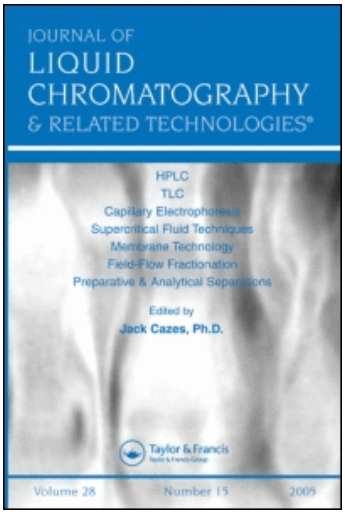

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CHLORAMPHENICOL AND NITROFURAN RESIDUE ANALYSIS BY HPLC AND PHOTODIODE ARRAY DETECTION IN MEAT AND FISH

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ABSTRACT

This paper describes a chromatographic method for the analysis of residues of chloramphenicol and nitrofurans in meat and fish. Chloramphenicol, furazolidone, furaltadone and nitrofurazone were extracted from the tissues with ethyl acetate. The extract was purified with petroleum ether and n-pentane and then analyzed with a liquid chromatograph and a photodiode array detector using a Chromspher C-8 column.

Chloramphenicol and nitrofurans were detected at 280 nm and 360 nm respectively. Blank samples fortified with chloramphenicol and nitrofurans at levels of 5, 10 and 20 μg/kg gave recoveries in the range of 69 to 88%. A quantitation limit of 1 μg/kg for nitrofurazone and furazolidone and of 2 μg/kg for chloramphenicol and furaltadone in meat and fish has been reached.

INTRODUCTION

Chloramphenicol (CAP) and nitrofurans (NF) have an antimicrobial activity. They are very often used for the treatment of diseases in animals (1,2). The most frequently NF used in Belgium are furazolidone, furaltadone and nitrofurazone. Furazolidone can act as growth promoter as well if it is added to feed in subtherapeutic doses. Many bacterial infections in fish have also been successfully controlled with CAP and NF (3-7). NF are known to be toxic. Furazolidone is suspected to be carcinogenic and mutagenic. CAP may be very toxic to humans and the FAO/WHO (Food and Agriculture Organization/World Health Organization) Committee on Food Additives proclaimed in July 1968 : "Chloramphenicol should not be used for any purpose that might result in the presence of residues in food for human consumption" (8).

In Belgium a zero tolerance level for CAP and NF in edible tissues is in force. It corresponds with the detection limit of analytical methods recommended. The Council Directive 86/469/ECC, Art. 9 and 16 (9) lay down to each member state a surveillance program for which analytical methods with a detection limit of 10 µg CAP/kg meat and of 5 µg NF/kg meat are recommended (10).

Many methods for the individual determination of CAP or NF in edible animal tissues have been published (11-17). Only some of them are easy to handle and result in a low detection limit (18). For the detection of CAP and NF residues in fish only a few analytical methods have been described (1,19-21).

In most circumstances animals are treated with premixes containing several antimicrobial substances. Therefore, to detect their residues multiresidue analytical methods have to be developed and validated. High-performance liquid chromatography (HPLC) and capillary gas-liquid chromatography (GLC) were used for the detection of more than 60 chemothera-

peutics, antiparasitics and growth promoters in feedstuff of animal origin (22-24). Gel permeation chromatography was used as clean-up procedure for the analysis of 40 veterinary drugs (25), while CAP, furazolidone and five sulfonamides were detected in eggs, meat and milk by HPLC (26). The disadvantage of multiresidue analyses is especially focused by intensive extraction and purification procedures and a lack of selecting and specifying the detection of the substances to be identified.

The method described in this paper has been developed for the detection of three NF and CAP. Extraction and purification procedures are simple and quick. For the detection we use a photodiode array detector which gives the possibility of continuously collecting data in a set wavelength range. This detection technique offers the opportunity to obtain a resolution of the interfering peaks with the products to identify. Another advantage of our method is the low detection level reached for the four compounds.

