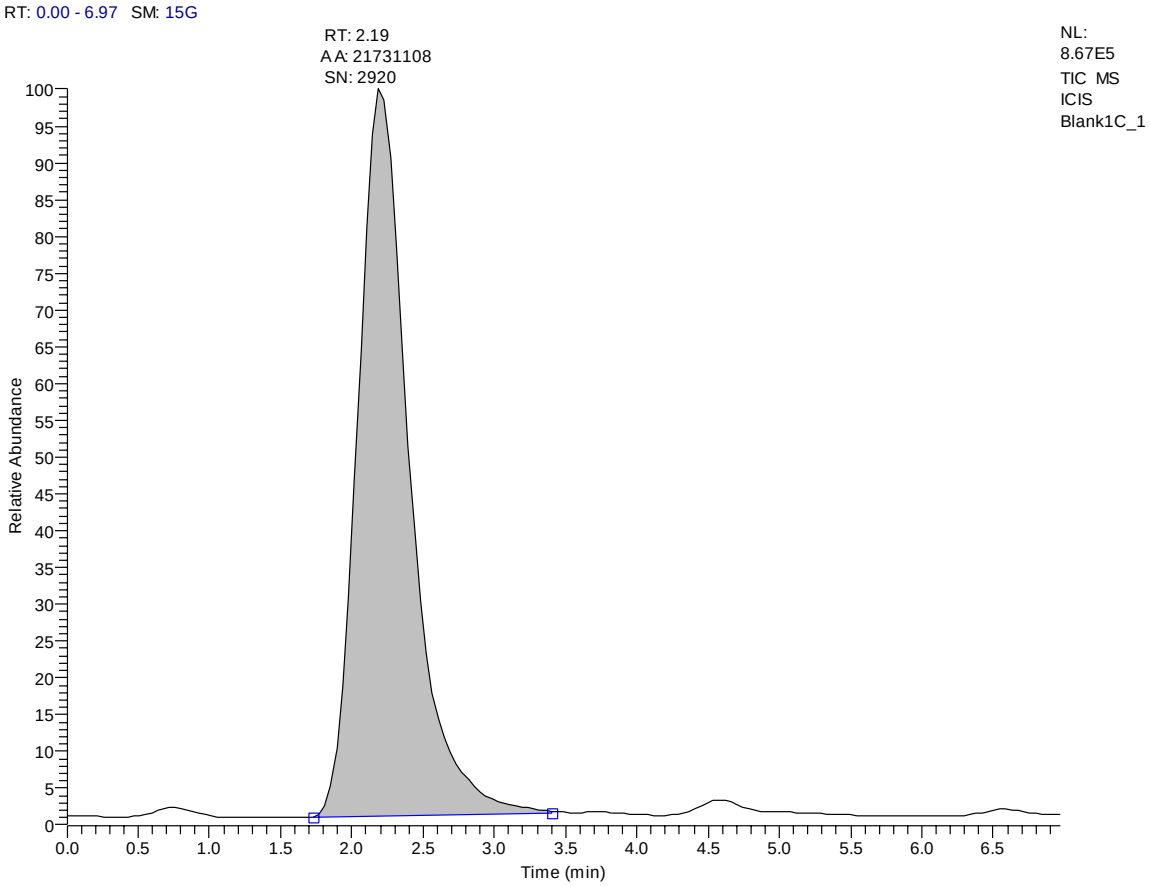


Fig. 1. TIC chromatogram of blank chicken meat extract showing large matrix peak eluting around 2–3 min.

in food samples (seafood, chicken, honey), based on LC–ESI–MS–MS. The rationale behind the method was to establish as simple method as possible, without compromising selectivity and sensitivity. As a landmark, the MRPL value of 0.3 μg/kg, established by EU, was taken. The sensitivity of the method should be much below this limit. The second aim was to detect CAP glucuronide (CAP-G), the main metabolite of CAP. This substance was never directly determined in food samples. In some studies [9,10], the samples were subjected to enzymatic hydrolysis with β-glucuronidase prior to extraction and LC–MS–MS determination of the total CAP. Recently, CAP-G was determined in rat liver fractions using LC–MS–MS [19].

2. Experimental

2.1. Materials and reagents

Chloramphenicol was supplied by Sigma–Aldrich (St. Louis, USA).

Chloramphenicol-D5 (98% purity) was supplied by Cambridge Isotope Laboratories, Andover, USA. Working solution of internal standard (IS) was prepared by diluting CAP-D5 to the concentration of 0.02 ng/μl in MeOH–H_2O (1:1).

β-glucuronidase Type HP-2 from *Helix pomatia*, was supplied by Sigma–Aldrich (St. Louis, USA).

Chloroform, HPLC grade, 99.9% purity, containing ethanol as preservative, was supplied by Fisher Scientific, USA.

Isooctane, Lichrosolv for liquid chromatography, min.: 99%, was supplied by Merck, Darmstadt, Germany.

Solid phase extraction (SPE) cartridges Bond Elut C18 LRC, 100 mg, were supplied by Gulf Scientific Co. (Dubai, UAE).

2.2. Food samples

Chicken meat, shrimp meat, and honey samples, used for validation, were tested for the absence of chloramphenicol using two independent immunoassay methods (ELISA Randox Labs, Crumlin, UK and ELISA Chloraquant, Biomedix, Diamond Bar, USA) prior to spiking. Food samples sent for the analysis on CAP were at first screened using ELISA procedures. Positive samples (immunoassay results above

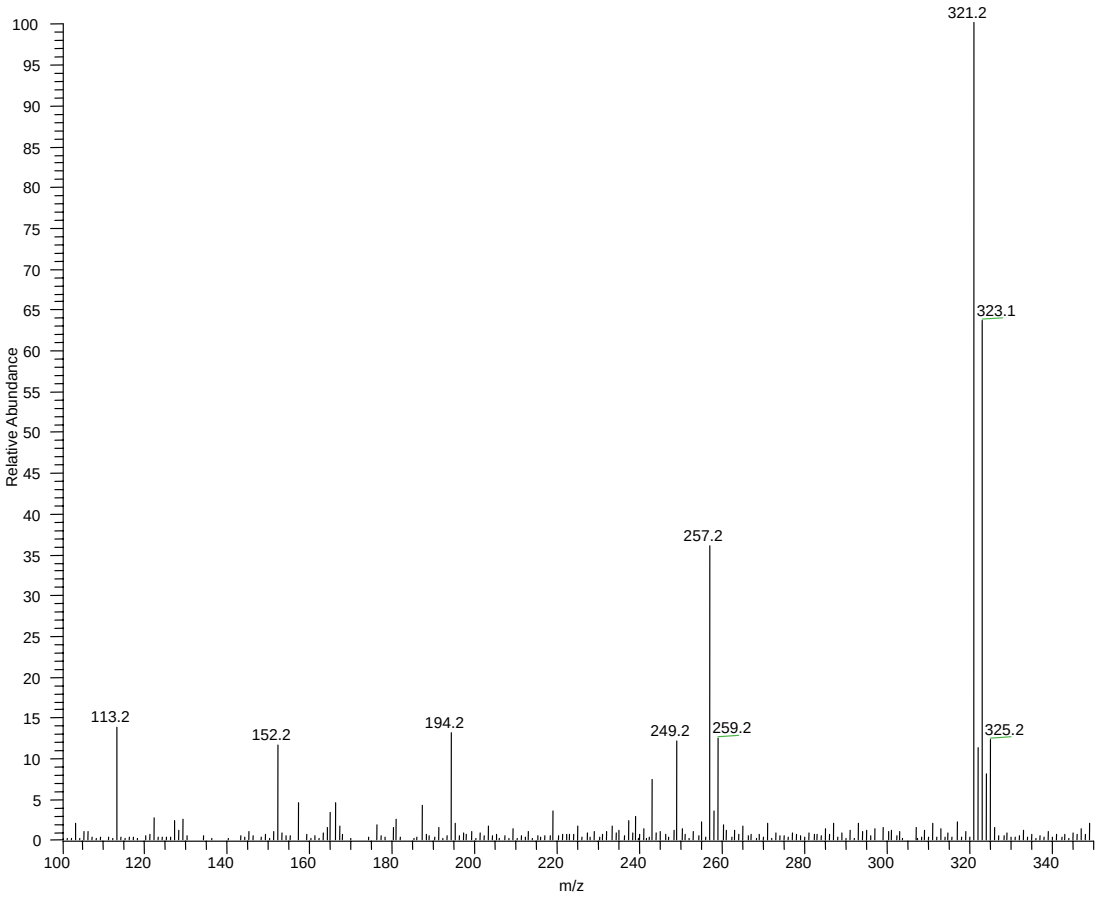


Fig. 2. Full scan spectrum of CAP. In-source collision energy 10 V.

