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Matrix Solid-Phase Dispersion Extraction and Gas Chromatographic Determination of Chloramphenicol in Muscle Tissue

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A method based on matrix solid-phase dispersion (MSPD) was developed for the gas chromatographic (GC) determination of chloramphenicol (CAP) residues in animal muscle tissue. Muscle tissue was blended with octadecylsilyl-derivatized silica (C_{18}). A column made from the C_{18}/muscle tissue matrix was washed with *n*-hexane and acetonitrile/water (5 + 95), after which CAP was eluted with acetonitrile/water (50 + 50) and partitioned into ethyl acetate. The final extract was evaporated, and a trimethylsilyl derivative of CAP was prepared with Sylon HTP and detected by GC with an electron capture detector (ECD) and a mass spectrometer. For quantitation, the internal standard used was the meta isomer of CAP (m-CAP) for GC-ECD. Muscle tissue samples were fortified at three concentration levels. At 5, 10, and 15 μg/kg levels the respective mean recoveries were 93, 96, and 98%, and the repeatabilities were 13, 11, and 3%. The detection and quantitation limits with ECD were 1.6 and 4.0 μg/kg, respectively. No statistically significant difference was observed in the efficiency of CAP extraction from muscle tissue of various animals (bovine, porcine, and poultry) by the MSPD technique.

KEYWORDS: Chloramphenicol; gas chromatography; matrix solid-phase dispersion; muscle tissue; residues

INTRODUCTION

Chloramphenicol (CAP) is a broad-spectrum antibiotic that has been widely used in veterinary medicine for the treatment of various infections. Today, however, its use in the treatment of food-producing animals is prohibited in many countries because of its proven toxicity to humans (*1*). The use of CAP for therapeutic and prophylactic purposes may result in the presence of its residues in edible animal tissues, which might pose a risk to the consumer's health (*1*). Due to possible abuse, a permanent control of CAP levels in foodstuffs of animal origin is indispensable. The structure of CAP is shown in **Figure 1**.

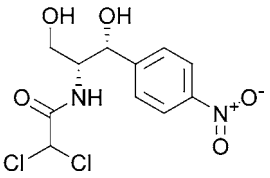

Figure 1. Chemical structure of CAP.

For the determination of CAP residues in foodstuffs of animal origin, various methods are used, based on different analytical techniques. The most commonly used methods for the determination of CAP in muscle tissue include gas chromatography (GC) (*2*−*6*) and liquid chromatography (LC) (*7*−*10*). In general, the use of LC facilitates the extract cleanup process, whereas lower detection levels are achieved by means of GC analysis with an electron capture detector (ECD) or a mass spectrometer (MS) (*4*). The procedures for CAP extraction from muscle tissue and those of extract cleanup reported in the literature are widely varied. Being rather time-consuming and complicated, they are impractical for testing a large number of samples within a reasonable period of time.

In this work, muscle tissue samples were prepared by means of the matrix solid-phase dispersion (MSPD) technique as first described by Barker et al. (*11*). The technique, which is very simple and fast, permits the simultaneous determination of a number of compounds in a single sample. In the past decade, MSPD has been increasingly applied for the preparation of different biological samples for the determination of residues of veterinary drugs and contaminants (*12*). The referent literature describes one application of this technique for the extraction of CAP residues from muscle tissue and their determination by LC with photodiode array detection (*13*). In the reported case, however, the CAP detection level was not low enough and CAP could not be detected in a 5 μg/kg spiked material.

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Our objective was to develop a method that would include the preparation of muscle tissue samples by the MSPD technique for the GC-ECD determination of CAP. The method was validated in terms of recovery, repeatability, and limits of detection and quantitation. Our aim was also to find whether the efficiency of CAP extraction by the MSPD technique depended on the type of matrix (muscle tissue of various animals) and to compare the results obtained by the MSPD technique to those obtained by the method previously used in this laboratory (*14*).

MATERIALS AND METHODS

Apparatus. Two chromatographic systems were used: (1) A gas chromatograph model ATI UNICAM 610 series was equipped with a splitless injector, a ^{63}Ni electron capture detector, and a fused silica capillary column DB-1, 30 m × 0.32 mm i.d., film thickness = 1 μm (J&W Scientific). The operating conditions were as follows: carrier gas, argon with 5% methane; splitless time, 1 min; injector temperature, 250 °C; detector temperature, 310 °C; column temperature, 120 °C, held for 1 min, raised to 280 °C at 20 °C/min, and held for 11 min; injection volume, 1 μL. (2) A gas chromatograph model ATI UNICAM AUTOMASS 615 GC SYSTEM 2 was equipped with a splitless injector, a quadrupole mass spectrometer, and a fused silica capillary column DB-1, 30 m × 0.25 mm i.d., film thickness = 0.25 μm (J&W Scientific). The operating conditions were as follows: carrier gas, helium; splitless time, 1 min; injector temperature, 250 °C; transfer line temperature, 280 °C; source temperature, 130 °C; column temperature, 120 °C, held for 1 min, raised to 280 °C at 20 °C/min, and held for 11 min; injection volume, 1 μL; electron ionization; ionization energy, 70 eV.

