VALIDATION OF THE GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF CHLORAMPHENICOL RESIDUES IN MILK

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Abstract

An effective gas chromatographic method coupled with tandem mass spectrometric detection and identification was developed for the determination of chloramphenicol (CAP) residues in milk. After a preliminary dissolution in acetonitrile and homogenization, the obtained extract was evaporated almost to dryness and dissolved in water and the solution was cleaned up on octadecyl solid phase extraction cartridges. CAP was determined, using chemical ionization in the negative mode, followed by separation on the capillary column DB-1MS. The whole procedure was validated for the identification and determination purposes. Milk samples were spiked with CAP solution at levels corresponding to 0.15–0.3–1.0 µg/L. At the studied levels, trueness ranged between 98.7 and 102.0% and within-laboratory reproducibility expressed as relative standard deviation was lower than 15%. The decision limit was estimated at the level of 0.083 µg/L and detection capability was 0.14 µg/L.

Key words: chloramphenicol, residues, milk, confirmatory method, validation.

Chloramphenicol (CAP) is an effective antibiotic that has widely been used since the 1950s in veterinary medicine. However, the clinical use of CAP may results in undesirable side effects, primarily blood dyscrasias. Particularly in humans the most common adverse side effect is dose related suppression of the bone marrow activity resulted in erythropenia, thrombocytopenia or leukopenia. Allergic hypersensitivity reactions such as skin rash and fever are the least often observed side effects of CAP therapy. Because of above effects, the use of CAP for the treatment of food producing animals is restricted in many countries and is totally banned in the European Union countries. Therefore, it is necessary to control CAP residues in animal tissues as well as in milk (1, 9, 12).

The use of wide variety of chemical based analytical strategies for the determination of CAP residues has been recently reported in the literature (2, 6-9, 13, 17). They include liquid chromatography-mass spectrometry (3, 13, 14, 17) and capillary gas chromatography-mass spectrometry (GC-MS) (11, 16). A limitation with some of these reported approaches is that the level of quantification is not sufficiently low to obtain the minimum required performance limit of 0.3 µg/L for CAP residues in the food product of animal origin set by the European Commission (5).

For this reason it was necessary to develop and validate analytical strategies which are capable of quantitatively confirming the identity of low levels (< 0.3 µg/L) of CAP in milk. The method reported in this paper were originally developed in response to a requirement for the determination of CAP residues in milk. Validation of the confirmatory method was performed according EU criteria (4). This includes the determination of decision limit and detection capability and associated data such as uncertainty.

Material and Methods

Materials. Acetonitrile was from Merck. Chloramphenicol was obtained from Sigma Co. BSTFA N, N-bis(trimethylsilyl)trifluoroacetamide + 1% TMCS (trimethylchlorosilane) was from Fluka. Water, acetic acid, methanol and BakerBond octadecyl C18 columns were from J.T. Baker. Trichloracetic acid was obtained from Ubichem and sodium acetate was purchased from P.O.Ch. Gliwice.

Stock solution and standards. Stock solutions of 1 mg/ml were prepared in acetonitrile and stored in the dark at < -16°C. The CAP working solutions at the level of 0.01 µg/ml were prepared in methanol and stored in the dark at < 6°C, no longer than six months.

Gas chromatography-mass spectrometry. The gas chromatograph (Trace GC 2000 Finnigan) was coupled to mass spectrometer Finnigan Polaris. Gas carrier: helium and methane. Chromatographic separation was performed in capillary column DB-1MS (J&W Scientific) (30 m x 0.25 µm x 0.25 mm). The injection and transfer line were kept at 280°C. The gas chromatography oven was programmed from 110°C to 200°C at the rate of 20°C/min and subsequently to
241°C at 8°C/min and finally to 280°C at 20°C/min and stay for 5 min.

The Finnigan Polaris MS detector, operating in NCI MS/MS mode: m/z 466 - as precursor ion and ions m/z 304, 322, 358, 394, and 430 as transition products for chloramphenicol was used.

**Extraction of milk.** A 5 ml portion of milk sample was diluted with 20 ml of acetonitrile, homogenized and centrifuged at 3 500 g for 10 min at about 6°C. The top layer was taken and evaporated until dry using nitrogen and dissolved in 6 ml of water. The whole solution was cleaned up by solid phase extraction (SPE) technique.

**Cleanup.** SPE C-18 cartridges were preconditioned with 3 ml of methanol and 3 ml of water. After percolation of the whole solution, the column was washed with 6 ml of water and 3 ml of (20%) methanol, and dried under depression for 5 min. CAP was eluted with 3 ml of (60%) methanol. The eluate was diluted with 5 ml of water, the solution was mixed and passed through a new C18 SPE column and elution was performed with 3 ml of methanol.

**Derivatization.** The eluate was dried under gentle nitrogen stream at 45°C. The dry residue was dissolved in 300 µl of methanol and shaken vigorously with a vortex to retrieve the whole residue. Then the solution was transferred to a vial, dried under gentle nitrogen stream and derivatized with 20 µl of BSTFA containing 1% of TMCS. The vial was heated at 60°C for 1 h and analysed by GC-MS.

