

# Amphenicols stability in medicated feed – development and validation of liquid chromatography method

Wojciech Jerzy Pietron<sup>2</sup>, Aneta Woźniak<sup>1</sup>, Katarzyna Pasik<sup>1</sup>,  
Wojciech Cybulski<sup>3</sup>, Dorota Krasucka<sup>1</sup>

<sup>1</sup>Department of Veterinary Pharmacy,

<sup>2</sup>Department of Radiobiology,

<sup>3</sup>Department of Pharmacology and Toxicology,

National Veterinary Research Institute, 24-100 Pulawy, Poland

wojciech.pietron@piwet.pulawy.pl

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## Abstract

A liquid chromatography–ultraviolet detection method for the determination of florfenicol (FF) and thiamphenicol (TAP) in feeds is presented. The method comprises the extraction of analytes from the matrix with a mixture of methanol and acetonitrile, drying of the extract, and its dissolution in phosphate buffer. The analysis was performed with a gradient programme of the mobile phase composed of acetonitrile and buffer (pH = 7.3) on a Zorbax Eclipse Plus C18 (150 × 4.6 mm, 5 μm) analytical column with UV (λ = 220 nm) detection. The analytical procedure has been successfully adopted and validated for quantitative determination of florfenicol and thiamphenicol in feed samples. Sensitivity, specificity, linearity, repeatability, and intra-laboratory reproducibility were included in the validation. The mean recovery of amphenicols was 93.5% within the working range of 50–4000 mg/kg. Simultaneous determination of chloramphenicol, which is banned in the feed, was also included within the same procedure of FF and TAP stability studies. Storing the medicated feed at room temperature for up to one month decreased concentration in the investigated drugs even by 45%. These findings are relevant to successful provision of therapy to animals.

**Keywords:** medicated feed, florfenicol, thiamphenicol, chloramphenicol, HPLC, drug stability.

## Introduction

Amphenicols are a group of chemical compounds including chloramphenicol (CAP), thiamphenicol (TAP), and florfenicol (FF). These antimicrobial agents are authorised both for human medicine and veterinary usage (Table 1) and are applied therapeutically in animal husbandry. Their mechanism of action is blocking the activity of the peptidyltransferase at the 50S ribosome subunit of bacteria thereby preventing protein chain elongation (1).

The first compound in this class, CAP, initially isolated as a natural product, was introduced into therapy in 1947. Currently, all amphenicols are made by chemical synthesis. Chloramphenicol affects mammalian protein synthesis, especially mitochondrial protein synthesis (23). Furthermore, CAP has been proven to be a cytotoxic, genotoxic, and haematotoxic

drug (2). Residues of CAP and its metabolites have been found in animal meat, including fish meat and meat products (1, 2). Veterinary medicinal products and medicated feeds containing CAP were banned in treatment of food-producing animals by the FDA in 1984. A ban has been also implemented in the EU since 1994 (23). More recently, CAP and its metabolites were evidenced as prohibited agents above permitted residue limits in various products, such as milk, honey, liver, poultry products, fish, and shrimps (28, 29, 32). For strict control of CAP residues, a minimum limit (MRPL) of 0.3 ng/g is required (6). The maximum residue limits (MRLs) are 1000 ng/g for the sum of FF and its major metabolite (4) and 50 ng/g for TAP (7). Toxicological concerns on thiamphenicol and florfenicol are less defined and these antimicrobials have been authorised for veterinary use (23, 28). Displaying pharmacodynamics, pharmacokinetics and

toxicological risk different to CAP, the oral preparations of FF and TAP have become the predominant form of administration to animals. Their therapeutic level in medicated feeds ranges between 200 and 4000 ppm.

According to legislation, the manufacturers are required to ensure that medicated feeds comply with §4 of Directive 90/167/EEC (8). Concentration has to be controlled for product homogeneity and more recently, the stability parameters of active substances in medicated feeds have also begun to be of interest (31).

Determination of amphenicols in biological matrices has been achieved by various analytical techniques (3, 9, 11, 13–17, 20, 24, 25, 27, 31, 32). A considerable number of methods have been developed for determination of amphenicols and their metabolites in tissues and biological fluids (9, 15, 20, 24, 21, 32). However, a limited number of papers dealing with animal feed formulations were found in available sources. The published methods for determination of amphenicols in feeds used the techniques of enzyme-linked immunosorbent assay (ELISA) (16, 17), planar chromatography (30), liquid chromatography (13, 14), and liquid chromatography tandem mass spectrometry (LC-MS-MS) (3, 25). The microbiological technique (9), ELISA (16, 17), and a procedure utilising biosensors (15) had to be chemically confirmed. Single quadrupole gas chromatography-mass spectrometry (GC-MS) was also adopted for the determination, confirmation, and concentration measurement of CAP, TAP, FF, and florfenicol amine (FFA) (11, 24).

The stability of amphenicols was studied in different biological formulations subjected to thermal factors using capillary electrophoresis (CE) with ultraviolet photodiode array detection (UV-DAD) spectrometry, in addition to gas chromatography with electron impact ionisation mass spectrometry (GC-EI-MS). Differences in thermal amphenicol stability in several matrices (water, salt water, soybean sauce, and chicken meat) were demonstrated while the samples were heated to 100°C for 2 h (11).

The official requirements for the control of amphenicols (TAP, FF) in medicated feeds mandate, *inter alia*, determination of drug concentration in order to evaluate the homogeneity of manufactured feedlots (8, 21). The rationale for choosing an analytical method was its easy utilisation in a routine laboratory. Our choice being so informed led us to focus on a liquid chromatographic technique with ultraviolet detection. It justified the aim of our study towards the development of a fast, simplified, operating procedure and convenient data manipulation based on the HPLC-UV method. The determination method derived by the authors seeks to make thiamphenicol and florfenicol more easily determinable, however, chloramphenicol was also included within scope of the research on feeds. This inclusion was only due to the specificity of examination and concerns over amphenicol stability

with the method developed for FF and TAP. For food producing animals, any use of CAP in therapy is strictly prohibited; therefore in any case it cannot be legally added to feed.