Reagents and Solutions. The trimethylsilyl derivatizing reagent Sylon HTP (hexamethyldisilazane/trimethylchlorosilane/pyridine, 3 + 1 + 9) was purchased from Supelco Inc. Chloramphenicol was from Sigma Chemical Co. Standard stock solution was prepared by dissolving 10.0 mg of CAP in 100 mL of methanol at 100 μg of CAP/mL. Standard spiking solution was prepared by diluting the stock solution with methanol at 0.2 μg of CAP/mL. The solution at 0.2 μg of CAP/mL was also used as the standard working solution for GC-MS identification of CAP. One hundred microliters of the solution was evaporated with nitrogen and derivatized as described under Derivatization. The internal standard working solution (m-CAP) at 1 μg of m-CAP/mL, dissolved in ethanol, was a gift from the Federal Institute for Health Protection of Consumers and Veterinary Medicine (Berlin, Germany). The reference material used was BCR-445, lyophilized porcine muscle tissue with the certified value of 8.9 μg of CAP/kg incurred material (Community Bureau of Reference).

Extraction Materials. Bulk C_{18} (octadecylsilyl derivatized silica, 40−63 μm) was purchased from Merck. The C_{18} was cleaned by sequentially washing a 50 mL column containing bulk C_{18} (20 g) with 2 column volumes each of *n*-hexane, dichloromethane, and methanol and then dried. Plastic syringe barrels of 10 mL, washed with hot soapy water, rinsed with demineralized water, and air-dried, were used as extraction columns.

Sample Preparation. *MSPD Method (Method A).* Muscle tissue was minced by means of a mixer, and 2 g of the sample was weighed into a glass grinding mortar. A 30 μL amount of internal standard working solution was added. For recovery experiments, 50, 100, or 150 μL of standard spiking solution was added to the sample to obtain three spiking levels (5, 10, and 15 μg of CAP/kg).

Method Used Thus Far (Method B). A 30 μL amount of the internal standard working solution was added to 3 g of minced muscle tissue. For recovery experiments, 75, 150, or 225 μL of standard spiking solution was added to obtain the identical spiking levels as with the MSPD method.

Extraction and Cleanup. *Method A.* A 3 g amount of C_{18} was added to the sample and blended by means of a glass pestle until a homogeneous mixture was obtained. Two filter paper disks were inserted at the bottom of the syringe, followed by 0.5 g of C_{18} and C_{18}/muscle tissue matrix blend, and covered with another filter paper disk. The column content was pushed with a syringe plunger toward the bottom, making sure not to compress it too much. The column was washed with 10 mL of *n*-hexane and 12 mL of acetonitrile/water (5 + 95). CAP was eluted with 10 mL of acetonitrile/water (50 + 50) into a tube. A 5 mL amount of water-saturated ethyl acetate was then added into the eluate and stirred with a vortex mixer for 1 min. The sample was centrifuged at 1700*g* for 10 min, and the top layer was separated for evaporation. The extraction with ethyl acetate was then repeated. The combined organic phase was subsequently evaporated on a rotary evaporator to a volume below 0.5 mL and dried up with nitrogen. A 0.5 mL amount of methanol was then added to the dry residue, and the sample was put in an ultrasonic bath for 5 min. The extract was transferred into a 1.5 mL tube and well dried under a gentle stream of nitrogen.

