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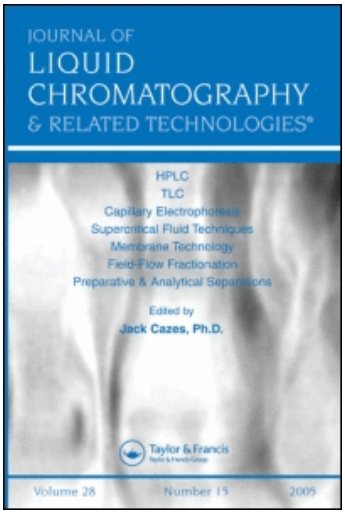

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Chloramphenicol Residues in Food Samples: Their Analysis and Stability During Storage

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Chloramphenicol Residues in Food Samples: Their Analysis and Stability During Storage

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ABSTRACT

In this study, two methods for the determination of chloramphenicol (CAP) residues in chicken muscle and milk, are proposed. Chromatographic determination was carried out by reversed phase HPLC with a UV photodiode array detector. Confirmation of CAP was performed by comparison of UV spectra. Limit of detection was $0.4\,ng\,g^{-1}$ in milk and $2\,ng\,g^{-1}$ in muscle samples. The methods were validated and the mean recovery was 78.9% with CV of 9.36% for milk samples, and 86.8% with CV of 7.87% for chicken muscle. Furthermore, the effects of different storage temperatures on the stability of CAP were tested. The results showed a high stability in muscle during refrigeration and freezing storage conditions, but milk freezing produced significant losses of CAP.

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Key Words: Chloramphenicol; Residues; Stability.

INTRODUCTION

The use of veterinary drugs with food producing animals is an accepted and established practice in developed countries. Inherent to this use, however, is the problem of the residues, which may remain in their tissues and in the excreta and, more specifically, in milk.

Hence, the advantage of a pharmacologic intervention must be weighed against the disadvantage of the appearance of residues in edible products and their risk for the consumer. The two main risks relate to hypersensitivity reactions and to resistance acquisition.

Chloramphenicol (CAP) is a broad spectrum antibiotic used against bacterial infections in chicken, pigs, calves, and cows. For some years, it has been known that CAP can cause serious health problems in man. Chloramphenicol exerts adverse effects on the bone marrow, being responsible for pancytopenia in man.

Thus, since 1994, the use of CAP is totally banned within the European Union.[1] In order to effectively monitor the occurrence of residues, specific and sensitive methods are required.

For screening purposes, enzyme-linked immunosorbent assays (ELISAs) are very suitable at low $\mu g\,kg^{-1}$ level, but confirmation at these low concentration levels, seems to be a major problem.

For quantitation of CAP, high performance liquid chromatographic methods have been reported,[2] using solid phase extraction or antibody-mediated clean-up (AMC) as sample pretreatment. Gas chromatographic analysis can be performed only after derivatization of CAP and often a high sensibility ($<5\,\mu g\,kg^{-1}$) can be achieved.[3]

Confirmation methods for positive samples are necessary because of the possibility of interfering matrix compounds that may produce false positive results. Diode Array UV–VIS detection, as a confirmation technique for the presence of CAP, may be used since its ultraviolet absorption is suitable for direct determination without derivatization.

Few studies on the effect of storage of CAP residues have been published. In the majority of these studies, CAP degradation was examined in muscle, liver, and kidney,[4] but not in milk.

It is well known that CAP is rapidly metabolized by oxidation, catalyzed by the cytochrome P-450 present in liver, kidney, and also in muscle. For this reason, metabolism of CAP might occur "in vitro" in tissues removed from the animal. Addition of piperonyl butoxide was suggested by some authors[5] to inhibit cytochrome P-450 activity and prevent metabolism taking place during sample preparation stages.

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The purpose of this study is to investigate the stability of CAP in milk and chicken muscle in the function of time and temperature of storage, using HPLC with Diode Array detection as a confirmatory technique.