## Material and Methods

**Reagents and chemicals.** Certificated reference material (CRM) thiamphenicol (CAS: 15318-45-3), florfenicol (CAS: 3231-34-2), and chloramphenicol (CAS: 56-75-7) were obtained from Sigma-Aldrich (Germany). Acetonitrile, methanol, and orthophosphoric acid were from POCh (Poland) and sodium hydroxide was procured from Sigma-Aldrich (USA). All chemicals were of HPLC or analytical grade. Cellulose syringe filters 0.22 µm were sourced from Sartorius (Germany) and ultra-pure water was supplied by the Milli-Q system (Millipore, USA).

**Standard solution.** A standard stock solution of each amphenicol in methanol at concentration 1 mg/mL and 20 mg/mL was prepared and stored for up to six months at -18°C. Working standard solutions for the calibration curve were prepared by appropriate dilutions of the stock solutions in phosphate buffer (0.02 M, pH 7.3) and their stability was tested once a month while refrigerated ( $5 \pm 3^\circ\text{C}$ ). Purity and water content of the standard substances were considered when the stock solutions were prepared.

**Sample preparation.** Samples were obtained from different local manufacturers of medicated feeds for swine through the agency of the Veterinary Inspectorate. Amphenicol-free samples, with status confirmed by analysis, were used for preparation of blanks and spiked control samples. Commercial sow feeds constituted the matrix for stability assessment and contained 16% of protein, 3.5% of fat, and 6% of ash and fibre among others. All samples were ground with a ZM 200 centrifugal mill (Retsch, part of Verder Scientific, Netherlands) with a 1.5 mm sieve before analysis. Control samples were prepared by adding 100 µL of 20 mg/mL standard to the 2 g of blank feed samples and leaving for at least 1 h to allow equilibration to take place.

**Extraction and clean-up.** Homogenised feed samples (2.0 g) were precisely weighed into propylene centrifuge tubes (50 mL) and 25 mL of acetonitrile and methanol mixture (1:1 v/v) was added. Firstly, the sample in the tube was moistened with solvent extract, this step was followed by 1 min shaking on a vortex mixer, and then extraction was continued on a KS 501 horizontal-shaker (IKA-Werke, Germany) at 250 rpm. After 30 min, the sample was centrifuged (6K15, Sigma Laborzentrifugen, Germany) for 10 min at  $4000 \times g$ , while the tube was cooled to 10°C. Supernatant in 1 mL volume was transferred to a 6 mL conical vial and dried under a gentle stream of nitrogen, maintaining the vial temperature in the range of 45-50°C. Then, 4 mL of phosphate buffer, pH 6.5, was

added to the residue and it was then shaken for 1 min on a vortex mixer. The reconstituted extract was filtered through a syringe filter (modified cellulose, 0.45  $\mu\text{m}$ ) and 20  $\mu\text{L}$  was analysed by HPLC.

**HPLC-UV analysis.** The chromatography equipment was the Agilent Series 1200 HPLC system (Agilent Technologies, Germany) equipped with degasser, binary pump, autosampler, column oven, and ultraviolet detector and controlled by ChemStation software (Agilent). Separation of the analysed compounds was achieved on a Zorbax Eclipse Plus C18 (150  $\times$  4.6 mm, 5  $\mu\text{m}$ ) analytical column (Agilent) thermostatted at 35°C with gradient elution of the mobile phase containing acetonitrile and phosphate buffer adjusted to pH 7.3. Solvents were mixed on the chromatographic system pump in a gradient elution: from 20% acetonitrile hold for 2 min and linearly changed to 50% within 12 min. Then the initial condition was restored and held for 5 min for equilibration of the system before the next injection. Analysis was performed at 1 mL/min flow rate. Absorbance of the compounds was measured with wavelength set at  $\lambda = 220$  nm.

**Validation of the method.** The analytical procedure was validated according to the recommendation of Commission Decision 2002/657/EC (5). The criteria for specificity, linearity, precision, repeatability, and recovery were as detailed hereafter. Firstly, the specificity of the method was evaluated from 12 blank samples of five different types of feeds for swine and poultry, and examined by determining the limits of detection (*LOD*) and limit of quantification (*LOQ*). The method's linearity was evaluated by calculation of the regression line by the least squares method and the coefficient of determination ( $R^2$ ). Linearity for amphenicols in feed was determined with the five concentration levels of 25, 50, 100, 1000, and 4000 mg/kg, the concentrations being exclusively of blank matrix. The precision of the method was tested at 100, 1000, and 4000 mg/kg concentration levels by spiking appropriate amounts of amphenicol standards (2 mg/mL and 20 mg/mL) to 2 g of feed. On the next day, the spiked samples were vortexed and analysed. Repeatability was learned by analysis of one sample in six repetitions during one day, whereas within-laboratory reproducibility was assessed by a weekly operator change for the analysis of any particular sample. The recoveries were determined on spiked samples analysed in order to evaluate the precision of the method.

**Stability research.** The stability of the working standard solution was tested weekly over a period of two months. A stock solution was tested in the same manner as a working standard by injection of freshly prepared dilutions. For checking the stability of active ingredients in the matrix feed, samples were fortified at

1000 mg/kg and divided into three groups stored in the dark at room temperature, in a refrigerator, and in a freezer. Two samples from each group were taken and analysed at the weekly interval, thus examining stability of amphenicols in the matrix over one month.