Method B. An 8 mL amount of acetonitrile/4% aqueous sodium chloride (50 + 50) was added to the sample and stirred thoroughly. After stirring, the sample was centrifuged at 4000*g* for 10 min. Five milliliters of *n*-hexane was then added to the separated supernatant, stirred thoroughly, and centrifuged at 1700*g* for 5 min. The top layer was removed, and extraction with *n*-hexane was repeated. After the separation of the top layer, 5 mL of water-saturated ethyl acetate was added to the lower aqueous phase and stirred thoroughly. After the centrifugation at 1700*g* for 5 min, the top layer was separated and extraction with ethyl acetate was repeated. The combined organic phases were evaporated to dryness, and the dry residue was dissolved in 3 mL of acetonitrile/water (5 + 95). The C_{18} column was washed with 5 mL of each methanol, chloroform, and methanol and with 10 mL of water. The extract was transferred in the column, and the column was washed with 5 mL of acetonitrile/water (5 + 95). CAP was eluted with 3 mL of acetonitrile/water (50 + 50). From the eluate CAP was extracted twice, each time with 5 mL of water-saturated ethyl acetate. After each extraction, it was centrifuged at 1700*g*, the top layer was separated, and the combined organic phases were evaporated to dryness. CAP was silylated directly in a tube after the evaporation of ethyl acetate, the reagent was evaporated, and subsequently 100 μL of hexane was added. CAP was determined by means of gas chromatography with a mass spectrometer as detector, and deuterated CAP was used as the internal standard (*14*). The course of the procedure following the last ethyl acetate evaporation was modified in our laboratory and was comparable to that of the MSPD method after ethyl acetate evaporation.

Derivatization. CAP was silylated by adding 50 μL of the silylating reagent to the dry residue and stirred on a vortex mixer for 10 s. A further 50 μL of *n*-hexane was then immediately added and stirred for further 10 s (if the extract turned turbid, it was centrifuged at 1700*g* for 5 min). A 1 μL amount of the extract was injected into the gas chromatograph.

Method Validation. The linearity of the method was evaluated by analysis of calibration graph samples. These were prepared by fortifying blank samples of bovine muscle tissue with CAP and m-CAP immediately after weighing. The 40, 50, 100, 150, and 200 μL amounts of standard spiking solution were added to the calibration graph samples prepared by the MSPD method and 30, 75, 150, 225, and 300 μL amounts of the same solution were added to those prepared by method B. For each point of the calibration graph six samples were prepared. The concentrations ranged from 4 to 20 μg of CAP/kg for the MSPD method and from 2 to 20 μg of CAP/kg of muscle tissue for method B. The peak area ratios between CAP and m-CAP (y) were plotted against the corresponding concentrations (x) by applying the least-squares method.

The accuracy of both methods was checked using the samples of muscle tissue to which a known CAP quantity had been added. The measured CAP levels were expressed as percentages of the CAP quantities added (recovery). Considering that the maximum residue limit (MRL) for muscle tissue in Croatia is 10 μg of CAP/kg (*15*), checks were made at three levels corresponding to 0.5, 1, and 1.5 times the MRL (5, 10, and 15 μg of CAP/kg of muscle tissue) with six samples for each level (*16*). For each level the mean value (X_m) and relative standard deviation (RSD) were calculated. The accuracy of the MSPD method was also checked on the basis of the analysis of four samples of the reconstituted reference material with the certified value of 8.9 μg of CAP/kg.

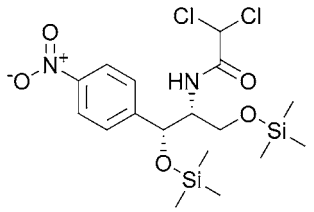

Figure 2. Chemical structure of disilylated trimethylsilyl-CAP derivative.

Table 1. CAP Recovery from Muscle Tissue Samples Fortified at Three Levels, Prepared by the Two Methods

CAP added, μg/kg	recovery %			
	method A		method B	
	X_m, %	RSD, %	X_m, %	RSD, %
5	100[a] (93[b])	10[a] (13[b])	99[a]	4[a]
10	96[a]	11[a]	104[a]	3[a]
15	98[a]	3[a]	105[a]	3[a]

[a] Bovine muscle tissue ($n = 6$). [b] Bovine, porcine, and chicken muscle tissues ($n = 18$).

The repeatability of the method was assessed using the calculated RSD values.

To determine the limit of detection (LOD), 20 muscle tissue samples with a CAP content below the LOD were analyzed. The samples were prepared by both methods. The detection limit was calculated as the apparent content corresponding to the value of the mean plus 3 times the standard deviation for 20 blank determinations (*17*), and the calibration line was based on the peak height ratio of CAP and m-CAP.

The limit of quantitation (LOQ) was determined as the smallest measured content of the analyte in a sample that could be quantified with a specified degree of accuracy and precision (*16*). LOQ corresponded to the lowest concentration point on the calibration graph.

CAP Identification. In samples prepared by the MSPD method CAP was identified by means of a mass spectrometer after GC separation. Electron ionization was applied, and the samples were tested for the presence of disilylated trimethylsilyl-CAP-characteristic ions (*m*/*z* 208, 225, and 242) (*2*). The analysis included the standard working solution of CAP and the extract of a bovine muscle sample prepared by the MSPD method, to which 10 μg of CAP/kg had been added. The structure of disilylated trimethylsilyl-CAP is shown in **Figure 2**.