EXPERIMENTAL

Apparatus

An Ultra-Turrax from Janke & Kunkel (Germany) and a Vortex super mixer from Lab-Line Instruments (Illinois, USA) were used to homogenize the sample preparations. Centrifugations were achieved with a centrifuge GLC-2B from Sorvall (Dupont, USA). The solvents were evaporated using a Rotavapor-R from Büchi (Switzerland) and a Reacti-Therm heating module from Pierce (Illinois, USA). HPLC analyses were performed with a 9010 liquid chromatograph from Varian (USA) and an automatic sample injector model 231 from Gilson (France). Chloramphenicol and nitrofurans were detected si-

multaneously with a photodiode array detector 991 from Waters (Massachusetts, USA). The results were acquired by an APC IV personal computer from NEC corporation (Massachusetts, USA). Chromatograms and spectra were printed by a printer plotter 990 from Waters (USA).

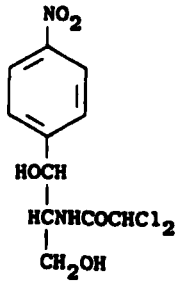
Solvents and reagents

Ethyl acetate, petroleum ether (30-60°C), acetonitrile and hexane were delivered by Baker (NJ., USA), n-pentane, acetic acid glacial 100%, ethanol and ammonium acetate by Merck (Germany) and sodium sulphate anhydrous by UCB (Belgium). All reagents were of analytical grade. For the extraction of nitrofurans an ammonium acetate buffer 1 M, pH 4.8 was prepared. The eluent for the HPLC analyses consisted of a 0.01 M ammonium acetate buffer pH 4.3 and acetonitrile (80 + 20, v/v). Buffer and acetonitrile were filtered and degassed by helium before use.

Standards and standard solutions

Chloramphenicol (CAP)

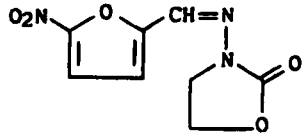
2,2-Dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide. CAP was purchased from Lepetit (Milano, Italy). The standard stock solution of CAP contained 1 mg/ml ethanol, the working standard solution 1 µg/ml ethanol.



Nitrofurans (NF)

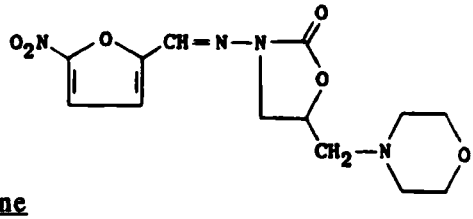
Furazolidone

3-[[(5-Nitro-2-furanyl)methylene]amino]-2-oxazolidinone



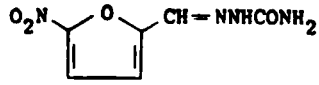
Furaltadone

5-(4-Morpholinylmethyl)-3-[[(5-nitro-2-furanyl)methylene]-amino]-2-oxazolidinone



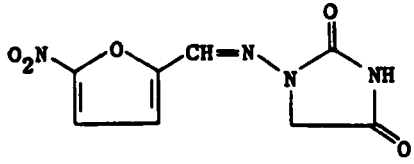
Nitrofurazone

2-[(5-Nitro-2-furanyl)methylene]hydrazinecarboxamide



Nitrofurantoin (internal standard)

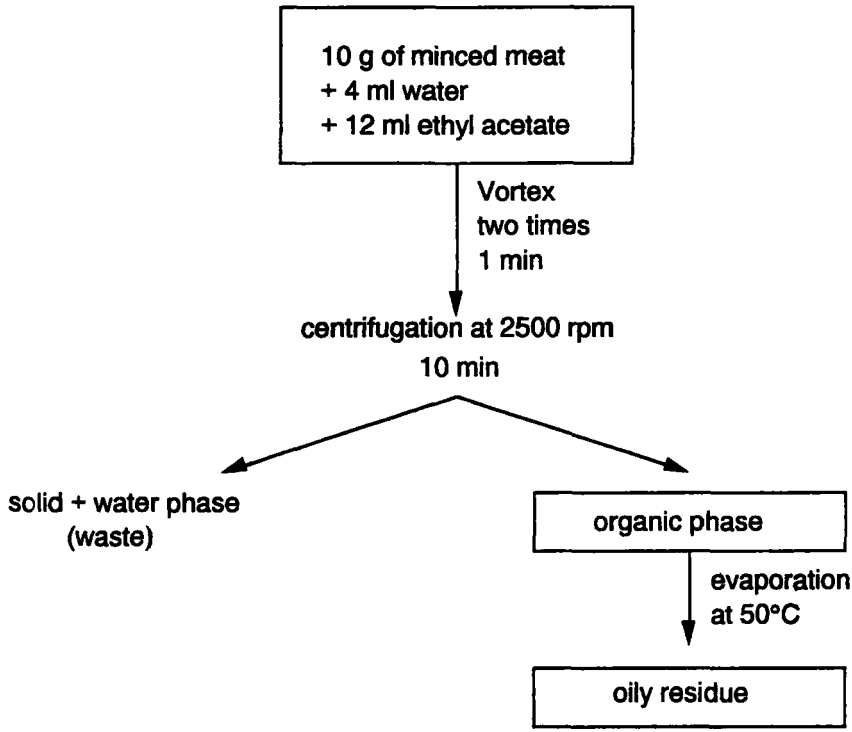
1-[[(5-Nitro-2-furanyl)methylene]amino]-2,4-imidazolidine-dione