## Results

The reversed-phase column Zorbax Eclipse Plus C18 and gradient of the phosphate buffer and acetonitrile were chosen to separate chromatographically thiamphenicol, florfenicol, and chloramphenicol within 12 min. The best separation and peak shape were achieved using phosphate buffer adjusted to pH 7.3. A typical chromatogram obtained from a spiked feed sample is presented in Fig. 1.

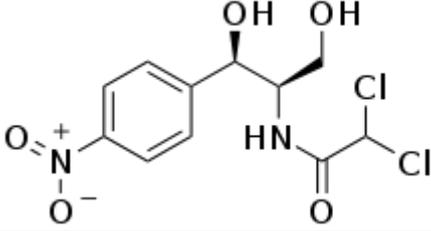
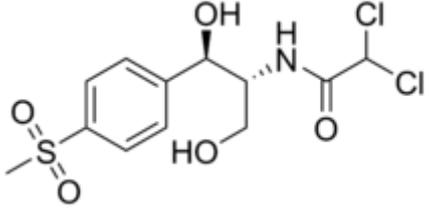
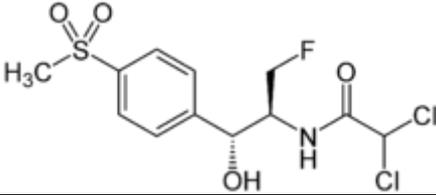
Under selected conditions, TAP, FF, and CAP were eluted at 4.17, 8.13, and 8.90 min respectively and displayed high UV absorption at 220 nm, while no interference of the matrix was observed (Fig. 1). The stability of the chromatographic method was checked by calculation of the standard deviation (S.D.) of retention times for all analytes, which were lower than 1.0 between compared days. The relative standard deviation (R.S.D.) percentage of the retention times was no higher than 3% measured over two months (Table 2).

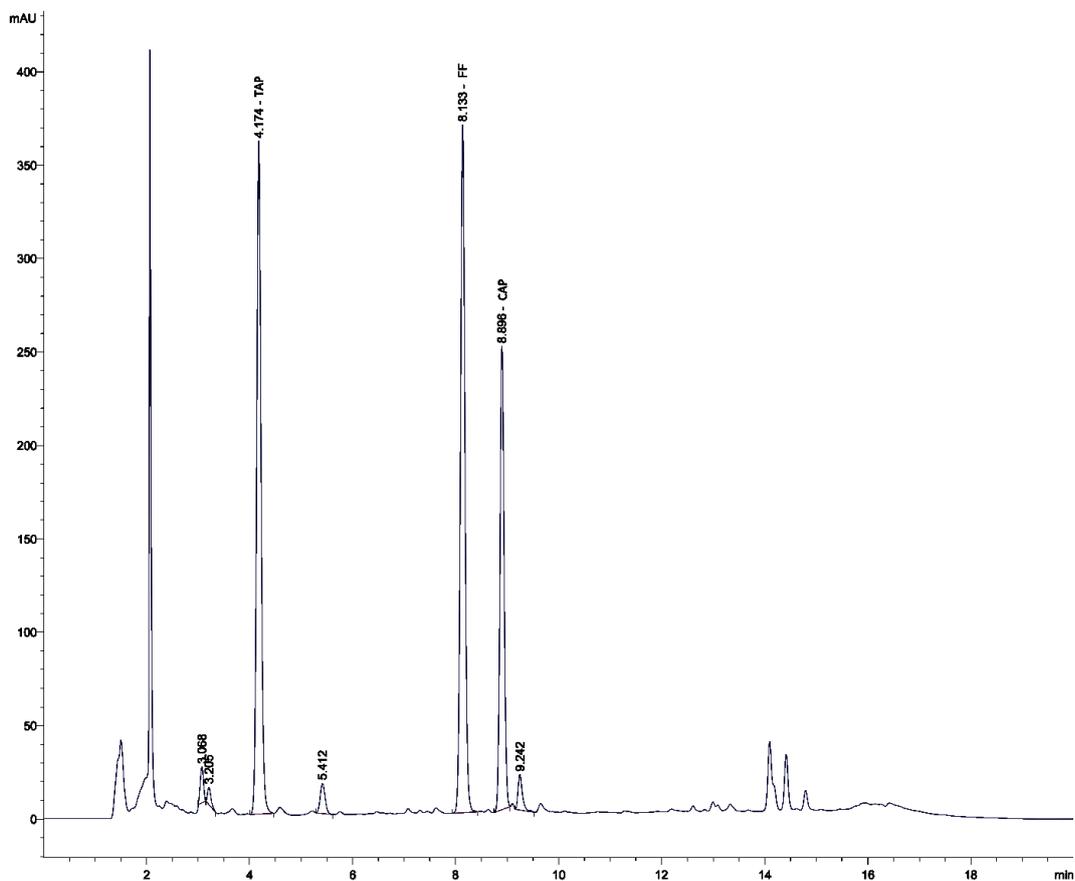
The linearity of the detector was evaluated using 1-100  $\mu\text{g/mL}$  standard concentrations for amphenicols and the coefficients of determination were higher than 0.998. Linearity and regression calculations for the spiked samples, in the range 50–4000 mg/kg, were separately performed for each validation day. No significant differences (data not shown) were observed, and therefore mean calibration curves were determined. The individual coefficients of the regression equation are presented in Table 2.