EXPERIMENTAL

Material and Reagents

Water was purified by demineralization (MilliQ, Millipore); sodium acetate, sodium chloride from Panreac. (Montplet and Esteban, Barcelona, Spain.); ethylacetate (for organic trace analysis), acetonitrile and methanol (HPLC grade), acetic acid (for analysis), dichloromethane (for residue analysis), *n*-hexane (for organic trace analysis), chloroform (for residue analysis), anhydrous sodium hydrogen phosphate and anhydrous sodium sulfate from Merck (Darmstadt, Germany). Standard chloramphenicol was purchased from Fluka (Buchs, Switzerland). Silica and C18 Sep Pak cartridges of Waters (Milford, USA) were used.

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Preparation of Standard Solutions

A CAP standard solution was prepared by dissolving 100 mg of CAP in 100 mL of methanol. Working standard solutions for HPLC were prepared in the range of 100–600 ng mL^{-1} by diluting the standard solution with the mobile phase.

Apparatus

The HPLC equipment consisted of a 515 pump (Waters, Milford, USA), a 717 autosampler (Waters, Milford, USA), and a 990 Photodiode Array detector (Waters, Milford, USA).

The separation was performed on a 4μ NovaPak C18, column (100 × 8 mm I.D., Waters, Milford, USA) with 0.01 M acetate buffer pH 4.5—acetonitrile (70 : 30 v/v) as mobile phase. The chromatograph was operating at room temperature.

The other instruments used were an ultrasonic bath (Selecta, Madrid, Spain), a pH-meter (Beckman, Fullerton, USA), a Macrotonic centrifuge (Selecta, Madrid, Spain), a rotatory evaporator (Büchi, Flawil, Switzerland), an Ultraturrax T25 (IKA, Jankel-Kunkel, Staufen, Germany), and a sample concentrator with nitrogen stream supplier (Techne, Ltd, Oxford, Cambridge, UK).

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Milk Sample Preparation and Storage Conditions

During the whole stability study, amber glass was used for the storage of the milk samples. Samples were prepared as follows:

An 1.5 L pool of different whole milk samples was prepared.

To an 800 mL aliquot of this pool, 6000 ng of CAP (10 mL of a standard solution of 0.6 $\mu g\,mL^{-1}$) were added to obtain a concentration of 7.5 $ng\,mL^{-1}$ of CAP. This spiked sample was ultrasonicated during 20 min to assure its total homogenization and divided into 40 samples of 20 mL each.

The rest of the milk pool was stored as 20 mL aliquots of blank samples, which were used for the recover control in every stability assay time-point.

Samples were analyzed during storage at refrigeration temperature ($4^{\circ}C \pm 2^{\circ}C$) for a period of 11 days and for 1 and 6 months under frozen conditions at $-18^{\circ}C \pm 5^{\circ}C$.

For stability studies during both frozen and refrigeration storage, five samples were analysed on each time-point: three spiked samples (7.5 $ng\,mL^{-1}$) and two blank samples (20 mL), each of them with the addition of 300 μL of a 0.3 $ng\,mL^{-1}$ CAP standard solution (4.5 $ng\,g^{-1}$) on the day of assay, for the control of the recovery value.

On day 0 the assay was performed on a non-frozen milk sample.

Chicken Muscle Sample Preparation and Storage Conditions

Polypropylene tubes of 50 mL with screw caps were used for storage of muscle samples during the stability study. Samples were prepared as follows:

A thoroughly homogenized pool (about 500 g) of different chicken muscle samples was prepared and aliquots of 10 g were weighed in 50 different polypropylene tubes.

Stability was studied at a level of 15 $ng\,g^{-1}$. To each of 30 tubes 150 ng of CAP (500 μL of a standard of CAP solution of 0.3 $\mu g\,mL^{-1}$) were added by means of injection with a microsyringe into each of the minced meat portions, which then were thoroughly mixed.

The other 20 tubes were stored as blank samples, which were used for the recovery control at each time-point.

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Samples were analyzed during storage at refrigeration temperature ($4^{\circ}C \pm 2^{\circ}C$) for a period of 8 days and after 1, 2, and 3 months under frozen conditions at $-18^{\circ}C \pm 5^{\circ}C$.

For stability studies during both frozen and refrigeration storage, five samples were analyzed on each time-point: three spiked samples ($15\,ng\,g^{-1}$) and two blank samples, each of them with the addition of 200 ng of CAP ($20\,ng\,g^{-1}$) on the day of assay for the control of the recovery value.