Furazolidone, nitrofurazone and nitrofurantoin were purchased from Norwich Eaton (Belgium), furaltadone from Lab Smeets (Belgium). The standard stock solutions contained 1 mg NF/ml acetonitrile, the working standard solutions 1 μg NF/ml acetonitrile. The working standard solutions were used to spike the samples and to prepare dilutions containing 0.025 to 0.4 μg NF and CAP/ml eluent for the standard curves.

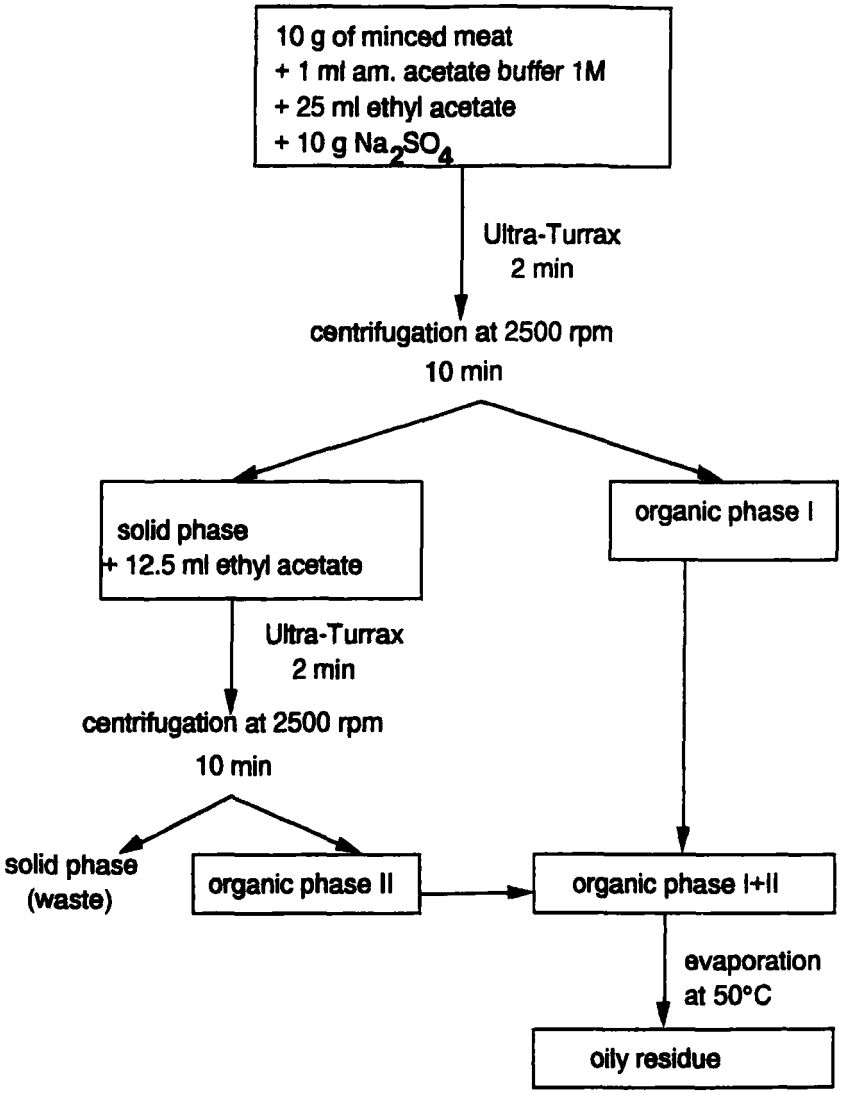
Sample preparation

Extraction procedure of chloramphenicol from meat and fish



10 g minced meat, 4 ml water and 12 ml ethyl acetate were vigorously mixed in a closed 100 ml glass centrifuge tube. After 10 min a second mixing action was repeated. The mixture was then centrifuged in a GLC-2B centrifuge at 2500 rpm during 10 min. The organic phase was transferred into a 10 ml glass tube and evaporated by a stream of nitrogen at 50°C in a Pierce Reacti-Therm heating module. About 400 µl of an oily residue were left in the tube.