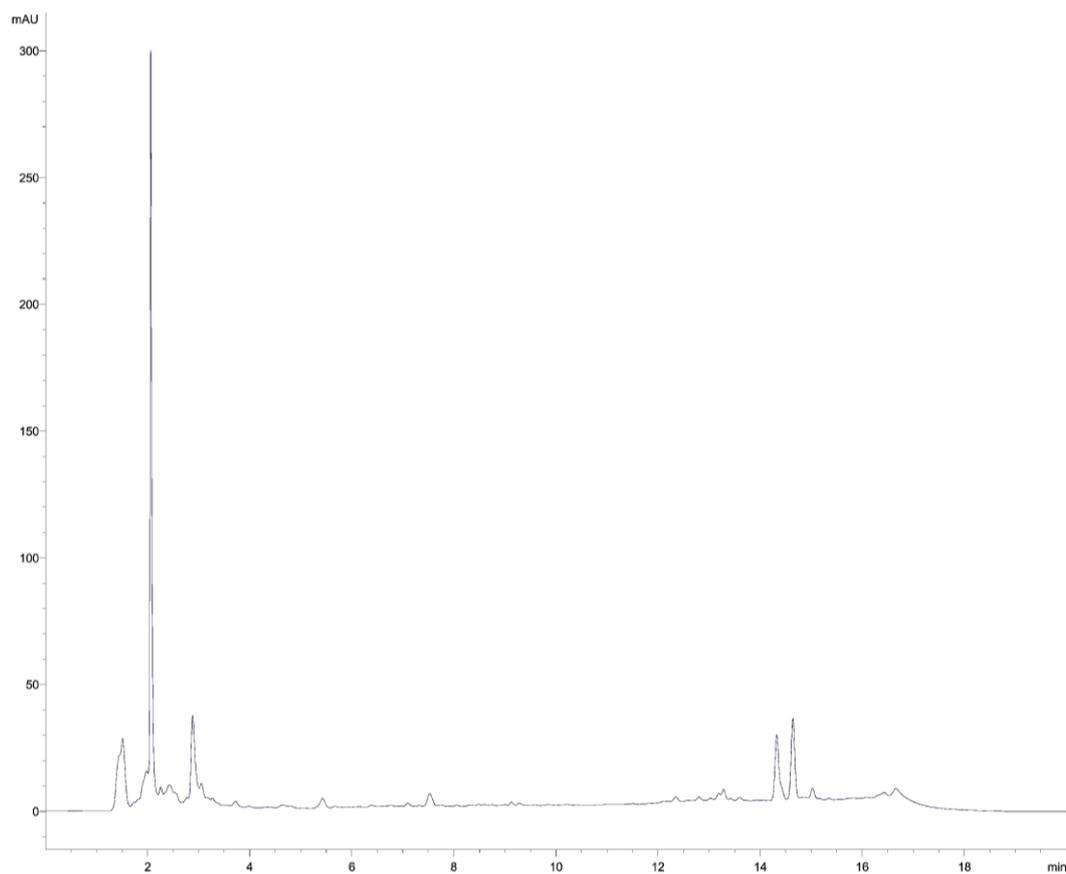
The R.S.D. was selected as the main precision parameter of the method. All R.S.D.s of repeatability and within-laboratory reproducibility were below 8% and 12% respectively (Table 3). Analysed compound recoveries were calculated as average values obtained within the estimation of method precision and were in the range of 88%–101% (Table 4).

For calculation of method specificity, gradients of matrix calibration curves and standard deviations were studied using samples containing analytes at 25 mg/kg. *LOD* and *LOQ* were calculated according to the equation  $LOD = 3.3 \cdot SD_{low}/a$  and  $LOQ = 10 \cdot SD_{low}/a$ , where  $SD_{low}$  was defined as the standard deviation for samples containing the analytes at 25 mg/kg and “a” as the slope of the matrix calibration curve. Calculated results are presented in Table 5. The chromatogram of an amphenicol-free sample is presented in Fig. 2.

**Table 1.** Amphenicol classification

Type of amphenicol	Structural formula	Description
Chloramphenicol		Chloramphenicol (CAP) is a bacteriostatic antimicrobial, considered as a prototypical broad-spectrum antibiotic alongside the tetracyclines. It is effective against a wide variety of Gram-positive and Gram-negative bacteria. This antibiotic treatment can cause bone marrow toxicity (10, 22).
Thiamphenicol		Thiamphenicol (TAP) is the methyl-sulfonyl analogue of chloramphenicol. It is insoluble in water, but highly soluble in lipids. It has a similar spectrum of activity to chloramphenicol. TAP is widely used as a veterinary antibiotic. Furthermore, TAP is used in humans in China, Morocco, Italy, and Brazil. TAP is not readily metabolised in cattle, poultry, sheep, or humans (12).
Florfenicol		Florfenicol (FF) is a fluorinated synthetic analogue of thiamphenicol. FF is indicated for treatment of bovine interdigital phlegmon (foot rot, acute interdigital necrobacillosis, and infectious pododermatitis) associated with <i>Fusobacterium necrophorum</i> and <i>Bacteroides melaninogenicus</i> and for the treatment of bovine respiratory disease associated with <i>Mannheimia (Pasteurella) haemolytica</i> , <i>Pasteurella multocida</i> , and <i>Haemophilus somnus</i> (26).

