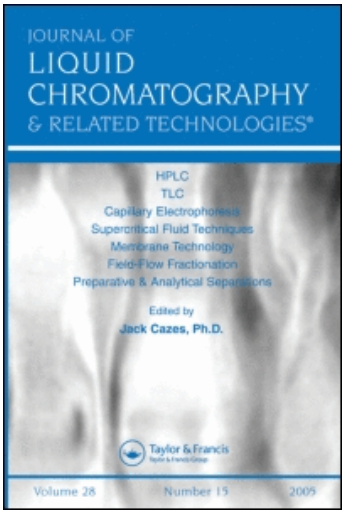

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Determination of the Metabolites of Nitrofuran Antibiotics in Meat by Liquid Chromatography-Mass Spectrometry

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ABSTRACT

A liquid chromatographic-mass spectrometry (LC-MS) method for the simultaneous determination of metabolites from furaltadone, furazolidone, nitrofurantoin, and nitrofurazone in meat is described. Samples were extracted with trichloroacetic acid (TCA) and further clean-up was achieved with solid phase extraction (SPE) after derivatisation with 2-nitrobenzaldehyde (NBA). The derivatised matabolites were eluted with acetonitrile–water and extracted with chloroform. The organic layer was evaporated to dryness and the residue was dissolved in water. The limits of detection varied from 0.2 to 0.5 ng/g.

Key Words: Nitrofuran antibiotics; Meat; Liquid chromatography-mass spectrometry.

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INTRODUCTION

The drugs furaltadone, furazolidone, nitrofurantoin, and nitrofurazone belong to the group of nitrofuran antibacterial agents that have previously been widely used in Europe in the prevention and treatment of gastro-intestinal infections caused by *E. Coli* and *Salmonella spp*.[1] in pigs, calves, and poultry. However, the 40th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1992 failed to establish an acceptable daily intake (ADI) for furazolidone. This was based on the evidence of carcinogenic and genotoxic effects of the parent drug and lack of data of the quantity and toxicity of the metabolites, including the bound residues.[2] Nitrofurazone was also evaluated at the same meeting with the same result.[3] Due to lack of data concerning the toxicity of furaltadone, nitrofurantoin, and nitrofurazone, the European Union (EU) prohibited the use of nitrofuran antibiotics (except furazolidone) in food-producing animals, by listing them in Annex IV of the Council Regulation 2377/90.[4] Furazolidone was included in Annex IV in 1995 since the manufacturers could not provide any favourable data justifying further use.[5] Since January 1997, the use of all nitrofurans to food-producing animals in the EU is no longer allowed.[4]

Nitrofuran antibiotics are rapidly metabolised in edible tissue, both in vivo and in vitro, with half-lives in the range of few hours.[6,7] Subsequently, analytical methods based on detection of the intact parent drug would be ineffective for monitoring compliance with the ban. Studies conducted with ^{14}C-labelled furazolidone have shown that protein-bound residues are formed, some of which contain the intact side chain 3-amino-2-oxazolidone (AOZ).[8–10] Analogue metabolites are later reported for the other three nitrofurans, 5-methylmorpholino-3-amino-2-oxazolidone (AMOZ) from furaltadone, 1-amino-hydantoin (AHD) from nitrofurantoin, and semicarbazide (SEM) from nitrofurazone.[11] These protein-bound nitrofuran metabolites may persist in edible tissue for a considerable time after treatment, and they can be released from the tissue proteins under moderately acidic conditions. Therefore, it is still possible to detect the metabolites even if the concentration of the parent drug is below their detection limit. EU requires that the analytical method used to determine the nitrofuran metabolites should be able to detect at least 1 μg/kg of each of the metabolites, AHD, AMOZ, AOZ, and SEM.[12] This so-called minimum required performance limit (MRPL), is used in order to harmonise the analytical performance of methods for determining substances for which no permitted limit can be established. Most analytical methods reported in the literature are based on UV or mass spectrometric (MS) detection of the 2-nitrobenzaldehyde (NBA) derivatives of some, or all,[1] of the four metabolites.[13–15] Neither of these reported methods could detect all four metabolites at the MRPL.

The intention of the present study, was to develop a simple and sensitive liquid chromatographic-mass spectrometry (LC-MS) method for the determination of AHD, AMOZ, AOZ, and SEM in meat down to the concentration levels required by EU.