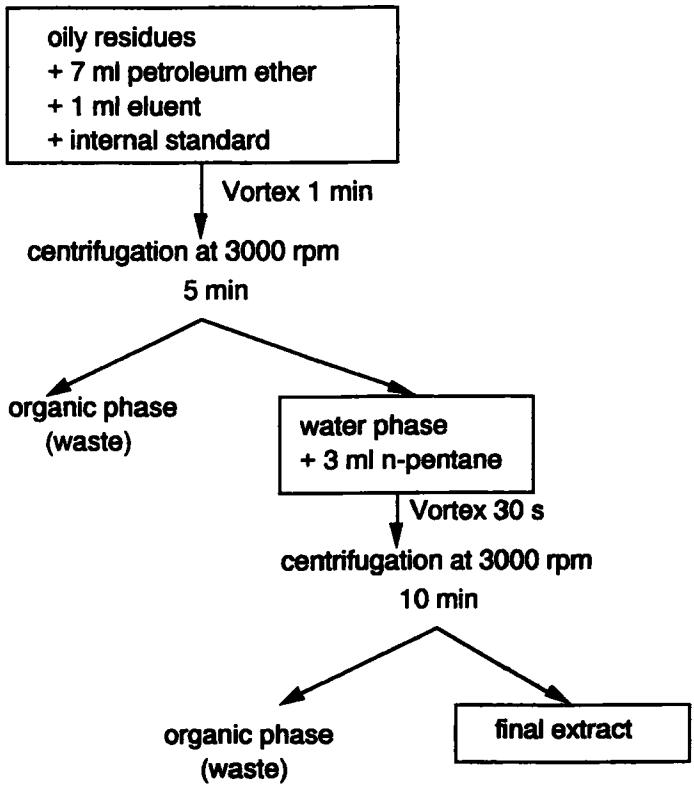
Extraction procedure of nitrofurans from meat and fish



10 g minced meat, 1 ml ammonium acetate buffer 1 M, pH 4.8, 25 ml ethyl acetate and 10 g sodium sulphate were mixed

in a 100 ml glass tube by means of an Ultra-Turrax during 2 min. The mixture was centrifuged in a GLC-2B centrifuge at 2500 rpm during 10 min. The organic phase was transferred into a brown round bottom flask. Light may damage nitrofurans. The solid material was mixed again with 12.5 ml ethyl acetate during 2 min and after centrifugation the organic phase was joined to the first part of the extraction. The extract was evaporated at 50°C using a Rotavapor-R. An oily residue was left.

Purification procedure of chloramphenicol and nitrofurans



The oily residue in the flask containing the NF was dissolved in 7 ml petroleum ether by shaking it. The flask was held during 1 min in an ultrasonic bath. The residue was then transferred into the 10 ml glass tube containing the residue of CAP. 1 ml eluent and 100 ng nitrofurantoin (internal standard) were added. The content of the tube was then shaken vigorously on a Vortex during 1 min. After centrifugation at 3000 rpm during 5 min the organic phase was discarded, 3 ml n-pentane were added to the water phase, the tube was shaken on a Vortex during 30 s and then centrifuged at 3000 rpm during 10 min. The organic phase was discarded and from the final extract 500 µl were transferred into a 2-ml autosampler vial. For some tissues fatty particles can be left at the surface of the final extract. In these cases an extra purification step with 1 ml hexane was necessary.

Chromatography

For the analyses of the extracts two Chromspher C-8 columns (100 x 30 mm, cat. no.: 28262) from Chrompack (USA) were joined together. The precolumn contained Perisorb RP-8 (30-40 µm) from Merck (Germany). The columns were conditioned at room temperature during 15 min using as eluent a 0.01 M ammonium acetate buffer pH 4.3 and acetonitrile (80 + 20, v/v). The flow rate of the eluent was 0.8 ml/min and the detection wavelengths were 280 nm for chloramphenicol and 360 nm for nitrofurans. For the analysis of the samples 100 µl of the extract were injected.