RESULTS

Linearity. The calibration graphs for CAP determination by the two methods were linear over the concentration ranges tested (4−20 and 2−20 μg of CAP/kg, respectively). CAP determination by the MSPD method followed the equation $y = -0.020 + 0.003x$ (correlation coefficient $r = 0.9992$) and by method B $y = 0.026 + 0.003x$ ($r = 0.9975$).

Accuracy. The results of accuracy testing are presented in **Table 1**. **Figure 3** shows the chromatograms of the bovine muscle tissue samples prepared by the MSPD method without any CAP added and with 15 μg of CAP/kg added.

The accuracy of the MSPD method was also evaluated by analyzing the certified reference material. The measured CAP content was expressed as a percentage of the certified value. The mean value was 108%, with an RSD of 3%.

The relationship between different matrices (muscle tissues of different animals) and the efficiency of CAP extraction by the MSPD technique was examined on samples of chicken and pork tissues (six samples of each tissue) to which 5 μg of CAP/kg was added. CAP recovery was 90%, with an RSD of 15%, for the chicken tissue and 90%, with an RSD of 13%, for pork. It was established that there was no significant difference in the efficiency of CAP extraction from muscle tissue samples

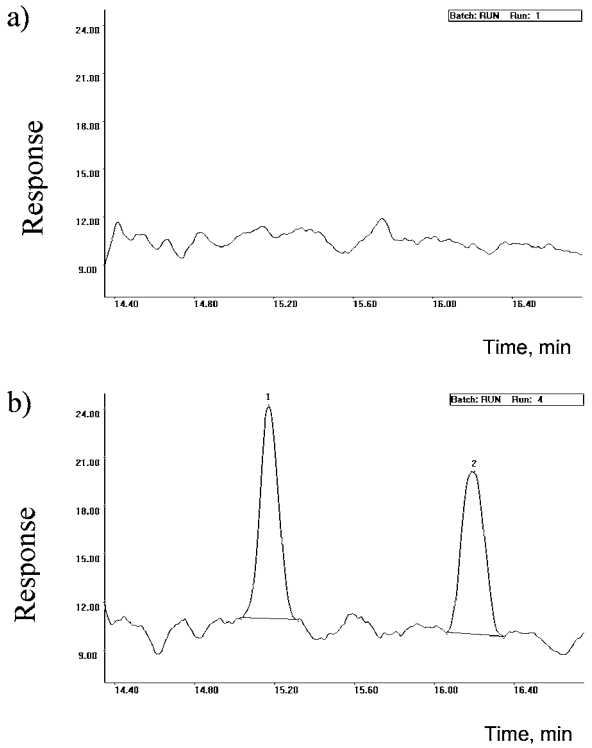

Figure 3. GC-ECD chromatograms of muscle tissue samples prepared by the MSPD method: (a) blank bovine muscle tissue; (b) bovine muscle tissue fortified with CAP at 15 μg/kg. Peak 1, m-CAP; peak 2, CAP.

taken from different animals at the level of 5 μg/kg (Student's *t* test, $P > 0.05$). On the basis of this observation, 93% recovery was calculated as the mean value for bovine, porcine, and chicken muscles at the level of 5 μg of CAP/kg (**Table 1**).

Comparison of the two methods failed to demonstrate any significant difference in terms of the efficiency of CAP extraction from bovine muscle tissue at levels of 5 and 10 μg of CAP/kg (Student's *t* test, $P > 0.05$).

Repeatability. Repeatability results are presented in **Table 1** (RSD values). Considering that no statistically significant difference was noted in the efficiency of the MSPD method as a function of different matrices, the repeatability of 13% at the level of 5 μg of CAP/kg was calculated from the results for bovine, porcine, and chicken muscle tissues (**Table 1**).

Detection Limit. The CAP detection limits were 1.6 μg of CAP/kg for the MSPD method and 0.7 μg of CAP/kg, for method B.

Quantitation Limit. The quantitation limits for CAP were set at 4 and 2 μg of CAP/kg levels for the MSPD method and method B, respectively. They were determined by analysis of spiked tissue samples ($n = 6$) at concentration levels of 4 μg of CAP/kg for the MSPD method and 2 μg of CAP/kg for method B. The respective CAP recoveries were 103 and 99%, with an RSD of 5%.

CAP Identification. The peak detected in the sample extract had the same retention time as the peak detected in the standard CAP working solution with identical characteristic ions (**Figure 4**).