0.1 ng/g of chloramphenicol equivalent) were then subjected to LC–MS analysis.

2.3. Sample preparation

2.3.1. Chloramphenicol extraction procedure from chicken and seafood

Around 10 g of sample was minced using Ultra-Turrax T-18 Disperser (IKA-Werke, Staufen, Germany) at 25,000 rpm. One hundred fifty microliters of working IS solution was added to 3 g minced mass and mixed thoroughly. The sample was homogenized for 1 min with 6 ml ethyl acetate and centrifuged 15 min at 5000 × *g*. Four milliliters upper phase was collected and evaporated under nitrogen. The residue was reconstituted in 2 ml isooctane/$CHCl_3$ (2:3) and thoroughly mixed. One milliliter of Tris buffer, 0.05 M, pH 7.4 was added, the sample was vortexed for 1 min and centrifuged at 16,000 × *g* for 5 min. An amount of 0.7 ml of supernatant was collected for LC–MS analysis and 25 μl was injected into LC–MS.

2.3.2. Chloramphenicol extraction procedure from honey

One gram honey was dissolved in 4 ml of 0.01 M ammonium carbonate buffer (pH 9.3) under heating (50 °C) and 50 μl of working IS solution was added. SPE cartridges were equilibrated with 5 ml water, 5 ml methanol, and 5 ml ammonium carbonate buffer. Honey solution was applied on the SPE cartridge and passed slowly (1 ml/min). After rinsing with 5 ml ammonium carbonate buffer and vacuum drying for 5 min, CAP was eluted with 3 ml methanol under gravity force. The solvent was evaporated to dryness under nitrogen, the residue was reconstituted in 200 μl acetonitrile–water (1:1), centrifuged 3 min at 16,000 × *g*, and the supernatant was collected to microvials for LC–MS determination. Twenty five microliters was injected into LC–MS.

2.3.3. Search for CAP glucuronide

Since CAP-G is not commercially available, CAP was administered to three male Wistar rats originating from the

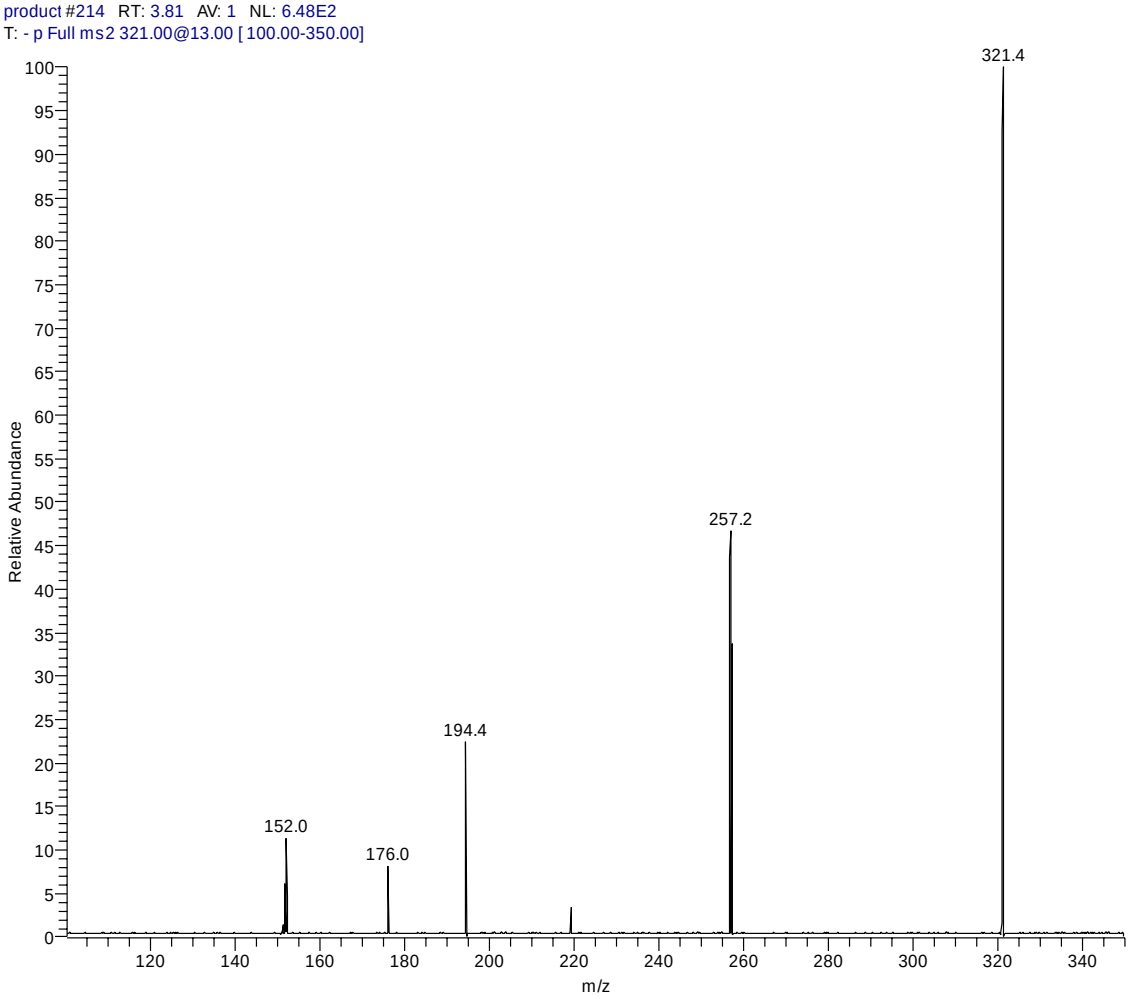


Fig. 3. CAP product scan of *m*/*z* 321. Collision energy 10 V.

animal pool of the Comparative Medicine Department of the Research Centre (300 ± 10 g body weight each) in dose of 100 mg/kg, using gastric tube. The animals were kept in metabolic cages and the urine specimens were collected for 24 h and the specimens were frozen until determination. It was assumed that the urine would contain CAP and CAP-G as well. Urine samples were centrifuged 5 min at 16,000 × *g* and the supernatant was diluted 1:10 with water. After centrifugation for 5 min at 16,000 × *g*, 10 μl of the supernatant was directly injected into LC–MS. In order to check the presence of CAP glucuronide, diluted 1:10 urine samples were treated with β-glucuronidase (30 μl per to 0.5 ml of diluted urine sample) and incubated for 5 h at 37 °C. The samples were then cooled and centrifuged for 5 min at 16,000 × *g*. Ten microliters of the supernatant was injected into LC–MS.

For the isolation of CAP and CAP-G from semi-liquid or solid samples, a solid phase extraction procedure, used also for honey samples, was applied. The same method has been used previously for isolation of opiate glucuronides and other drugs from biological material [20,21].

2.4. HPLC

CAP and CAP-G were separated on Superspher RP-18 column 125 mm × 3 mm, particle size 4 μm (E. Merck, Darmstadt, Germany). For CAP determination, a mobile phase consisting of acetonitrile (ACN) and ammonium formate buffer 10 mM, pH 3.0 (AMF) in proportion 40:60 was used, at flow rate of 0.3 ml/min. The run time was 7 min.