RESULTS AND DISCUSSION

For our experiments beef, porc and cultured trouts have been used as extraction material. To obtain an optimum ex-

TABLE 1

Correlation Coefficients and Confidence Intervals of CAP and NF

Added drug	Correlation coefficient	Confidence interval
Chloramphenicol	0.9988	± 1.2
Nitrofurazone	0.9988	± 2.2
Furazolidone	0.9997	± 4.0
Furaltadone	0.9989	± 2.7

traction of the NF several precautions have to be taken :

1. The extraction procedure must be carried out at a pH of 4.5-5.5. This working condition was obtained by adding 1 ml ammonium acetate buffer 1M.
2. The ethyl acetate phase must be dried with sodium sulphate.
3. Good recoveries are only obtained if the tubes on the Vortex are vigorously agitated.
4. An internal standard has to be added to the water phase to check the efficiency of the purification step.

The calibration curves for CAP and the NF were obtained by plotting the peak area versus the concentration of the standards. All plots were linear in the range of 0.02-0.4 µg/ml

TABLE 2

Recovery Data of CAP and NF in Meat and Fish by HPLC and Photodiode Array Detection

	Added drug	Added quantity (μg/kg)	Mean (n=5) (μg/kg)	Standard deviation	Coefficient of variation (%)	Recovery (%)
Meat :	Chloramphenicol	5	3.78	4.86	6.40	75.60
		10	7.20	1.47	2.00	72.20
		20	15.30	5.24	6.80	76.40
	Nitrofurazone	5	4.14	2.84	3.43	82.70
		10	8.42	2.66	3.16	84.20
		20	17.58	2.02	2.30	87.90
	Furazolidone	5	4.32	4.55	5.20	86.40
		10	8.51	4.20	4.90	85.10
		20	15.26	5.29	6.90	76.30
	Furaltadone	5	3.58	0.99	1.40	71.50
		10	7.06	2.59	3.66	70.60
		20	13.78	3.27	4.70	68.90
Fish :	Chloramphenicol	5	3.90	3.44	4.40	78.00
		10	7.43	2.19	2.95	74.30
		20	14.26	1.20	1.57	71.30
	Nitrofurazone	5	3.76	2.60	3.50	75.20
		10	8.55	4.47	5.23	85.50
		20	17.40	3.35	3.94	85.20
	Furazolidone	5	4.31	1.84	2.15	86.30
		10	8.49	5.89	6.90	84.90
		20	17.60	0.82	0.95	85.90
	Furaltadone	5	3.64	1.70	2.36	72.70
		10	7.05	2.58	3.66	70.50
		20	16.64	3.05	3.65	83.20

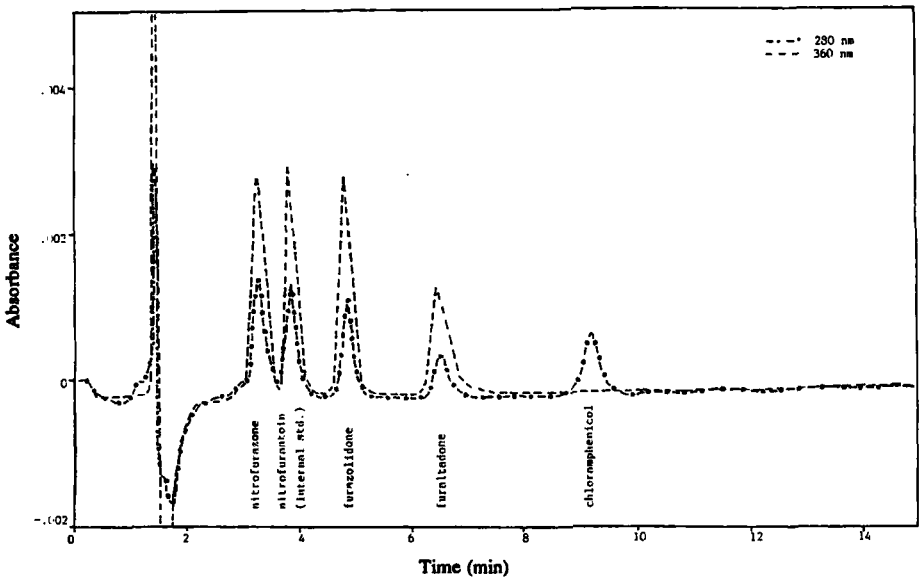


FIGURE 1 Chromatogram of a Standard Solution containing 10 ng CAP and 10 ng of each NF/100 ul Eluent.

working solution. The correlation coefficients and the confidence intervals for CAP and NF are given in table 1.