**Fig. 1.** Chromatogram of a sample spiked at 100 mg/kg



**Fig. 2.** Chromatogram of an amphenicol-free sample

**Table 2.** Linearity of the detector (standard solutions) and method measured in a week interval during two months

Compound	$t_R$ (min)		a	b	$r^2$
TAP	4.19	solution	110.7 (0.18)	14.1 (23.3)	0.99991 (0.003)
	(0.11)	matrix	0.897 (0.91)	-8.75 (46.7)	0.99980 (0.014)
FF	8.15	solution	113.3 (0.09)	13.3 (24.2)	0.99997 ( $10^{-04}$ )
	(0.07)	matrix	0.923 (0.52)	-9.41 (31.6)	0.99988 (0.007)
CAP	8.91	solution	70.8 (0.17)	24.4 (9.01)	0.99850 (0.003)
	(0.05)	matrix	0.921 (1.24)	-12.3 (46.6)	0.99983 (0.00772)

$t_R$  – retention time; ( ) – relative standard deviation %

**Table 3.** Precision of the method

Concentration (mg/kg)	Repeatability precision (R.S.D.%; n = 6)			Reproducibility precision (R.S.D.%; n = 18)		
	100	1000	4000	100	1000	4000
TAP	4.77	1.21	0.92	11.8	4.29	3.05
FF	1.07	1.71	0.57	7.54	3.85	3.84
CAP	7.99	1.72	1.35	10.7	4.91	5.60

**Table 4.** Recovery parameters (%) (n = 18)

Concentration (mg/kg)	TAP	FF	CAP
100	96.4	100.1	88.5
1000	88.1	92.7	92.8
4000	90.7	93.0	93.5
Average	91.7	95.3	91.6
R.S.D.%	4.63	4.40	2.96

**Table 5.** Method specificity

Compound	$LOD$ (mg/kg) $= 3.3 * SD_{low} / a$	$LOQ$ (mg/kg) $= 10 * SD_{low} / a$
TAP	1.86	5.64
FF	4.10	12.41
CAP	2.60	5.63

$SD_{low}$  - standard deviation for samples containing analytes at 50 mg/kg;  $a$ -slope of method calibration curve

Amphenicols were the most stable when the medicated feed was stored in a freezer. In this condition concentrations of FF and CAP did not change, and only TAP concentration decreased about 10% over one month (Fig. 3). For the medicated feed stored in a refrigerator, concentration of all investigated analytes decreased about 15%–25% (Fig. 4). When the medicated feed was stored at room temperature in the dark, the degradation of the drugs was faster as confirmed by the weakening of the concentration by about 30%–45% over four weeks (Fig. 5).

## Discussion

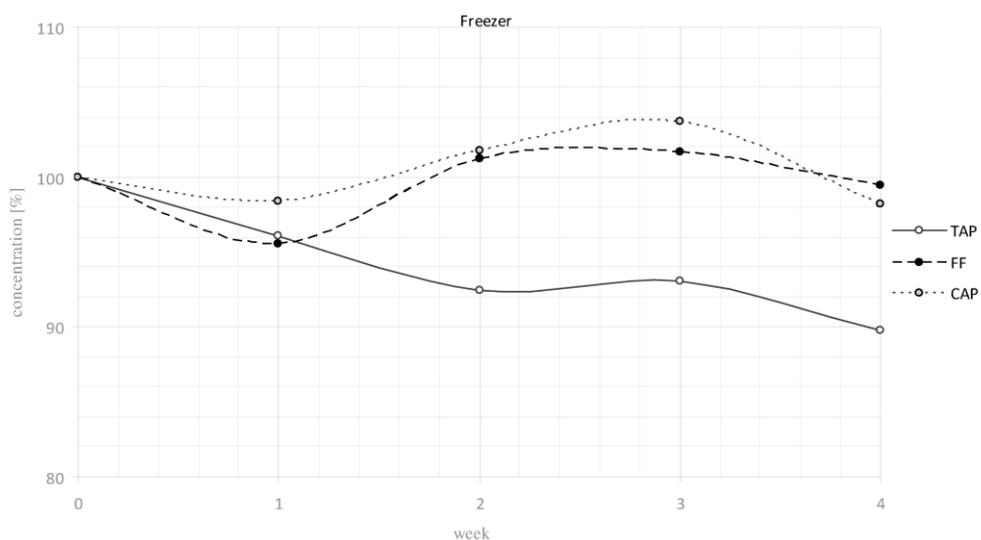
The literature contains limited information about the amphenicol group of antibiotics in respect to their stability in medicated feeds as well as in respect to optimisation of determination procedures and the traits of amphenicol extraction from the feed matrix..

Complete and selective extraction of the investigated compounds is the most crucial during analytical study. In this study, the mixture consisting of MeOH : MeCN (1:1 v/v) successfully enabled one-step nonetheless the complete extraction (19). Though Boscher *et al.* (3) suggested, that the best solvent for TAP and FF extraction in multi compounds analysis was a mixture of MeOH : MeCN : McIlvaine buffer, pH 6.0, (37.5:37.5:25, v/v/v). In this case, addition of the buffer to the mixture was required for the chelation of ions disturbed during tetracycline determination (18). However, in that method, ultrasound-assisted extraction (UAE) optimised to 60°C, 7 min, and two cycles was also applied. Moreover, the authors suggested the possibility of using different methods such as pressure liquid extraction (PLE) with the same solvent without impact on the results. The recoveries in the range of 85%–100% presented in that paper were similar to those obtained by our simplified one-step method. Hayes *et al.* (14) proposed multi-step extraction of FF by MeCN : H<sub>2</sub>O (1:1 v/v) mixture, improved by using UAE at 45–50°C for 5 min. Then, the extraction was continued by shaking for 10 min and all steps of the process were repeated. After cleaning up by SPE, the