On day 0 the assay was performed on non-frozen chicken muscle.

Preparation of Glassware

Contamination of reagents or instrumentation has to be strictly avoided and, therefore, glassware is cleaned thoroughly previous to analysis. Eluents are prepared freshly and the analytical column is used only for CAP analysis.

Extraction and Clean-up of Milk Samples

Twenty milliliter of homogeneous milk was brought to pH 7.0 ± 2 with 2 mL of 0.3 M phosphate buffer (pH 10). A C18 Sep Pak cartridge (Waters) was washed respectively with 5 mL of methanol and 5 mL of water. The sample extract was gently pressed through the cartridge with a disposable syringe and the cartridge was washed with 5 mL of water and 5 mL of acetonitrile in water (5%).

Chloramphenicol was eluted from the cartridge with 10 mL of acetonitrile in water (30%). Then, 2 mL of ethylacetate was added to the eluate and the mixture was shaken. The upper layer was transferred to a clean tube and the extraction with ethylacetate was repeated twice. The combined organic phases were evaporated till dryness in a sample concentrator at 50°C under a gentle stream of nitrogen. The dry residue was dissolved in 0.5 mL of water and 0.8 mL of a mixture of hexane/chloroform (1 : 1, v/v) was added. After stirring for 12 s, the mixture was centrifuged during 5 min. An aliquot of the supernatant was injected into the chromatographic system. A flow diagram is presented in Fig. 1(A).

Extraction and Clean-up of Chicken Muscle

Muscle tissue was prepared, removing visible fat as far as possible, and homogenized in a Sorval Omnimixer. A 10 g sample was weighed into a centrifuge tube and mixed with 30 g of anhydrous sodium sulfate and 30 mL of ethyl acetate during 1 min in a Omni-Mixer.

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The mixture was centrifuged during 10 min at 4000 rpm and the upper organic layer was transferred to a round bottom flask. The remaining aqueous phase was extracted once more with 30 mL of ethyl acetate.

The combined organic phases were evaporated till dryness under a stream of nitrogen at 50°C and taken up three times in 5 mL of dichloromethane.

A Silica Sep Pak cartridge (Waters) was washed, respectively, with 5 mL of acetonitrile in water (20%), 5 mL of acetonitrile, and 5 mL of