Recovery data, standard deviations and coefficients of variation for CAP and NF in spiked porc and trout muscle tissues are given in table 2. For each drug three different levels corresponding with 5, 10 and 20 µg/kg tissue were analyzed. The average value for each level has been calculated from 5 replicates. Recoveries varied from 69 to 88% for the materials tested. The quantitation limits for CAP and NF in meat and fish determined on spiked tissues were between 1 and 2 µg/kg. At this level the peaks of the compounds to be identified were undoubtly distinguished from the noise of the baseline.

With UV detection the absorption maxima of CAP and NF are characterized at a wavelength of 280 nm and 360 nm res-

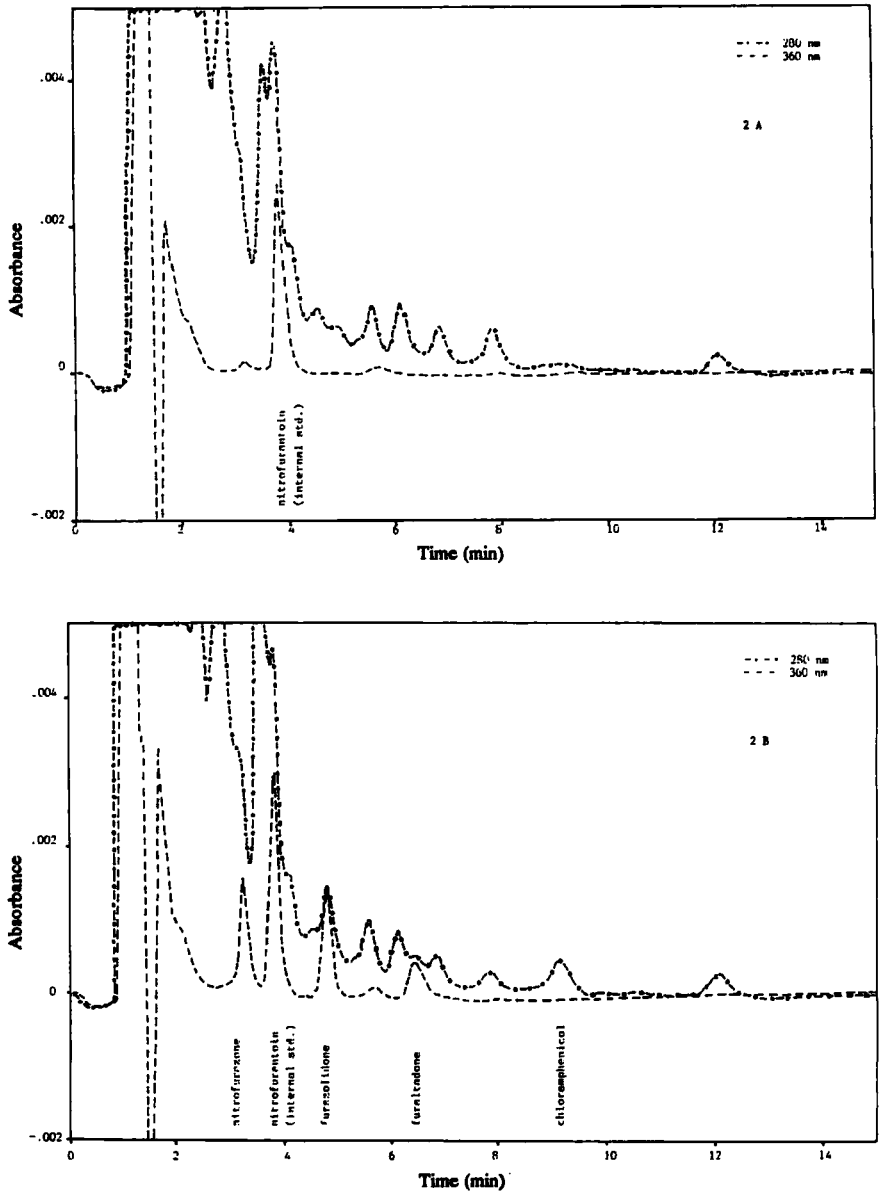


FIGURE 2 A) Chromatogram of a Blank Meat Sample containing 10 ug Nitrofurantoin/kg. B) Chromatogram of a spiked Meat Sample (- -) 5 ug CAP/kg; (- - -) 5 ug of each NF/kg; Internal Standard: 10 ug Nitrofurantoin/kg.