EXPERIMENTAL

Materials and Reagents

Samples of drug free chicken and swine meat, egg, and shrimp were used in the spiking experiments.

All chemicals and solvents were of analytical or HPLC grade. The standard of AHD, AMOZ, AOZ, and the NBA derivatives of AHD (NPAHD), AMOZ (NPAMOZ), AOZ (NPAOZ), and SEM (NPSEM) were supplied by Chemical Synthesis Services (Belfast, Northern Ireland, UK, http://www.css-almac.com). SEM was supplied by Aldrich Chemical Company, Inc. (Milwaukee, WI). Stock standard solutions of 1 mg/mL of each of the metabolites were prepared in water.

Working standard solutions comprised of 0.1 μg/mL of each metabolite, were made by mixing aliquots of these stock solutions and diluting them with water. Stock standard solutions of NPAHD, NPAMOZ, and NPAOZ (1 mg/mL) were prepared in methanol, while NPSEM (100 μg/mL) was prepared in acetone–tetrahydrofurane (6 + 4). All solutions were left in an ultrasonic bath for 5 min. The mixed working standard solutions (0.1 μg/mL) were made by aliquots from these stock solutions and diluted with water.

Stock and working standard solutions were stored at +4°C. Trichloroacetic acid (TCA) was supplied by Merck (Darmstadt, Germany), and 85% TCA was prepared by dissolving 85 g TCA in 15 g water and stored at +4°C. NBA and dimethylsulphoxide (DMSO) were supplied by Sigma (Steinheim, Germany).

Solution A, consisting of 0.5 M $Na_2HPO_4 \cdot 2H_2O$, was made by dissolving 44.5 g of $Na_2HPO_4 \cdot 2H_2O$ in ca. 450 mL of water at 35°C. The solution was cooled to room temperature before the pH was adjusted to 6.0 with 85% phosphoric acid. The pH adjustment was repeated after further dilution to volume (0.5 L).

Solid phase extraction (SPE) columns Oasis® HLB 3 cc (60 mg) were purchased from Waters (Ireland) and Spin-X centrifuge filter units (0.22 μm, nylon type) (Costar, NY) were used for filtration.

Chromatographic Conditions

The LC-MS instrumentation used for the method development, consisted of a Series 200 quaternary pump and autosampler (Perkin Elmer, Norwalk,

CT) and an API 100 single quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada) equipped with a Turbo-Ion Spray ion source. The turbo probe of the interface was maintained at 200°C and the probe air flowrate was 6 L/min. The N_2 nebulizer and curtain gas was 5 and 10 L/min, respectively. The MS was set to collect single-ion data in negative ion mode at m/z 246.9 for NPAHD for 7 min and then in positive ion mode at m/z 335.2, 236.1, and 209.2 for NPAMOZ, NPAOZ, and NPSEM, respectively, for 19 min. The electrospray voltage was set at −3000 V in negative ion mode and 4500 V in positive ion mode. The entrance electrode voltages and the position of the ion spray inlet were adjusted to provide optimum intensity for the molecular ions. The acquired data were entered into a Model 8500 Apple Power Macintosh, and processed with either Multiview 1.4 or MacQuant 1.6 software packages (Applied Biosystems), for spectral information and quantification data processing.

The analytical column (stainless steel 3.0 × 50 mm) and the guard column (3.0 × 20 mm) was packed with 3.5 μm, particles of XTerra® MS C_{18} from Waters (Milford, MA), and they were operated at a constant temperature of 23°C. The guard column was connected to an A-318 precolumn filter online with an A-102X frits (Upchurch Scientific, Oak Harbor, WA). The mobile phase consisted of a mixture of two solutions, B and C. Solution B consisted of 985 mL water + 15 mL acetonitrile + 100 μL NH_3 (25%), while solution C was acetonitrile. The mobile phase operating conditions are shown in Table 1. After separation, the LC eluent was split approximately 1 : 20 before entering the MS interface.

Sample Pretreatment

Volumes of 1 mL water or standard (the total volume should always be 1 mL), 3 mL hexane, and 2 mL 85% TCA were added to 3 g of sample. The mixture was homogenized for approximately 15 sec with an Ultra-Turrax

Table 1. Mobile phase operating conditions.