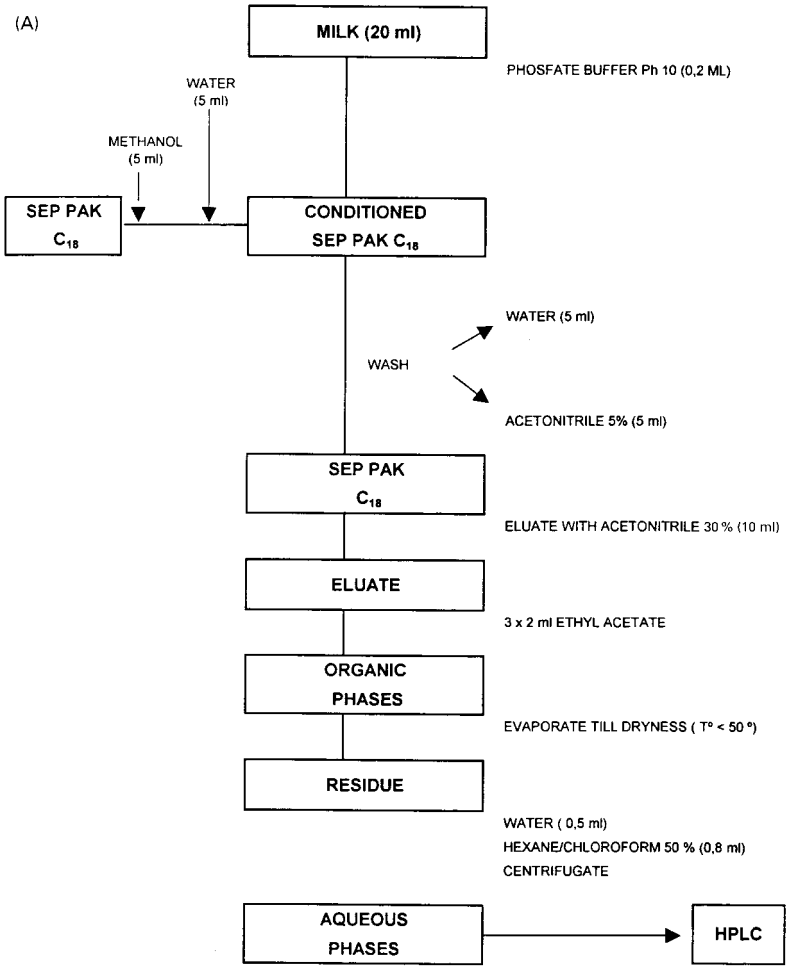

Figure 1. Flow diagram of the determination of CAP in (A) milk, and (B) chicken muscle.

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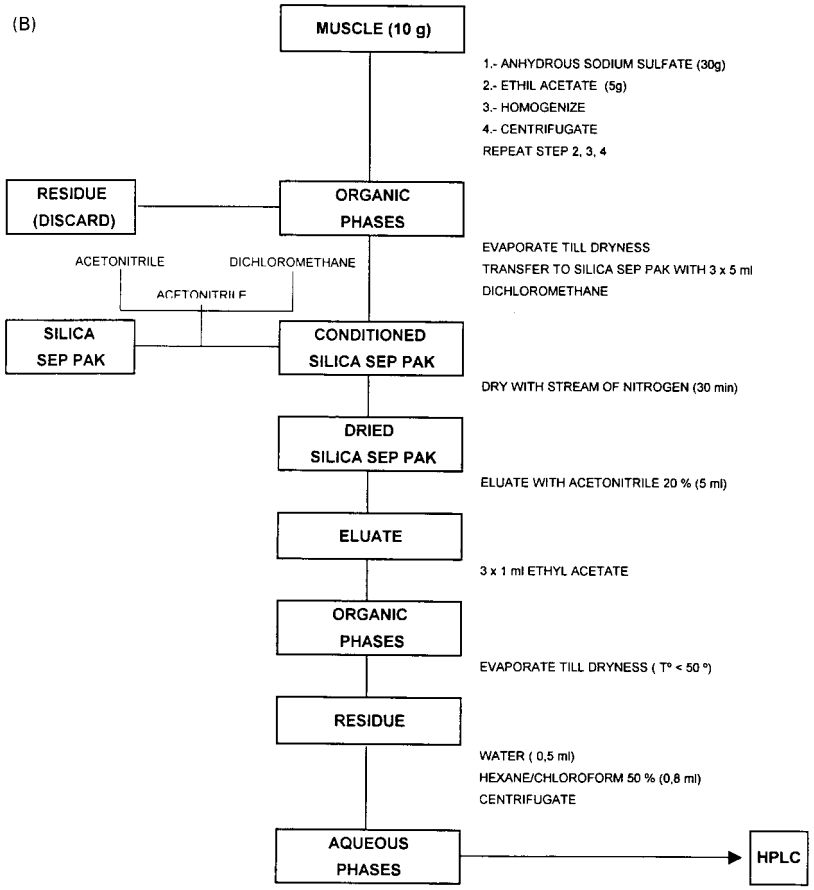

Figure 1. Continued.

dichloromethane, and then dried by forcing, during 30 min, a gentle stream of nitrogen through the cartridge. The sample extract (15 mL) was gently pressed through the cartridge with a disposable syringe and the cartridge was washed with two 5 mL portions of dichloromethane.

After drying with a stream of nitrogen (about 30 min), CAP was eluted from the cartridge with acetonitrile in water (20%). Then, 1 mL of ethyl acetate was added to the eluate and the mixture was shaken.

The upper layer was transferred to a clean tube and the extraction with ethyl acetate was repeated twice. The combined organic phases were evaporated till dryness in a sample concentrator at 50°C under a gentle stream of

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nitrogen. The dry residue was dissolved in 1 mL of water and 1.6 mL of a mixture hexane/chloroform (1 : 1 v/v) was added. After stirring for 15 s, the mixture was centrifuged during 5 min for layer separation. Chloramphenicol was determined by injecting 50 µL of the upper aqueous phase in the chromatographic system. A flow diagram is presented as Fig. 1(B).