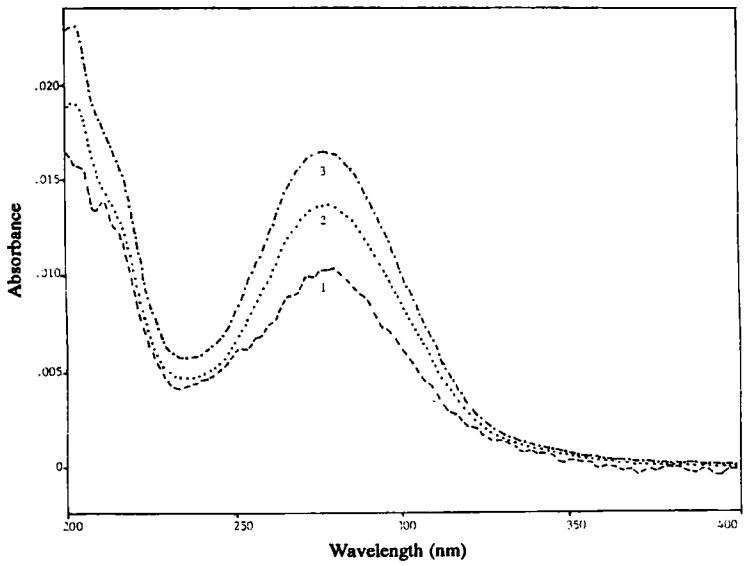


FIGURE 3 Ultraviolet Spectra of CAP. 1. CAP Standard (10 ng/100 ul Eluent); 2. Meat Sample containing 150 ug CAP/kg (150 ng/100 ul Extract). 3. Meat Sample containing 180 ug CAP/kg (180 ng/100 ul Extract).

pectively. The 4 compounds can be detected simultaneously. Figure 1 shows a chromatogram of a standard solution containing 10 ng CAP and 10 ng of each NF/100 µl. The analysis of a blank meat sample containing the internal standard is shown in figure 2A. A chromatogram of a blank meat sample fortified with 5 µg/kg of CAP and the nitrofurans is illustrated in figure 2B. The flow rate and the mobile phase were optimized in a way that the interfering peaks detected did not disturb the quantitation of the 4 substances. The retention time for CAP was 9.1 min. For the detection of nitrofurans a wavelength of 360 nm has been chosen. At this wavelength a high specificity for the detection of nitrofurans was obtained because no matrix effects were observed on the chromatogram of the meat sample analyzed. Retention times of 3.3 min for nitrofurazone,

3.8 min for nitrofurantoin (internal standard), 4.8 min for furazolidone and 6.4 min for furaltadone were registered. The chromatograms obtained for the extracts of fish were similar to those of meat.

The method has been applied for routine analysis with a surveillance program based on beef and porc tissues. Recently a program has been set up for the detection of these drugs in tissues of cultured fish. 600 samples of meat have been analyzed for NF. 1 sample contained 4 µg furazolidone/kg. 2000 samples of meat have been analyzed for CAP. 10 samples (0.5%) contained CAP between 2 and 180 µg/kg. Each positive sample has been confirmed by comparing the spectrum of the peak of the extract with the spectrum of the corresponding standard, which is shown in figure 3. The spectrum of 10 ng CAP/100 µl working solution has been superposed with the spectra of 2 meat samples containing 150 and 180 µg CAP/kg which corresponds to 150 and 180 ng/100 µl injection volume. The scale of absorbance of each spectrum on the graph was adjusted automatically by the computer to allow the comparison of the shape of the three spectra. Confirmation of the 4 drugs with photodiode array detection is possible until a level of 5 µg/kg product.

Finally, the results of this experiment show that the proposed method is reliable and suitable for the simultaneous residue analysis of CAP and NF in meat and fish.

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