For the separation of CAP and CAP-G on the same column, a mobile phase consisting of ACN–AMF (30:70) was applied, at flow rate of 0.3 ml/min. The run time was 12 min.

2.5. ESI–MS–MS

A TSQ quantum triple stage quadrupole instrument, together with Surveyor LC quaternary pump and Surveyor autosampler (Thermo Finnigan, San Jose, USA), was used. The instrument was optimized for CAP using automated optimization procedure in syringe infusion mode as provided by the manufacturer. Following source parameters were applied: spray voltage: 3200 V, sheath gas: 35 units, auxiliary gas: 20 units. Collision gas pressure was 1.5 mTorr, tube lens

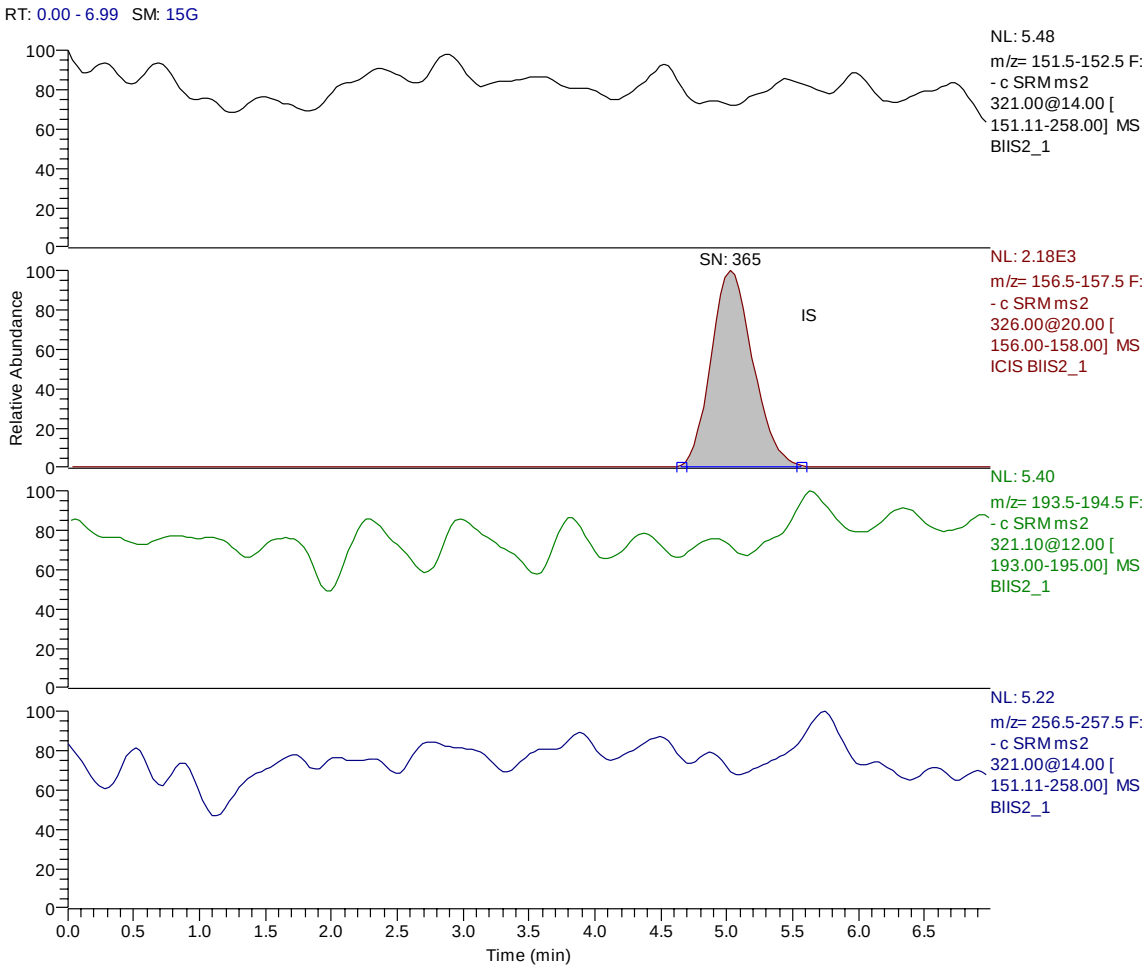


Fig. 4. Mass chromatogram of blank chicken meat extract spiked only with IS.

offset was optimized for CAP. Table 1 shows the MRM transitions monitored for particular compounds as well as collision energies applied.

Full scan LC–MS (*m/z*: 50–500) of blank extracts showed large, broad matrix peak eluting between 2 and 3 min (Fig. 1). Therefore, in order to protect the instrument, the mobile phase flow was diverted to waste for the first 3 min after injection.

2.6. Validation

2.6.1. Study of matrix effects

The matrix effects (possible suppression or enhancement of the signal) was studied for CAP for all material used, i.e. for the extracts of chicken meat, shrimps, and honey. In the case of rat urine, both urine sample diluted 1:10 with water and urine extract obtained after SPE were used. This study was performed in following ways:

- Blank samples of chicken meat, honey, and rat urine (three samples of each material) were extracted as described above. The extracts, as well as diluted rat urine specimens, were injected into the HPLC column under the same conditions as for routine samples. Through a tee-joint installed post-column, CAP solution (100 ng/ml of mobile phase) was infused from the syringe at the infusion speed of 2 μl/min.
- Blank extracts of chicken meat, honey, and rat urine, reconstituted with 25 μl of mobile phase were mixed (1:1) with CAP solution in mobile phase to the final concentration of 1 and 5 ng/ml. Twenty five microliters of the mixture were injected into LC–MS. Then 25 μl of pure CAP solution (1 and 5 ng/ml in mobile phase) were injected and the peak areas for each transition were compared.

In experiments involving post-column infusion of CAP, the mobile phase flow was not diverted for the first 3 min, but directed to the source from the beginning. All other analytical conditions (mobile phase composition and flow rate, transitions monitored) for matrix experiments were identical as for analyzed samples.