Chromatographic Conditions

Analysis of CAP was carried out in the chromatographic conditions as described under apparatus. For quantitative purposes, monitoring was performed at 278 nm, while wavelenght range was set on 220 till 360 nm, permitting spectrum comparison of the CAP peak of the sample with that of the standard.

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Validation of the Analytical Methods

The precision of the proposed methods was determined by analysis of spiked samples in the range of 2.2–15 $\mu g\,kg^{-1}$ for milk and 10–60 $\mu g\,kg^{-1}$ for chicken muscle. This parameter was evaluated using at least three known concentrations of analyte and expressed as coefficient of variation.

Linearity of the UV detector response for CAP was determined from a set of three working standards over the whole range of sample concentrations.

Chloramphenicol Residue Stability

Samples were analyzed using the validated analytical procedures. Concentrations of CAP, during storage at different temperatures and time, were measured and corrected for the recovery factor of CAP of the same day.

RESULTS AND DISCUSSION

Chromatography and Selectivity

Well-defined chromatographic peaks of CAP can be obtained on a 4µ NovaPak C18 (100 × 8 mm I.D.) column [Fig. 2(A)]. Representative chromatograms of drug-free milk and chicken muscle are shown in Fig. 2(B), (C), respectively. These chromatograms indicated that no endogenous compounds interferred at the CAP retention time. None of the milk and chicken samples

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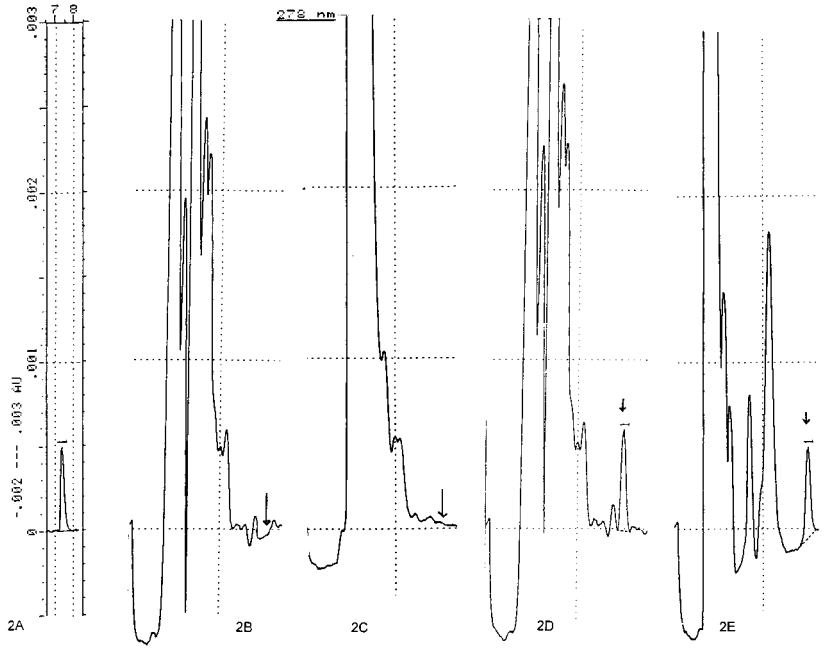

Figure 2. (A) Chromatogram of CAP standard (5 ng injected amount). (B) Chromatogram of blank milk sample. (C) Chromatogram of blank chicken muscle sample. (D) Chromatogram of milk sample spiked with 4.5 ng g^{-1} CAP. (E) Chromatogram of chicken muscle sample spiked with 15 ng g^{-1} CAP.

used in the validation process or in the stability study showed any interferences at the retention time of CAP.

Representative chromatograms of milk and chicken muscle spiked at 4.5 and 15 μg kg^{-1}, respectively, are shown in Fig. 2(D), (E).

Chloramphenicol has proved to be quantified and has been confirmed in muscle and milk at levels of 10 and 2.2 μg kg^{-1}, respectively. Detection limits are defined as 3 × Señal/Noise in blank sample are, as low as 2 μg kg^{-1} in muscle and 0.4 μg kg^{-1} in milk samples.