DISCUSSION

Extraction of drug residues from a tissue is a demanding task because it involves a very complex biological matrix. Conventional extraction and extract cleanup techniques are usually time-consuming and complicated. Besides, they require large volumes

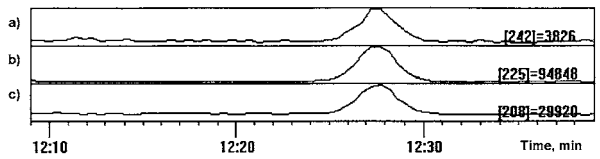

Figure 4. Selected ion chromatograms of a bovine muscle tissue sample fortified with CAP at 10 μg/kg and prepared by the MSPD technique: (a) *m/z* 242; (b) *m/z* 225; (c) *m/z* 208.

of solvents and make the preparation of a large number of samples within a reasonable time impractical.

Most methods for GC determination of CAP in muscle tissue include several steps (*2*−*6*). Tissue homogenization is followed by the addition of a suitable solvent for extraction. After centrifugation, the extract is separated and a defatting solvent is added. CAP is then re-extracted from the extract. Further cleanup is usually performed either by means of liquid−liquid extraction or by solid-phase extraction. The final extract is evaporated and CAP is silylated. Acetonitrile (*2*, *4*, *5*) and ethyl acetate (*3*) are most commonly used for CAP extraction from muscle tissue. Lipids are usually removed with *n*-hexane (*4*, *5*). Solid-phase extraction (*4*) generally serves for the extract cleanup. Some authors combine the extract cleanup on a silica gel column with LC purification (*3*) or cleaning by immunoaffinity chromatography (*5*).

The method used in our laboratory so far has been rather time-consuming and complicated to perform because it involves six liquid−liquid extractions and seven cetrifugation and extract separation cycles. Our aim was therefore to facilitate sample preparation by applying the MSPD technique.

The mechanisms included in the MSPD technique make it possible for sample homogenization, destruction of cellular structure, extraction, and cleanup to be carried out in a single procedure. Further extract cleanup prior to the analyte determination may be performed but need not be necessary (*11*).

In MSPD procedures, the proportion of tissue and solid phase is usually 1:4 (0.5 g of tissue and 2 g of solid phase), but in our case it was slightly modified (2 g of tissue and 3 g of solid phase) because of the small quantities of CAP that had to be detected while maintaining the quantities that are acceptable for this particular technique (*11*). The interfering compounds were removed by the column washing with *n*-hexane and an aqueous solution of acetonitrile. After the evaporation of the final extract, CAP was silylated before being injected in the gas chromatograph. The reagent used for silylation was hexamethyldisilazane/trimethylchlorosilane/pyridine (3 + 1 + 9). The silylation reagent is usually removed (wholly or partly) by evaporation before the final solvent is added (*4*, *5*). However, the silylated CAP may be lost (*18*). We have omitted this step because a loss of silylated CAP was noted, which seriously affected the sensitivity of the method.

The efficiency of CAP extraction (**Table 1**) by both methods, at all three levels, was in accordance with the criteria as defined in the European Communities recommendations (*16*). According to the criteria, the recoveries at those levels should range from 70 to 110%. Our results show the accuracy and repeatability of the MSPD method to be fully comparable to those of the highly efficient methods described by Hummert et al. (*7*) and Delepine et al. (*9*).

The detection and quantitation limits for CAP determined by the MSPD method (1.6 and 4 μg of CAP/kg) were higher than those by the method used so far (0.7 and 2 μg of CAP/kg). However, considering that the MRL for animal muscle tissue in Croatia is 10 μg of CAP/kg, the MSPD method is considered to be acceptable for the determination of CAP residues in muscle tissue. The detection limit of a method is subject to a number of factors, such as sample quantity, efficiency of the extraction technique, final volume of the extract, quantity of the extract injected in the instrument, sensitivity, and certain specific features of the detector unit used, as well as the presence of interfering compounds (*19*). Besides, methods for calculating LOD values have not been standardized. Therefore, comparison of the results published in the literature is hardly possible as most of the factors influencing the LOD differ from one paper to another (*5*, *7*, *9*, *13*).

The GC-MS identification of CAP confirms the suitability of the MSPD method for the determination of CAP in muscle tissue and the sensitivity of the mass spectrometer as a gas chromatographic detector for the identification of CAP at the level of 10 μg of CAP/kg.

Comparative results show that the MSPD method facilitates selective and efficient extraction of CAP from animal muscle tissue. Being fast and easy to use, this method permits analysis of a larger number of samples (seven samples per sequence compared to four by the former method) using a small quantity of solvents.

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