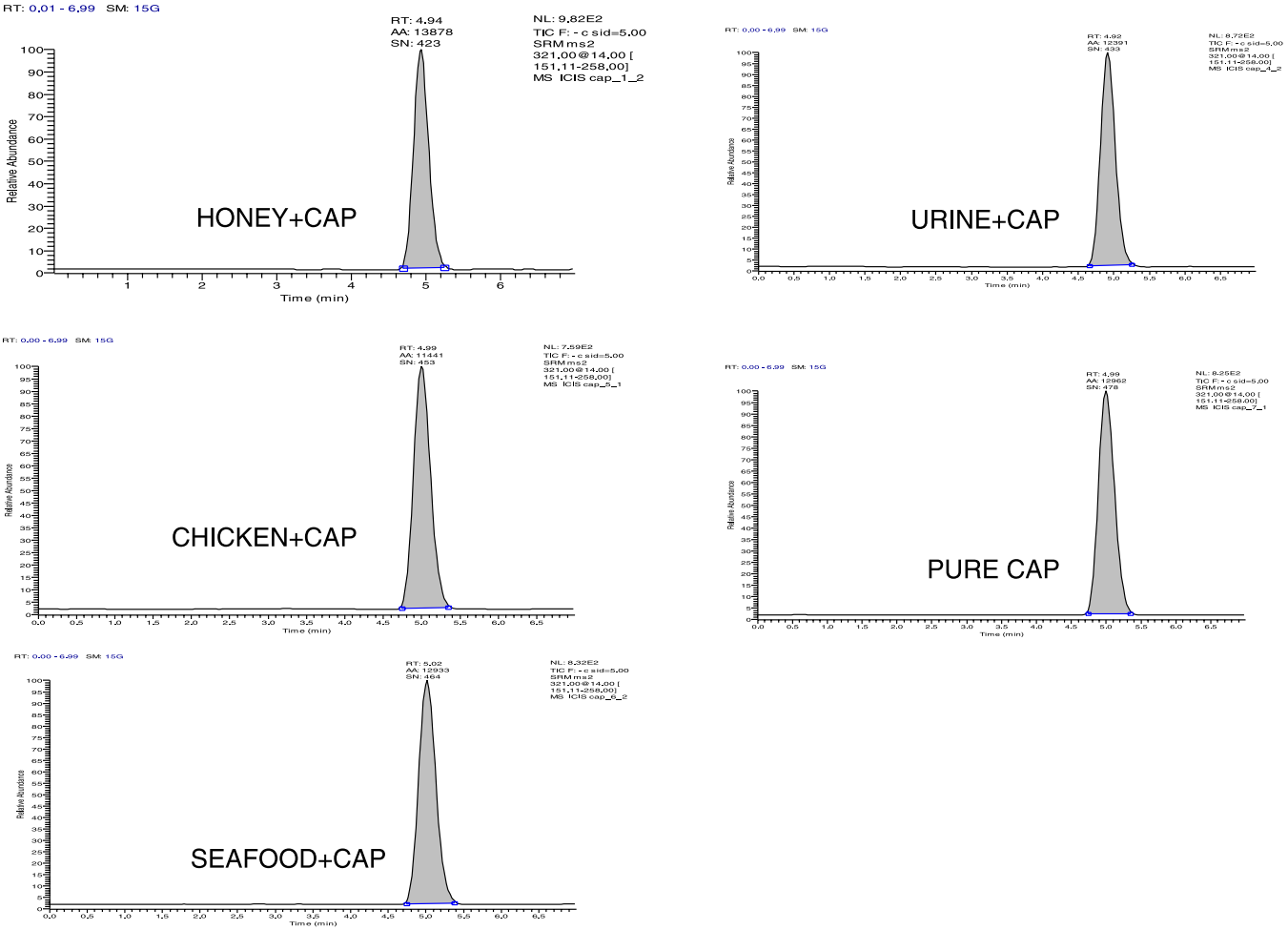


Fig. 5. Comparison of signal intensities for 25 pg of CAP (TIC of all transitions) injected together with blank extracts and with mobile phase only. No differences in intensities were observed.

2.6.2. Validation of CAP determination in food samples

Validation experiments were performed in the concentration range of 0, 0.1, 0.2, 0.5, 1.0, 5.0, and 10 ng/g CAP in chicken meat, shrimp meat, and honey. All experiments were performed on three different days, always in duplicate. Each reconstituted extract was injected in duplicate. All validation parameters (linearity, limit of detection (LOD), limit of quantitation (LOQ), and confidence range (CR)) were calculated using a BEN 2.0 software [22] for the calculations the analytical limits according to the DIN 32645 [23,24]. The significance level was set at 99%.

Day-to-day precision as well as accuracy was determined for chicken meat and honey samples spiked with CAP to the concentrations of 0.5, 2, and 10 ng/g. Three different samples for each concentration step were used. These experiments were performed by two different persons.

2.6.3. Validation of CAP-G determination

Since CAP-G was not available as a reference standard, the only possible validation procedure was the comparative assessment of the recovery of this compound from rat urine using SPE. This was done through the comparison of results, obtained for CAP and CAP-G analyzed directly in diluted urine samples, with the results obtained for the same samples after extraction.

2.6.4. Stability of CAP and postulated CAP-G in frozen samples

Since the chicken and seafood samples were submitted for examination in the frozen state, the stability of CAP in frozen and thawed samples were tested. Three different blank chicken meat samples were homogenized and spiked with CAP to the concentration of 2 and 10 ng/g. The samples were divided into portions and kept frozen at −20 °C. From this material, the specimens were taken for examination after 7, 30, and 60 days. Additionally, rat urine samples, taken from rat after CAP administration, were kept frozen for 60 days. During this time, urine specimens were thawed at days 10, 30, and 60 and CAP and CAP-G was determined after dilution 1:10 with mobile phase containing IS (2 ng/ml) and centrifugation. The peak area ratios CAP:IS (*m*/*z* 152:157) and CAP-G:IS (*m*/*z* 321:157) were monitored.

3. Results and discussion

3.1. Optimization of LC–MS–MS method

Figs. 2 and 3 show the full scan spectrum of CAP and product scan of the ion *m*/*z* 321 (deprotonated quasi molecular ion of CAP). All fragments, reported by other authors,

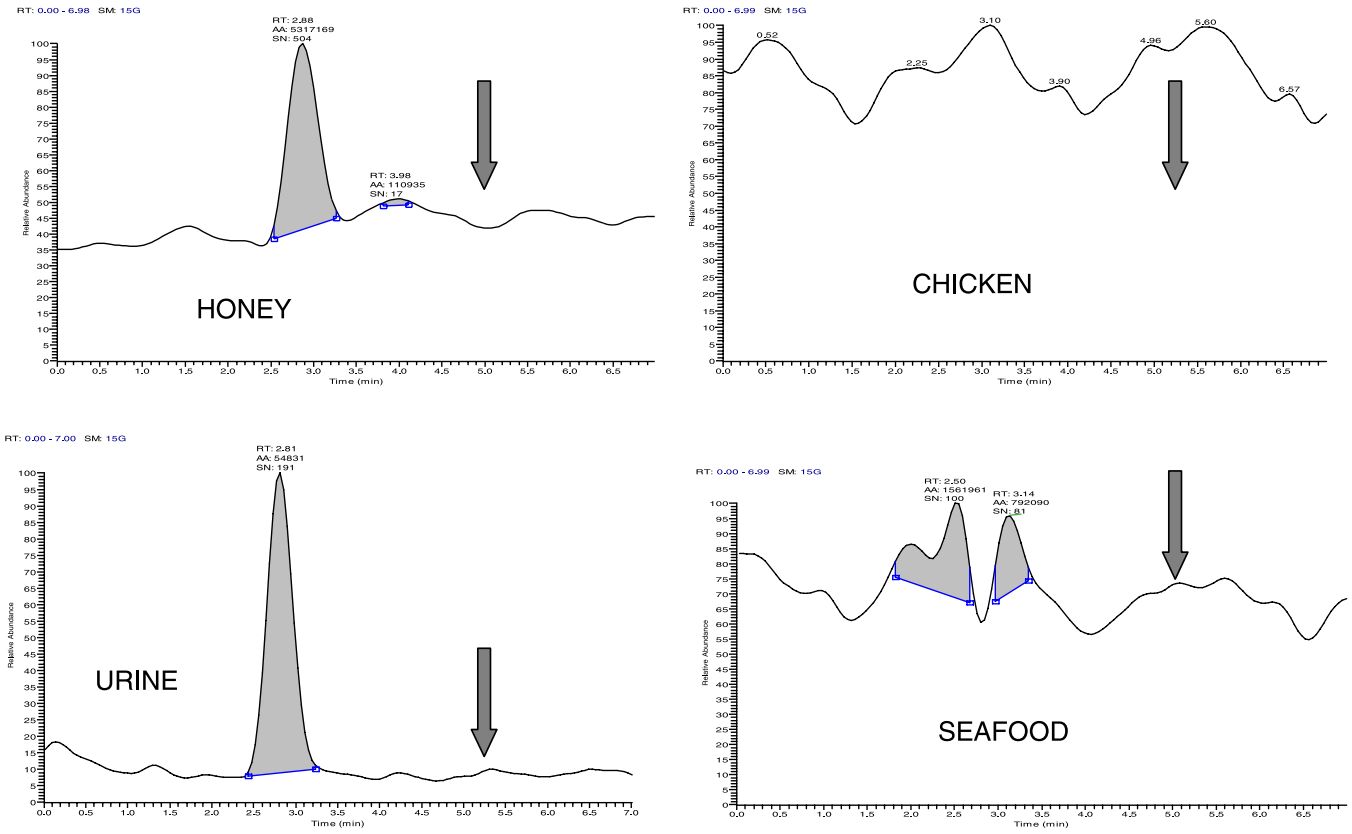


Fig. 6. Matrix peaks observed after injection of blank extracts on HPLC column with post-column infusion of CAP. TIC of all ions monitored for CAP. The arrows indicate expected Rt of CAP.

were detected. In the optimization procedure, performed under syringe infusion of CAP, the conditions for MRM were established (Table 1). The ion *m*/*z* 152, giving the strongest signal, was used as quantifier along with the ion *m*/*z* 157, originating from CAP-D5. It was demonstrated that CAP and CAP-D5 did not contribute mutually to their particular fragment ions (Fig. 4).