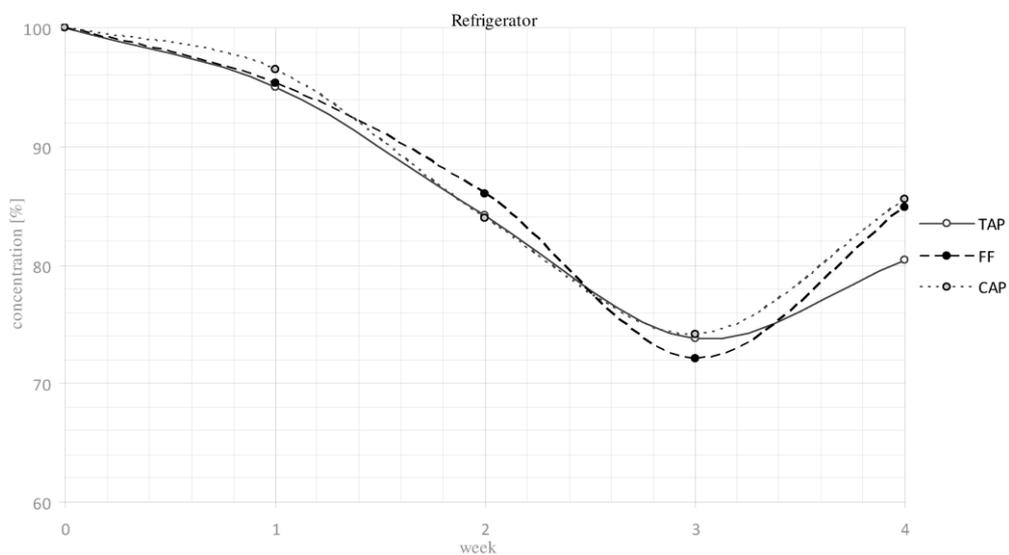
recoveries of florfenicol ranged from 98.1% to 101.1%.

Unsatisfactory recovery results (data not shown) were obtained in pre-testing the SPE clean-up method with the use of C18 and Oasis HLB cartridges. Similar findings were reported by Boscher *et al.* (3), who decided to apply dispersive SPE by adding primary and secondary amine exchange material (PSA) as a sorbent to clean up feed extracts. Furthermore, their GC-MS/MS technique needed four times dissolution of the eluates in order to reduce matrix effects (3). The Oasis HLB cartridges were also used by Shen *et al.* (24), followed by the reconstruction of the extract in 0.5 mL of MeOH, its dissolution in 10 mL of 4% sodium chloride solution, clean-up twice with hexane, and a pass through SPE columns. Then, evaporated eluates were derivatised with N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% TMCS and analysed by GC-MS without interference. SPE cartridges with active carbon (Envi-Carb, Sigma-Aldrich, USA) (14) and octadecyl (C18) were also investigated (20, 31). Using a C18 bed, a satisfactory extract purity and good recoveries were achieved only in the case of FF. For other amphenicols further optimisation of the clean-up step was needed. Feed extract impurity is bound to water present in the extraction solvent, *e.g.* a buffer, which intercepts a variety of polar compounds from the matrix. Hence a more or less advanced SPE cleaning-up technique is necessary as an additional clean-up step. A time saving and cost reduction are achieved when the SPE cleaning-up step is skipped. However, in the case of cross-contaminated feeds analysis use of the SPE cartridges is recommended to improve method specificity, since it is an essential step in examination of drug residues at trace level.

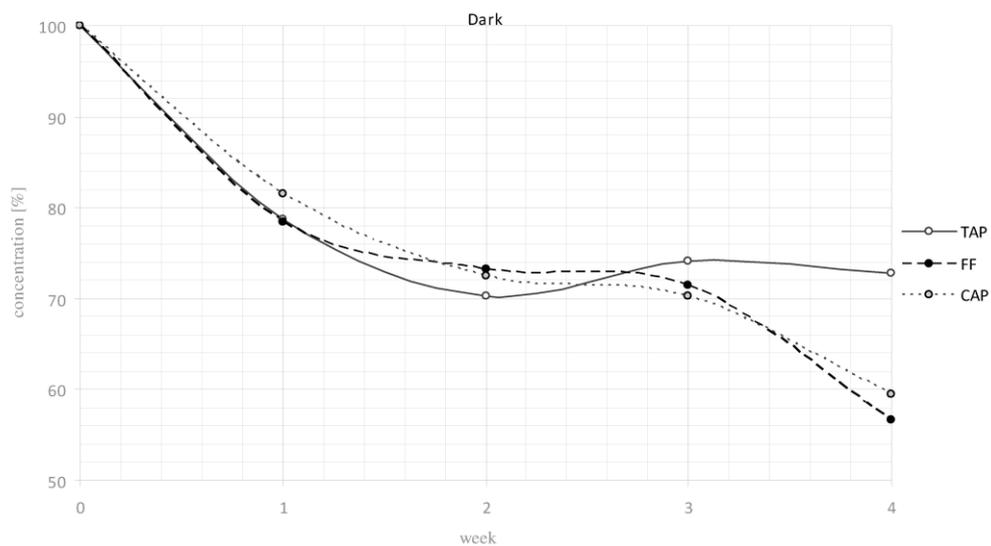
The chromatographic parameters specified here are similar to those of other HPLC methods for determination of amphenicols reported in a number of publications (3, 13, 14, 20, 24, 31, 32). The best separation between chloramphenicol and thiamphenicol was achieved with a mobile phase of low pH, although significant baseline drift was observed. When the pH was changed from 2.5 to 7.3, the impact on separation was rather small and at the



**Fig. 3.** Concentration of amphenicols in medicated feed stored in the freezer (-20°C)



**Fig. 4.** Concentration of amphenicols in medicated feed stored in the refrigerator (2-6°C)



**Fig. 5.** Concentration of amphenicols in medicated feed stored in dark (room temp.)

same time baseline drift was not present. The composition of mobile phase chosen and analytical column selected yielded satisfactory chromatographic separation and analyte peak shapes. The high stability of retention time and reproducibility of data with the mobile phases prepared as described proved that chromatographic separation was adequate for the analysis of drugs. The simplicity and rapidity of this instrumental method of FF and TAP determination in medicated feeds and CAP in feeds have been clearly demonstrated, since the HPLC with reversed-phase technique employed here was capable of separating all the amphenicols within 10 min.