Calibration

Calibration curves were linear, with a CV of $\leq 5\%$ of the mean response factor, defined as response/ng injected CAP, and correlation coefficients (r) of the regression curves higher or equal of 0.9995.

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The between-day coefficient of variation of the slope of the calibration curve was 1.75% for chicken muscle validation and 4.56% for milk samples validation.

Recovery

The recovery was determined by comparing results of analysis of the spiked samples with those of the standard and identity and purity of the peak of CAP was tested by means of diode array spectra. Comparison of spectra obtained from CAP peaks of fresh spiked chicken muscle and milk, frozen spiked chicken muscle, and CAP standard is presented in Fig. 3. No significant differences can be observed.

Mean recoveries of the proposed methods for milk and chicken muscle are presented in Tables 1 and 2.

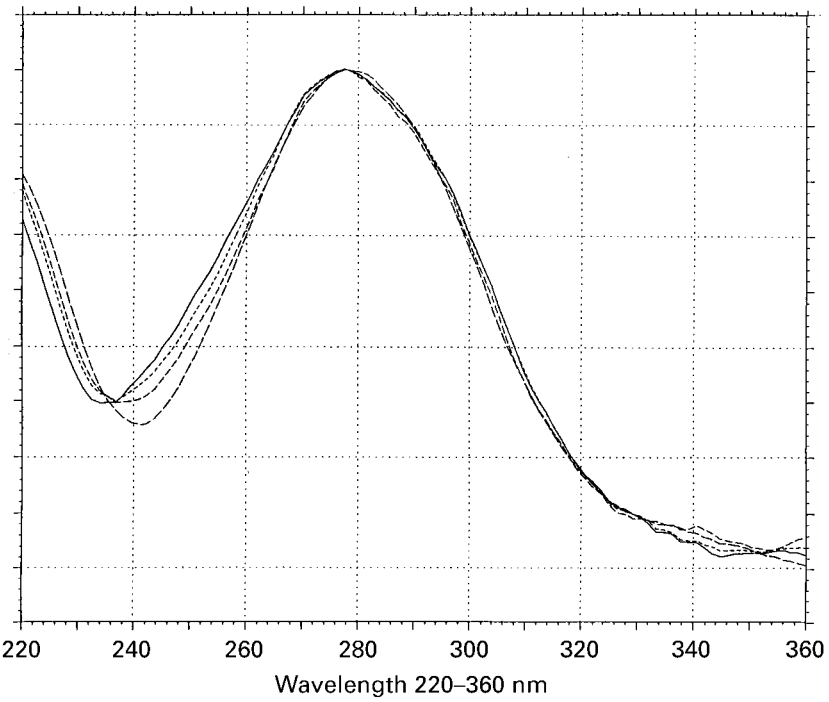

Figure 3. Comparison of spectra of CAP peaks in fresh and frozen spiked chicken muscle and milk extracts with that of CAP standard.

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Table 1. Recovery of CAP in milk samples.

Concentration ($\mu g\,kg^{-1}$)	Recovery (%) ($n = 5$)
	Mean ± SD
2.2	83.40 ± 8.10
4.4	79.62 ± 3.44
7.5	78.46 ± 7.14
15.0	74.38 ± 6.14

Overall mean recoveries greater than 79% and variation coefficient lower than <10% were obtained with both samples.

Precision

Intra-day precision of the method for the determination of CAP in chicken muscle was evaluated by analyzing muscle samples spiked at concentrations of 10, 30, and 60 $\mu g\,kg^{-1}$ in replicates ($n = 4$). Precision was based on the calculation of the coefficient of variation of the mean recovery at each spiked level.

Between-day precision was determinated by assaying spiked chicken muscle samples ($n = 7$) on four separate days and spiked milk sample ($n = 5$) on five separate days. Tables 3–5 show intra-day and between-day precision.

Stability in Chicken Muscle

Results of the stability assay in chicken muscle are summarized in Table 6. The results show that no significant differences in CAP contents were observed when muscle samples were stored at 4°C ± 2°C for five days.

Table 2. Recovery of CAP in chicken muscle samples.