3.2. Validation

3.2.1. Study of matrix effects

It is a common knowledge that poor sample preparation procedure and incomplete chromatographic separation may dramatically influence LC–MS–MS results. It should be noted that high selectivity of LC–MS–MS tempts to neglect both sample pretreatment and chromatography in order to accelerate the whole analytical run. Very often, isolation step is not utilized at all, but replaced by simple dilution of liquid sample ("dilute and shoot" approach). It is, therefore, recommendable to present the evidence that the results of detection and quantitation are not influenced by some uncontrolled factors originating from individual sample. Such evidence was provided in the present study using two approaches: by direct comparison of the signal intensity of CAP in the presence of different matrices, and by the observation of possible signal enhancement or suppression in post-column infusion experiments. Both approaches were used by other authors.

Müller et al. [25] studied the effect of coextracted serum matrix on the signal of test substances in positive and negative ionization mode. Signal suppression was observed for both ionization methods after injection of serum matrix. The authors stated that the suppression effects were caused by polar, non-retained matrix components, appearing on the beginning of the chromatogram, and were related to the extraction mode. Matuszewski et al. [26] stated that the matrix effects (mainly signal enhancement) were dependent on the individual plasma matrix and ionization source applied. When APCI was applied, the matrix effect was not visible. On the other hand, Mei et al. [27] found that APCI mode was more prone to matrix effect than ESI. They stated also that matrix effect is dependent on the source design and may be different in various brands of instruments. Zhou et al. [28] injected blank serum matrix samples into the

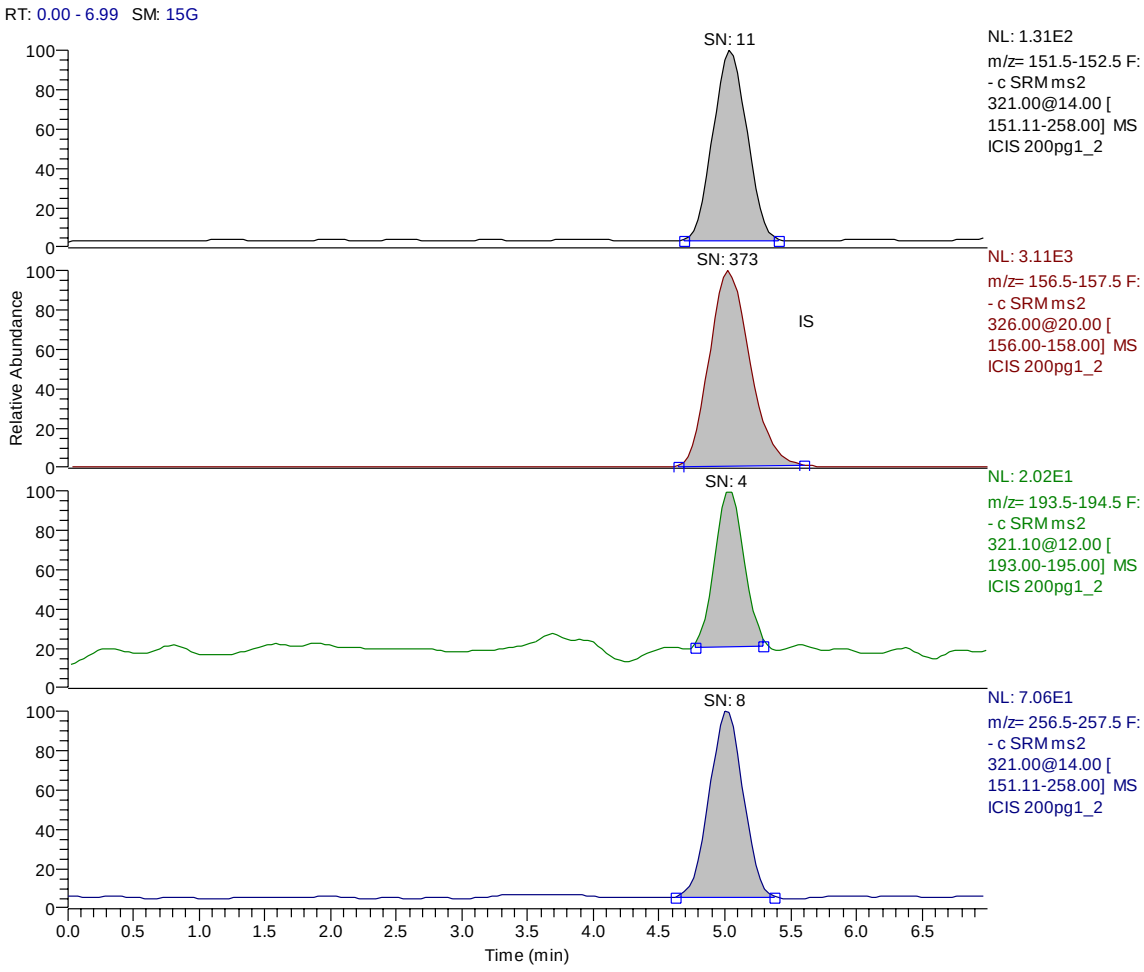


Fig. 7. Mass chromatogram of blank chicken meat extract spiked with CAP 0.1 ng/g and with IS.

HPLC column with the post-column infusion of four test compounds at three concentration levels. The areas of suppression were located along the whole chromatogram. On the solvent front, salts and other polar unretained species were present. Other endogenous compounds were eluted later, sometimes in very high concentrations, causing severe ion suppression, which was independent on the analyte concentration. Tang et al. [29] also studied matrix effects in post-column infusion experiments. Extracted blanks were injected while the ion transitions of the infused analytes were monitored. Both suppression and enhancement of ionization was observed. These phenomena were compensated by changing the ionization energy, ionization source, sample pretreatment method, or by including matrix ions in acquisition methods. Avery [30] compared the ion suppression effects caused by extracts of human and animal (rat, dog, monkey, rabbit, guinea pig) plasma and stated that each species showed different suppression. Therefore, the validation should be performed with samples originating from the same species. Dams et al. [31] studied the detectability of morphine extracted from urine, plasma, and oral fluid with four methods and analyzed with ESI or APCI–MS–MS. Observed matrix effects were dependent on all factors studied. From all above-mentioned studies three general conclusions may be drawn: the extracts should be of high quality, the chromatographic separation should not be neglected or sacrificed, and the matrix effects should be checked for all analyzed materials. Observation concerning matrix effect is valid only for the specimens and conditions involved.

The results of the present study show that the determination of CAP is not affected by the coextracted matrix components. Observed signal intensities of all monitored transitions were practically identical for all matrices and did not differ from the values for non-extracted drug (Fig. 5). Infusion experiments showed that in the elution range of CAP no compounds appeared which may affect the detection. Such compounds were visible in the first 3 min of chromatographic run. In the case of honey, diluted urine and urine extract, signal enhancement was observed for the early eluting compounds, whereas seafood extract showed mixed (enhancement and suppression) effects. In the case of chicken extract, practically no matrix effect was ob-