The method was successfully validated according to Commission Decision 2002/657/EC (5). The *LOD* and *LOQ* of the method were calculated from feeds spiked with amphenicols at low level (Table 4). Moreover, 12 blank feed samples were analysed and *LOQs* calculated as 10 x signal to noise from these samples, confirming the method specificity. Estimated *LOQs* were near 1 mg/kg, but in the reality this concentration level cannot be achieved. The coefficients of determination were close to 1.0 both for linearity of the detector and for the method. The average recoveries were higher than 90%, and were compared to those reported by Boscher *et al.* (3) and Hayes *et al.* (13), who worked at fortification levels lower than and similar to those used in our study. Validation results show that the method is suitable for its objectives, being linear in the concentration range between 50 and 4000 mg/kg in medicated feeds.

Active ingredient stability in the matrix is one of the most important quality parameters for the pharmaceutical industry and is required to be determined for all medicinal products. Medicated feeds are not classified as veterinary medicinal products, nevertheless, they also need to be assayed for concentration and stability of pharmacologically active agents. According to directive 90/167/EEC (8), feed mills are obliged to control the level of antimicrobials in the final products, as well as the drug residues in the cleaning feed due to its cross-contamination (8). The feed matrix is prone to speeding up the degradation of amphenicols, leading to the production of derived compounds. They are more or less deprived of any pharmacological property, nonetheless, the derivatives are implicated in adverse effects during treatment of animals (21). Outcomes of this study have indicated that the amphenicol stability in feed depended on the temperature and time of feed storing. The amphenicols were the most stable in the freezer and the most unstable when stored at room temperature in a dark jar. Only slight stability differences of no more than 10% existed between individual drugs in feeds stored in the freezer and the difference pointed to TAP concentration decreasing faster than FF and CAP. Amphenicols proved to be more unstable in feed than in standard solution when stored under the

same conditions; the solutions were unchanged over two months' storage in a refrigerator. The results for stability were comparable to the results published by Franje *et al.* (11) who presented that amphenicols degraded more in heated chicken muscles in comparison to heated water/salt water solutions. The heat stability of amphenicols was as follows: water  $\geq$  salt water > soybean sauce > meat. Franje *et al.* (11) demonstrated differences in amphenicol thermal stability: FF > TAP = CAP in water, FF = TAP > CAP in salt water, TAP  $\geq$  FF = CAP in soybean sauce, and TAP  $\geq$  FF = CAP in meat. Moreover, TAP was one of the FF degradation products, detected even in water solutions stored at room temperature for 24 h (11). From our results with feeds follows the conclusion that the matrix could increase the speed of the degradation process. The most notable is a one third decrease in the concentration of all investigated amphenicols over two weeks when feeds were stored at room temperature. Neglecting the above findings on amphenicol stability implies unsuccessful therapy with use of medicated feeds. Moreover, the transformation and instability of amphenicols in feeds are relevant to public health issues, *i.e.* the antibiotic residues in food of animal origin and/or an impact on acquiring antimicrobial resistance (21). Improving a simple, effective, and reliable method of amphenicol determination in medicated feeds gave a useful control tool for prudent antimicrobial treatment of animals.

The quantitative HPLC-UV method presented here for determination of thiamphenicol and florfenicol in medicated feeds was fully validated and proved to be suitable for the intended purpose, *i.e.* checking the drugs' stability and homogenous distribution within the matrix. Moreover, the presented method is able to separate chloramphenicol from legally-used amphenicols in the feeds, and in this way can diagnose off-label use of the banned antimicrobial agent. Though its results have to be proved by a confirmatory method. The analytical procedure authored in this article cannot be applied for determination of amphenicol residues without improvement. Otherwise the method does not meet specified analytical criteria (4, 6, 7). The definition of the amphenicols decomposition kinetics over the course of time in stored medicated feeds contributed a relevant conclusion on prudent use of the antibiotics in therapy of animals. However, some other parameters of the amphenicols stability should be further investigated.

## References

1. Andrée S., Jira W., Schwind K.H., Wagner H., Schwägele F.: Chemical safety of meat and meat products. *Meat Sci* 2010, 86, 38-48.