**Quantification of chloramphenicol.** The milk samples were spiked with chloramphenicol at the level of 0.15, 0.3, 1.0 ng/ml and processed through the extraction procedure described above. The external standard method was used for the quantitation of the obtained results. The recovery of chloramphenicol was evaluated by comparing the concentration found with standard solution. The precision of the method was measured using the same samples.

**Results**

The whole procedure was validated according to the quality standard ISO 17025 and 2002/657/EC Decision (4). Table 1 summarizes the data obtained from validation. A good linearity was obtained for calibration curves in matrix and a correlation coefficient was estimated. Repeatability was calculated from the analysis of six blank milk samples, spiked with CAP at each of three fortification levels (0.15, 0.3 and 1.0 µg/L) and performed by one operator on three separate occasions and these, three fortification levels were below 15%. The results for the decision limit (CCα) and the detection capability (CCβ) were calculated and recoveries were determined by comparing the peak areas of the milk samples spiked with corresponding amounts of CAP before and after extraction. The comparative chromatograms reported of extracts obtained from standard solution, blank milk samples, chloramphenicol-fortified milk samples highlighted the specificity and sensitivity of the presented method (Fig 1). Chromatogram of milk sample spiked with chloramphenicol at the level 0.3 µg/L is shown in Fig. 1b. The single peak in chromatogram indicates that there were no side products and no multiple derivatives.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
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<tbody>
<tr>
<td>Compound</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Linear regression equation (y = mx + b)</td>
<td>y = 12339.9x – 69.086</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9789</td>
</tr>
<tr>
<td>Linearity (working range), µg/L</td>
<td>0.15-2.0</td>
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<tr>
<td>Decision limit, µg/L</td>
<td>0.083</td>
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<tr>
<td>Detection capability, µg/L</td>
<td>0.14</td>
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<tr>
<td>Level of spiked samples milk, µg/L</td>
<td>0.15 0.3 1.0</td>
</tr>
<tr>
<td>Recovery, %</td>
<td>61.9 78.0 68.5</td>
</tr>
<tr>
<td>Repeatability</td>
<td></td>
</tr>
<tr>
<td>x, µg/L</td>
<td>0.093 0.237 0.685</td>
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<tr>
<td>x, s, µg/L</td>
<td>0.014 0.018 0.091</td>
</tr>
<tr>
<td>xii, RSD, %</td>
<td>15.0 7.8 13.4</td>
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<tr>
<td>Uncertainty combined (uc)</td>
<td>0.0056</td>
</tr>
<tr>
<td>Expanded (U)</td>
<td>2</td>
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<tr>
<td>Coverage factor (k)</td>
<td>0.15 ± 0.0011</td>
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</tbody>
</table>
Discussion

Several procedures for the determination of CAP residues in milk matrices have been published (3, 11, 17). Generally, organic solvents are used as extractant for quantitative procedures for CAP analysis, predominantly ethyl acetate which provided good recoveries, but many interfering compounds were co-isolated that did not allow CAP to be screened at low levels. Our procedure proposed an extraction of a milk sample with acetonitryle and SPE C18 with double C18 cartridges cleanup instead of the previously described ethyl acetate extraction (3, 11).

Beside detection with GC-MS in electron impact (EI) mode, CAP could also be determined using soft ionization techniques, i.e. negative chemical ionization (NCI), because of the Cl atoms in the molecular structure. The principal fragments of CAP-TMS for negative chemical ionization (NCI), explaining the specific mass spectrum, are shown in Fig. 2.
Fig. 2. Fragmentation of chloramphenicol in negative chemical ionization in MS/MS techniques.

The prepared GC-MS method considers the confirmatory purposes because of obtaining the following criteria: the ratio of the retention time of the analyte to that of the corresponding standard corresponded to that of the calibration solution within a ± 0.5% tolerance, all the selected ions – m/z 466 → 304, 466 → 322, 466 → 358, 466 → 394, 466 → 430 for CAP - should be present on the mass spectrum of the chromatographic peak; the relative abundance of these ions must correspond to that of the standard, with the acceptable deviations; the ions intensity must be, at least, three times greater than the base noise of the MS detector and the peak area ratios of the various transition reactions were within the tolerances set by the EU criteria (4). For quantification purposes, m/z 466 → 304 and 466 → 322 were chosen. The reproducibility of these ion ratios used for confirmatory purposes (m/z 466 → 304 and 466 → 322) was studied.

It was found that CAP residues could be efficiently extracted by acetonitrile. The mixture of BSTFA + TMCS (99:1) was selected as derivatization agents for GC-MS procedures. The comparative studies indicate that optimal conditions were obtained by derivatization at 60°C for 1 h. The validation study indicated that the developed method provide a reliable confirmatory strategy for the determination of CAP residues in milk matrices.

References