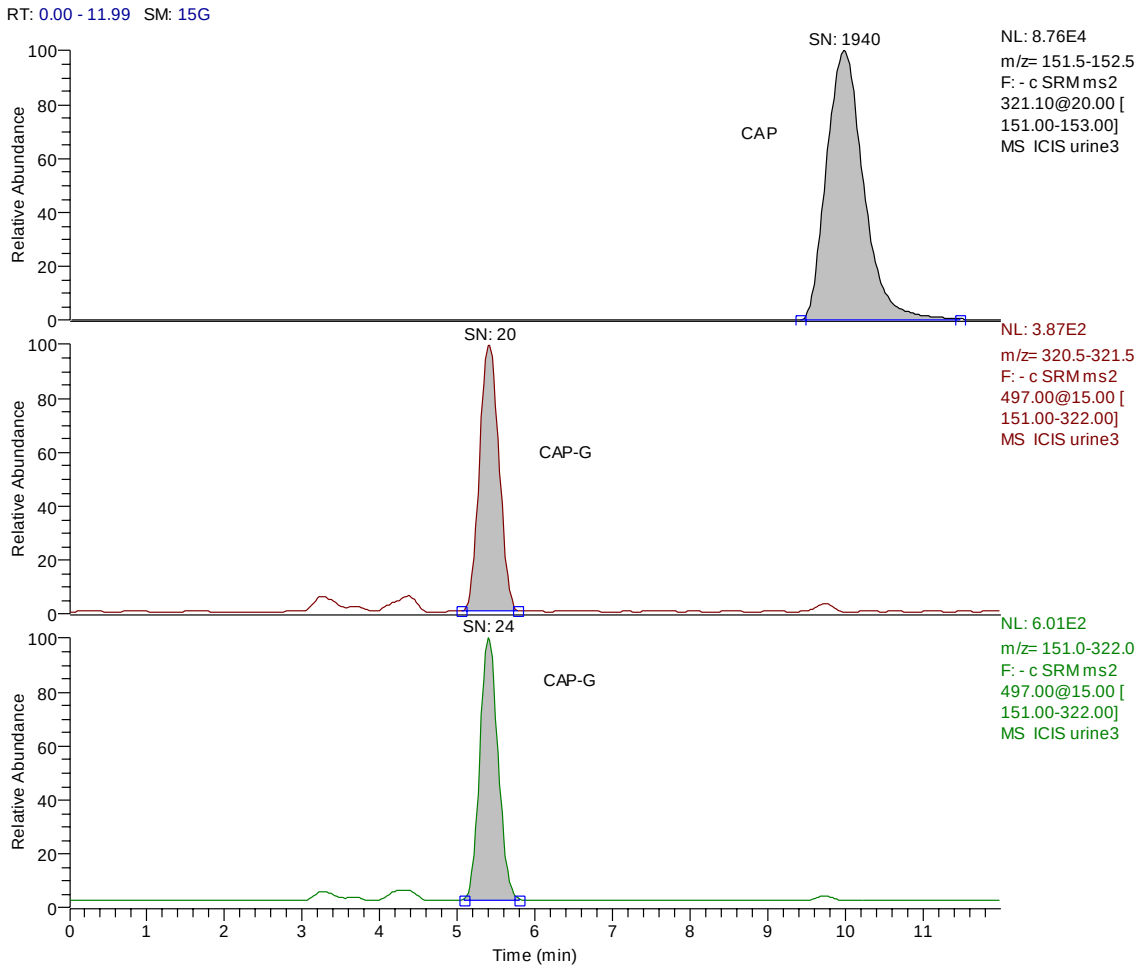


Fig. 8. Mass chromatogram of diluted 1:10 rat urine taken after administration of CAP. Peak at Rt 5.39 is a postulated CAP-G, peak at Rt 10.00 is a CAP.

Table 2
Results of validation of chloramphenicol determination in food samples

Material	LOD (ng/g)	LOQ (ng/g)	Linearity	Recovery
Chicken/seafood	0.1	0.2	$y = 2.12x - 0.17$ $r = 0.99551$	$46 \pm 9\%$ $n = 36$
Honey	0.05	0.1	$y = 1.99x + 0.01$ $r = 0.99821$	$63 \pm 4\%$ $n = 36$

served (Fig. 6). These findings confirmed the observation of other authors, that the matrix effects are specimen-specific. Interpretation of possible matrix influence on CAP-G detectability remains only in the sphere of speculation, since no experimental data were available without reference standard.

3.2.2. Validation of CAP determination in food samples

Tables 2 and 3 show the results of validation. The validation results for chicken meat and shrimps were presented together, since the samples were treated in the same way and the results obtained showed no differences. Generally, validation experiments showed that all three monitored ions were clearly visible from the level of 0.1 ng/g (Fig. 7).

Table 3
Day-to-day precision and accuracy ($n = 3$)

	Material added (ng/g)		
	0.5	2	10
Chicken			
Range	0.4–0.5	1.8–2.2	9.7–11
Mean ± R.S.D. (%)	0.43 ± 13	2.05 ± 7.3	10.46 ± 5.3
Recovery (%)	45	50	45
Honey			
Range	0.6, 0.4, 0.6	2.1, 2.2, 2.0	9.8, 9.9, 9.9
Mean ± R.S.D. (%)	0.53 ± 17	2.1 ± 3.9	9.87 ± 0.47
Recovery (%)	60	69	68

Day-to-day precision as well as accuracy was satisfactory at the whole concentration range tested. From our practice,

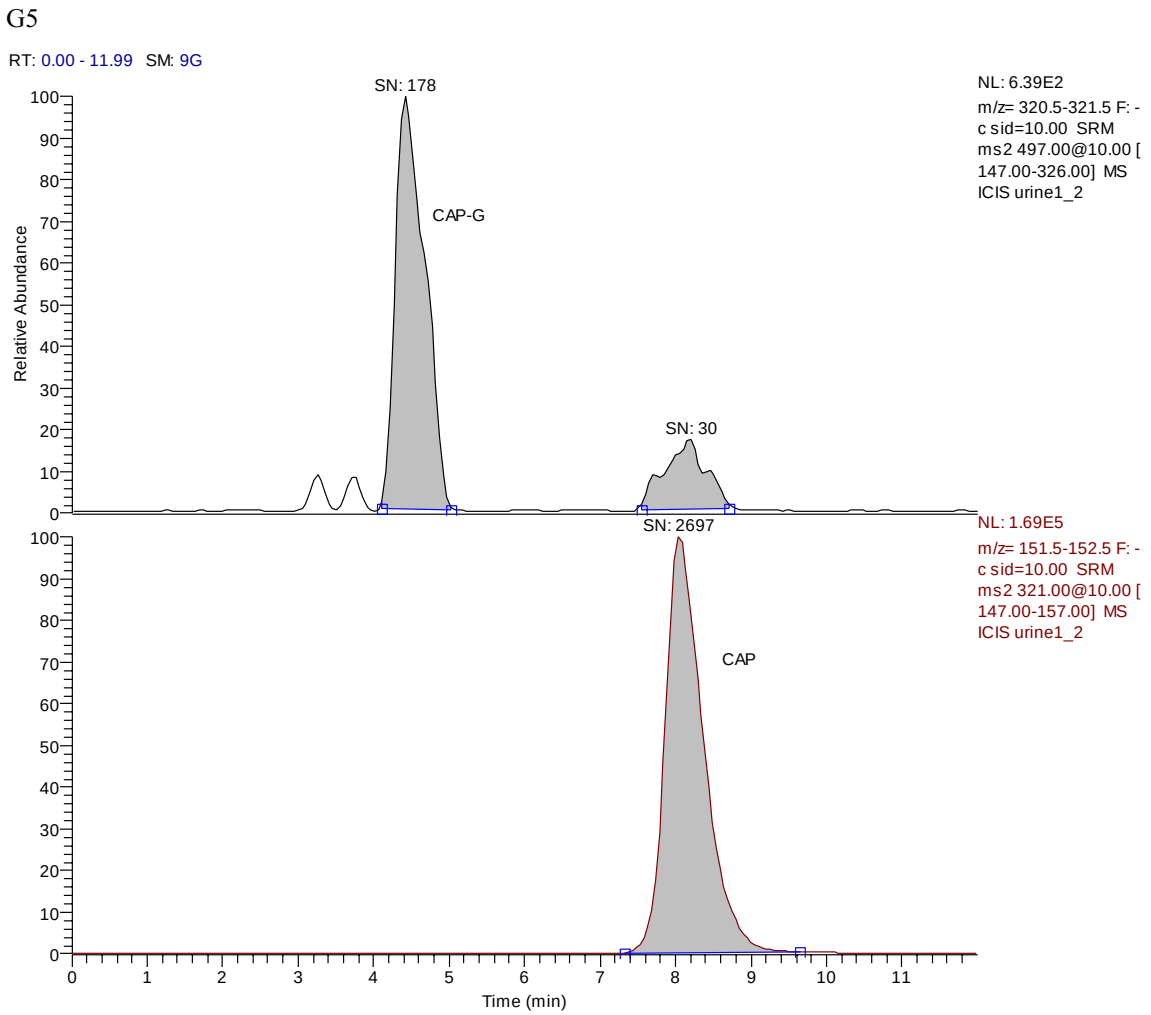


Fig. 9. Mass chromatogram of rat urine taken after administration of CAP and extracted with SPE.