Concentration ($\mu g\,kg^{-1}$)	Recovery (%) ($n = 7$)
	Mean ± SD
10	86.51 ± 6.59
30	87.15 ± 4.82
60	90.75 ± 5.17

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Table 3. Intra-day precision for analysis of CAP in chicken muscle.

Theoretical concentration ($\mu g\,kg^{-1}$)	Mean found concentration ($n=4$)	CV (%)
10	8.71	7.20
30	26.99	4.18
60	55.44	4.51

In our experiment, grinding and addition of standard was carried out before freezing the sample. Grinding destroys cells expòsing CAP to metabolic degradation. However CAP stability was quite good and no degradation was observed.

These results are in accordance with previously reported results in which no degradation of CAP was observed during a 180 day period at $-20^{\circ}C$ (personal, not published, results).

Measurement of CAP at day 8 was impossible due to interference compounds (a similar effect was detected previously by us in lyophilized samples), so the stability of CAP residues in muscle stored longer than 5 days in refrigeration is unknown.

Storage under frozen conditions during 30, 60, and 90 days did not produce significant losses or degradation of CAP residues in muscle.

Stability in Milk

Results of the stability assay in milk samples are summarized in Table 7. The results show that mean values of CAP concentrations observed in milk

Table 4. Between-day precision for analysis of CAP in chicken muscle.

Theoretical concentration ($\mu g\,kg^{-1}$)	Mean found concentration ($n=7$)	CV (%)
10	8.65	7.61
30	26.14	5.53
60	54.45	5.70

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Table 5. Between-day precision for analysis of CAP in milk samples.

Theoretical concentration ($\mu g\,kg^{-1}$)	Mean found concentration ($n = 5$)	CV (%)
2.2	1.83	9.61
4.4	3.50	4.25
7.5	5.95	9.00
15.0	11.15	8.27

during 11 days of storage did not present significant variations in comparison with original CAP spiked amounts ($7.5\,\mu g\,kg^{-1}$).

However, freezing has a clear influence on the recovery of CAP in milk samples. The mean CAP concentration appeared to reduce 24.5% and 23.5%

Table 6. CAP stability in spiked chicken muscle sample ($15\,ng\,g^{-1}$).

	Day			
	0	2	4	5
Stored under refrigeration conditions ($4°C \pm 5°C$)[a]				
	15.06	15.67	14.83	13.74
	15.06	14.33	13.35	14.67
	13.83	14.33	14.83	14.2
Mean	14.65	14.77	14.33	14.2
SD	0.579	0.631	0.697	0.379
	Month			
	0	1	2	3
Stored under frozen conditions ($-18°C \pm 5°C$)				
	15.06	14.53	14.51	15.19
	15.06	14.53	13.84	14.63
	13.83	15.12	14.51	14.63
Mean	14.65	14.72	14.28	14.81
SD	0.579	0.278	0.315	0.263

[a]Detection of CAP in chicken muscle after 8 days of refrigeration was impossible due to a high UV-absorption of matrix interferences.

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Table 7. CAP stability in spiked milk sample (7.5 ng g^{-1}).

	Day			
	0	3	8	11
Stored under refrigeration conditions (4°C ± 5°C)				
	7.05	6.96	7.03	7.91
	6.60	6.60	7.50	7.58
	7.60	7.10	7.34	7.41
Mean	7.08	6.88	7.29	7.63
SD	0.408	0.210	0.195	0.207
	Month			
	0	1	6	
Stored under frozen condition (−18°C ± 5°C)				
	7.05	5.58	5.19	
	6.60	5.27	5.36	
	7.60	5.43	5.51	
Mean	7.08	5.42	5.35	
SD	0.408	0.126	0.130	

after 1 and 6 months of storage at −18°C, respectively. We concluded that these decreases did not depend on the variability of the method, but may be due to real degradation of CAP or sequestration of the residues by coagulated proteins.

CONCLUSION

With the proposed analytical methods based on HPLC-Diode Array, CAP may be measured in muscle and milk samples at levels lower than 5 and 2 ng g, respectively. Spectra of the peaks may be used to confirm presence of CAP. These methods have been used for a stability study of CAP during different storage conditions. Chloramphenicol appeared a rather stable residue, although in milk samples stored under conditions where coagulation of the milk proteins may occur, measurable CAP decreased in about 25%.

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