2. Bilandžić N., Varenina I., Solomun Kolanović B.: Control of chloramphenicol in samples of meat, meat products and fish. *Meso* 2011, 13, 192–196.
3. Boscher A., Guignard C., Pellet T., Hoffmann L., Bohn T.: Development of a multi-class method for the quantification of veterinary drug residues in feeds by liquid chromatography-tandem mass spectrometry. *J Chromatogr A* 2010, 1217, 6394–6404.
4. Commission Decision 2001/1322/EC of 29 June 2001 Amending Annexes I and III to Council Regulation (EEC) No. 2377/90 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *OJL177/52*, 30.6.2001.
5. Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *OJ L* 221, 17.8.2002, pp. 16-17.
6. Commission Decision 2003/181/EC of 13 March 2003 on amending Decision 2002/657/EC as regards—of minimum required performance limits (MRPLs) for certain residues in food of animal origin. *OJ L71* 15.03.2003 pp. 17-18.
7. Commission Regulation (EC) No. 1805/2006 of 7 December 2006 Amending Annex I to Council Regulation (EEC) No 2377/90 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, as regards thiamphenicol, fenvalerate and meloxicam. *OJ L* 343 8.12.2006 pp. 66-68.
8. Council Directive 90/167/EEC laying down the conditions governing the preparation, placing on the market and use of medicated feeds in the Community. *OJ L* 92, 07.04.1990, pp. 42–48.
9. Dang P.K., Degand G., Douny C., Ton V.D., Maghuin-Rogister G., Scippo M.L.: Optimisation of a new two-plate screening method for the detection of antibiotic residues in meat. *Int J Food Sci Tech* 2011, 46, 2070–2076, doi:10.1111/j.1365–2621.2011.02718.x
10. Falagas M.E., Grammatikos A.P., Michalopoulos A.: Potential of old-generation antibiotics to address current need for new antibiotics. *Expert Rev Anti Infect Therapy* 2008, 6, 593–600. doi:10.1586/14787210.6.5.593. PMID 18847400.
11. Franje C.A., Chang S.K., Shyu C.L., Davisc J.L., Leea Y.W.: Differential heat stability of amphenicols characterized by structural degradation, mass spectrometry and antimicrobial activity. *J Pharmaceut Biomed* 2010, 53, 869–877.
12. Fuchs F.D.: "Tetraciclina e cloranfenicol". In: *Farmacologia clinica: fundamentos da terapêutica racional*. Edited by Fuchs F.D., Wannmacher L., Ferreira M.B., Guanabara Koogan, Rio de Janeiro, 2004. p. 375.
13. Hayes J.M.: Determination of florfenicol in fish feed by liquid chromatography. *J AOAC Int* 2005, 88, 1777–1783.
14. Hayes J.M., Gilewicz R., Freehauf K., Fetter M.: Assay of florfenicol in swine feed: interlaboratory study. *J AOAC Int* 2009, 92, 340–347.
15. Huet A.C., Fodey T., Haughey S.A., Weigel S., Elliott C., Delahaut P.: Advances in biosensor-based analysis for antimicrobial residues in foods. *Trend Anal Chem* 2010, 29, 1281–1294.
16. Luo P.J., Cao X.Y., Wang Z.H., Jiang H.Y., Zhang S.X., Chen X., Wang J.P., Feng C.M., Shen J.Z.: Development of an enzyme-linked immunosorbent assay for the detection of florfenicol in fish feed. *Food Agr Immunol* 2009, 20, 57–65.
17. Luo P.J., Jiang W.X., Chen X., Shen J.Z., Wu Y.N.: Technical note: development of an enzyme-linked immunosorbent assay for the determination of florfenicol and thiamphenicol in swine feed. *J Anim Sci* 2011, 89, 3612–3616.
18. Patyra E., Kowalczyk E., Kwiatek K.: Determination of chlorotetracycline and doxycycline in medicated feedingstuffs by liquid chromatography. *Bull Vet Inst Pulawy*, 2012, 56, 329-333.
19. Pietroń W.J., Cybulski W., Krasucka D., Mitura A., Kos K., Antczak M.: Determination of five sulphonamides in medicated feedingstuffs by liquid chromatograph with ultraviolet detection. *Bull Vet Inst Pulawy*, 2013, 57, 545-552
20. Pouliquen H., Morvan M.L.: Determination of florfenicol in freshwater, sediments and bryophyte fontinalis antipyretica by HPLC with fluorescence detection. *Chromatographia* 2005, 62, 225–231.
21. Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004. On official controls performed to ensure the verification of compliance with feed and food law animal health and animal welfare rules.
22. Rich M., Ritterhoff R., Hoffmann R.: A fatal case of aplastic anemia following chloramphenicol (chloromycetin) therapy. *Ann Int Med* 1950, 33, 1459–1467.
23. Riviere J.E., Papich M.G. *Veterinary Pharmacology and Therapeutics*. Wiley–BlackWell, Hong Kong 2009.
24. Shen J.Z. Xia X., Jiang H., Li C., Li J., Li X., Ding S.: Determination of chloramphenicol, thiamphenicol, florfenicol, and florfenicol amine in poultry and porcine muscle and liver by gas chromatography–negative chemical ionization mass spectrometry. *J Chromatogr B* 2009, 877, 1523–1529.
25. Shinoda N., Kojima F., Sugiura K.J.: Residuals. Simultaneous determination of residues of chloramphenicol and florfenicol in animal feed by liquid chromatography tandem mass spectrometry. *J Residuals Sci Tech* 2011, 8, 125–129.
26. Syriopoulou V.P., Harding A.L., Goldmann D.A., Smith AL.: *In vitro* antibacterial activity of fluorinated analogs of chloramphenicol and thiamphenicol. *Antimicrob Agents Chemother* 1981, 19, 294–297.
27. Sniegocki T., Posyniak A., Gbylik-Sikorska A., Zmudzki J.: Determination of chloramphenicol in milk using a QuEChERS-based on liquid chromatography tandem mass spectrometry method. *Anal Lett* 2014, 47, 568–578.
28. The Annual Report of The Rapid Alert System for Food and Feed (Rapid Alert System for Food and Feed, RASFF, 2007. doi:10.2772/63727
29. The Annual Report of The Rapid Alert System for Food and Feed (Rapid Alert System for Food and Feed, RASFF, 2013. doi:10.2772/33031.
30. Vega M.H., Jara E.T., Aranda M.B.: Monitoring the dose of florfenicol in medicated salmon feed by planar chromatography (HPTLC) *Jap J Planar Chromat* 2006, 19, 204–207.
31. Vue C., Schmidt L. J., Stehly G.R., Gingerich W.H.: Liquid chromatographic determination of florefnicol in the plasma of multiple species of fish. *J Chromatogr B* 780, 2002, 111–117.
32. Wen L.L., Ren J.L., Maw R.L.: Supercritical fluid extraction *in situ* derivatization for simultaneous determination of chloramphenicol, florfenicol and thiamphenicol in shrimp. *Food Chem* 2010, 121, 797–802.