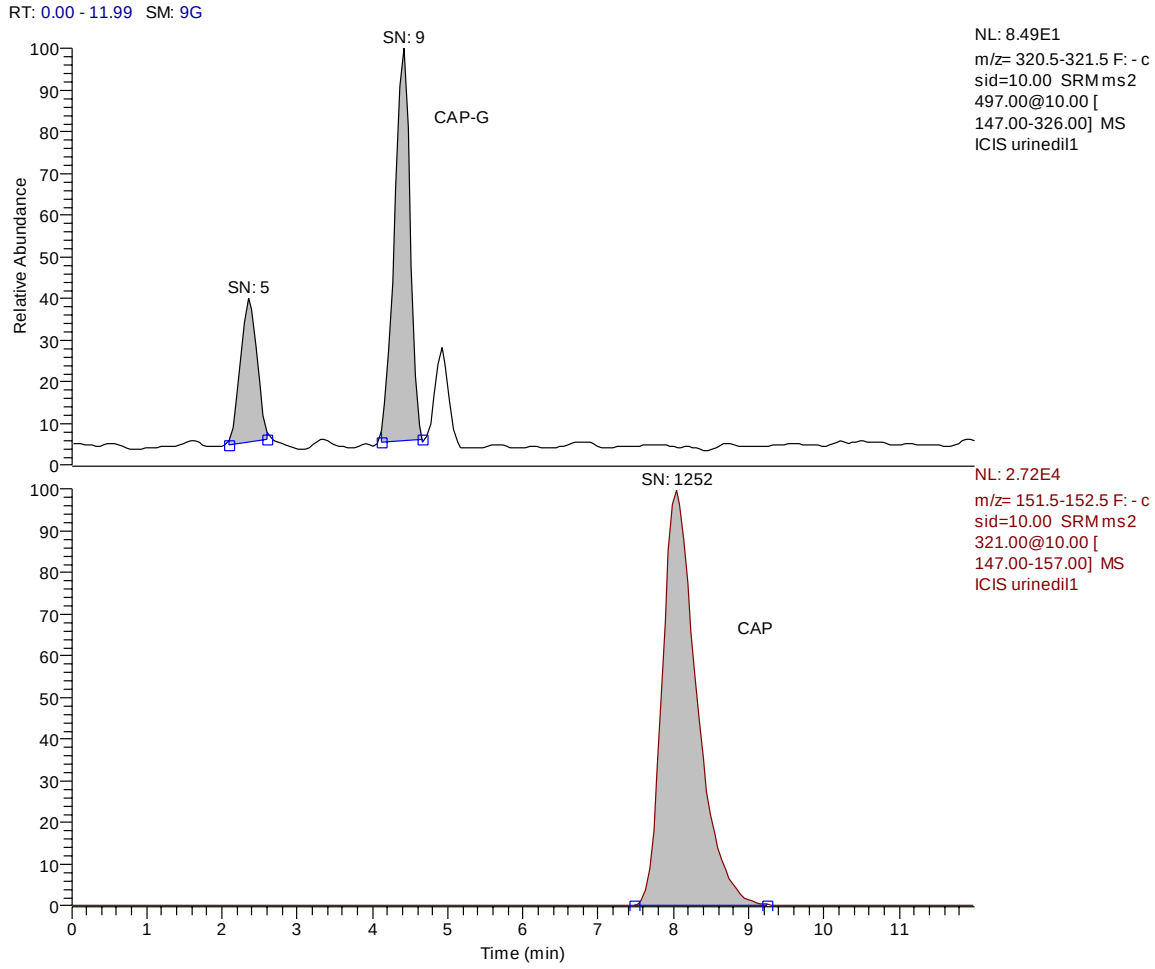


Fig. 10. Mass chromatogram of the same rat urine in Fig. 10, but only diluted 1:10 and injected directly into LC–MS.

this concentration range comprise practically all positive results.

Intensity ratios of fragment ions were measured from all analyzed 90 samples. Following mean values were found: for fragments 257/152, 0.79 ± 0.1, for fragments *m*/*z* 194/152, 0.31 ± 0.1. These ratios were not dependent on the concentration of CAP.

The mean retention time ratio CAP/CAP-D5 was 1.008 ± 0.003.

3.2.3. Validation of CAP-G determination

Mass chromatograms of diluted rat urine samples demonstrated in all three specimens the presence of two peaks; one identical with CAP, and the second one eluting earlier than CAP as a product of the transitions *m*/*z* 497 > 321 and 497 > 152. These transitions correspond to fragmentation of CAP-G (Fig. 8). After hydrolysis of urine samples with β-glucuronidase the peak of postulated CAP-G disappeared (Fig. 11). CAP-G and CAP were successfully isolated from rat urine specimens with SPE (Figs. 9 and 10). The recovery of CAP-G, calculated on the basis of comparison with direct urine injection, was $62 \pm 1.5\%$. This value, however, must be treated with caution, since no reference standard of CAP-G was available, and no matrix experiments for CAP-G was performed. CAP-G eluted much earlier than CAP and some interference of matrix compounds cannot be ruled out, particularly in diluted urine. Nevertheless, the study demonstrated that the method used allowed to detect CAP-G together with CAP after administration of CAP to the rat. The intensity ratios of transitions *m*/*z* 321 > 152 (CAP) to *m*/*z* 497 > 152 (CAP-G) were: 54, 130, and 190, for particular urine specimens. These results indicate that CAP was glucuronidated only in small fraction and mainly the unchanged drug was excreted with urine. It should be stressed, however, that these results are valid for rats and in other animals (e.g. shrimps) the metabolic rate may be different. In man about 48% of orally administered CAP is excreted as CAP-G [1].

3.2.4. Stability of CAP and CAP-G in frozen samples

The experiments demonstrated that CAP is stable in frozen chicken meat samples for at least 30 days. CAP and

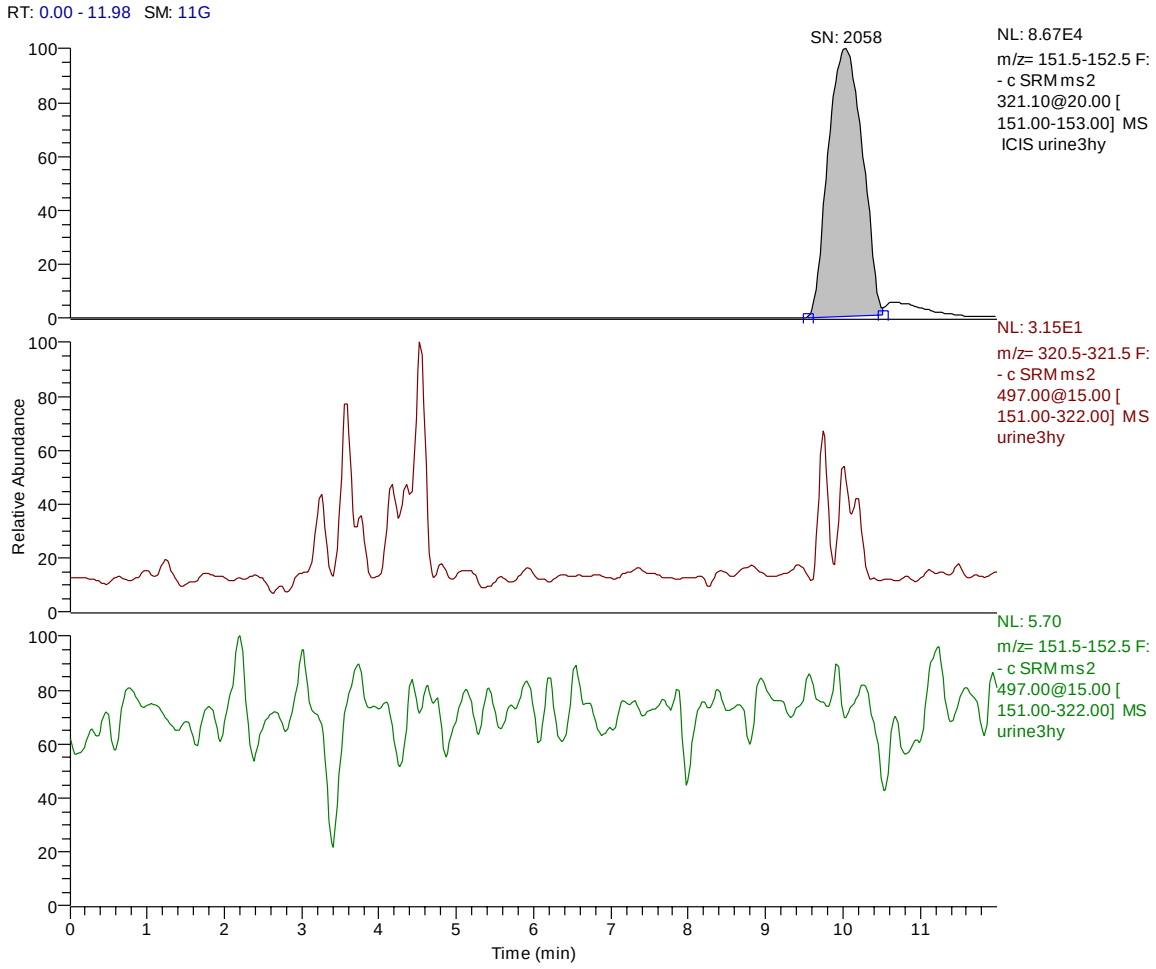


Fig. 11. Mass chromatogram of the same urine as in Fig. 8, but subjected to hydrolysis with β-glucuronidase. Only peak at Rt 10.04, corresponding to CAP, is visible.

CAP-G was stable in frozen urine samples for 60 days. It should be noted that the urine specimens were thawed as a whole and frozen again three times (at Days 10, 30, and 60) during this experiment. Meat samples were divided into small portions and thawed only once for particular experiment (Table 4).

Table 4
Stability of CAP and CAP-G in stored frozen samples, expressed as percentage of the starting value

	Material added (ng/g)	Day			
		0	7	30	60
Chicken CAP[a]	2	100	98	103	102
	10	100	106	98	99
Rat urine[b]		100	106	108	98

[a] For CAP in chicken meat, the concentration of CAP was measured.
[b] For CAP-G in rat urine, the ratio CAP-G/IS was measured.

3.3. Confirmation and quantitation of CAP

On the base of validation data, following criteria of positive finding of CAP were formulated:

- Rt within ±1% of deuterated IS.
- The presence of at three ions originating from CAP (152, used as quantifier, 194 or 257).
- The presence of ion originating from deuterated CAP (157).
- The intensity ratios of the fragment ions in the range ±2S.D. of the mean control values, i.e. 0.8 ± 0.2 for the ratio *m/z* 257/152, and 0.3 ± 0.2 for the ratio 194/152. These criteria are used in the current analytical practice. Fig. 12 presents mass chromatogram of honey sample, which contained CAP 3.1 ng/g.

From the present experience it may be stated that all positive immunoassay results, indicating CAP concentration higher than 1 ng/g, were confirmed with LC–MS. In the case when immunoassay result showed value below 0.2 ng/g, as a rule, no CAP was detected with LC–MS.

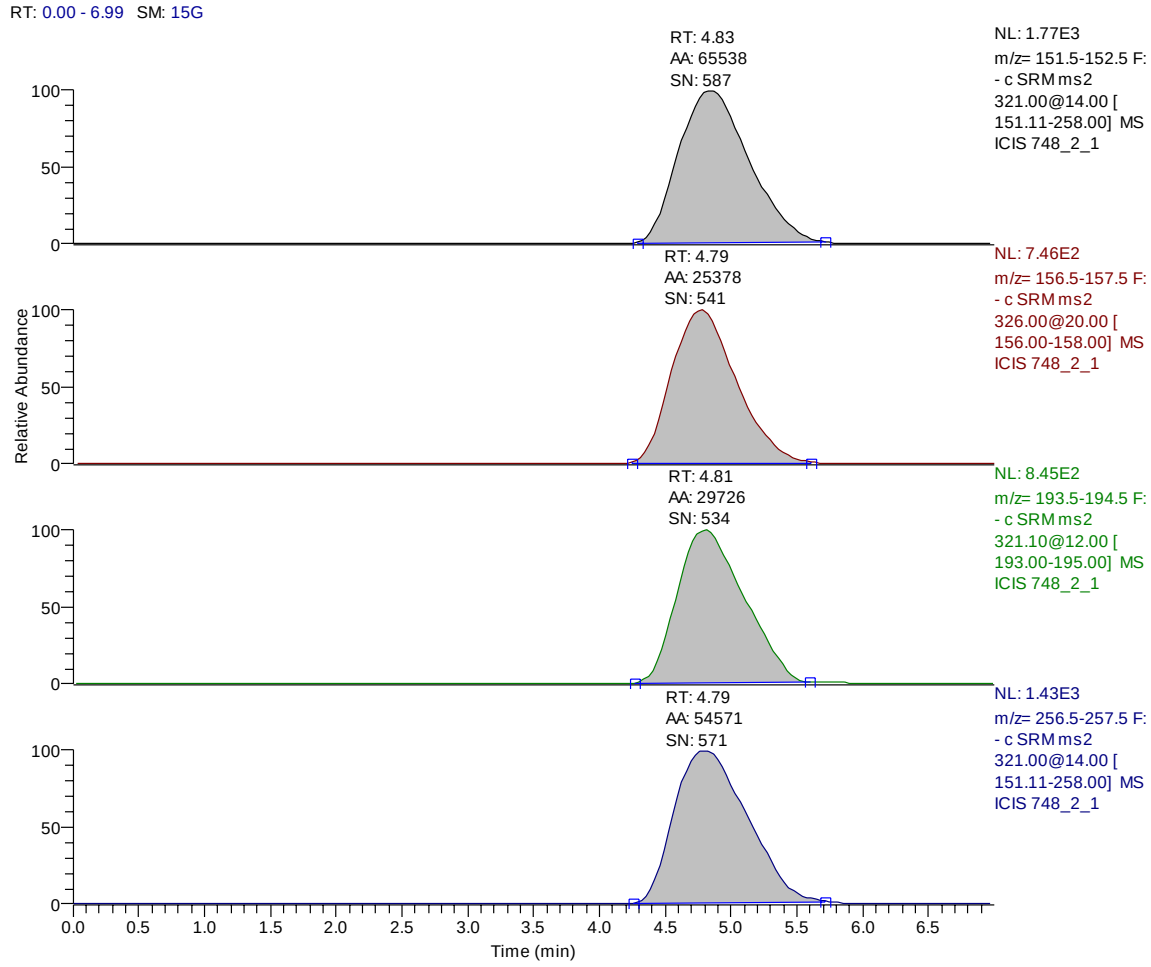


Fig. 12. Mass chromatogram of honey sample containing CAP 3.1 ng/g.

4. Conclusions

The method applied for determination of chloramphenicol in chicken, shrimp and honey is very simple and cost-effective and fulfils required sensitivity limit. It allows analyzing 20–30 samples per day. The preparation procedure for all materials may be fully automated. The method applied for honey may be also used for isolation of chloramphenicol glucuronide.

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