Step	Time (min)	Gradient curve	Flow (μL/min)	Solution B (%)	Solution C (%)
1	0.1	0	700	100	
2	12	1	700	71	29
3	6	0	600	100	
4	7	0	1,200	100	
5	1	0	700	100	

TP 18/10 (Janke & Kunkel KG, Germany). After centrifugation for 5 min at 5000 rpm, the upper layer (hexane) was discharged. From the water-based phase, 3 mL (corresponding to 1.5 g sample) was transferred to a glass-stoppered centrifuge tube and 100 μL freshly prepared NBA solution (0.1 M in DMSO) was added and the sample was mixed. The reaction mixture was kept for 16 hr (overnight) in a waterbath at 37°C. After this period of time, samples were removed from the waterbath and the pH value was adjusted to about 6.0 by addition of 2 mL solution A, followed by 4 mL 2 M NaOH, and 1 mL solution A again. The sample was blended and centrifugated for 4 min (4500 rpm). The supernatant was loaded on a conditioned Oasis column.

Clean-Up SPE Column

The column was conditioned with 3 mL methanol, followed by 3 × 3 mL water (column volumes) before the aqueous extract was applied. Conditioning and application of the sample took place under gravity flow (dropwise rate). The column was washed with 3 × 0.5 mL water at a vacuum of −5 in. Hg, and suctioned to dryness for 10 sec at a vacuum of −10 in. Hg. The elution of the analytes was achieved with 0.5 mL and 2 × 1 mL acetonitrile–water (70 + 30) at a vacuum of −5 in. Hg. After the eluting solvent had passed through, the column was suctioned to dryness for 5 sec. To the eluate, 3 mL $CHCl_3$was added and the mixture was shaken vigorously for approximately 15 sec. After centrifugation for 3 min (3500 rpm), the upper water layer was discarded. The organic layer was transferred to another glass-stoppered tube with a Pasteur pipette to avoid water residues.

The organic layer was evaporated to dryness under a stream of air at 40°C. To the dry residue, 500 μL of water was added, mixed with a whirlimixer for 6 sec, left in an ultrasonic bath for 3 min to reconstitute the residue, mixed, and then filtered through a Spin-X centrifuge filter.

Aliquots of 75 μL were injected into the LC-MS at intervals of 26 min for the determination of AHD, AMOZ, AOZ, and SEM.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for AHD, AMOZ, AOZ, and SEM were determined by spiking swine meat samples with standard solutions to yield 0.2, 0.3, 0.5, 0.6, 0.8, 1.0, 2.0, 5.0, and 7.0 ng/g. The recovery was determined by comparing the analyses of spiked meat with those of standard solutions of NPAHD, NPAMOZ, NPAOZ, and NPSEM. The linearity of the

standard curves for AHD, AMOZ, AOZ, and SEM in meat was calculated using peak height measurements.

RESULTS AND DISCUSSION

The standard curves were linear in the investigated areas from 1 to 7 ng/g for AHD, AMOZ, AOZ, and SEM. The linear correlation coefficients were $r = 0.999$ for AHD, AMOZ, and SEM, and 0.998 for AOZ. The recovery and repeatability values for AHD, AMOZ, AOZ, and SEM from swine meat are shown in Table 2.

Chromatograms obtained from drug-free meat samples, and from the corresponding samples spiked with AHD, AMOZ, AOZ, and SEM, are shown in Figs. 1–4.

The limits of detection for the metabolites were calculated as three times the peak-to-peak baseline noise ($S/N = 3$) from drug-free swine tissue, chicken tissue, shrimp, or egg. They were 0.5, 0.4, 0.2, and 0.3 ng/g for AHD, AMOZ, AOZ, and SEM, respectively, regardless of matrix.

The precision, recovery, and linearity of AHD, AOZ, AMOZ, and SEM in chicken, shrimp, and egg samples were not validated in this study, but extracts from those matrixes showed very similar baseline resolution to swine extracts.

The derivatisation procedure was found to be completed after a reaction time of approximately 15 hr, no further increase of analyte signals was observed after this period. This is in accordance with what is reported in the literature.[1,13]

The presence of NH_3 in the mobile phase improved the ion signal from the analytes when compared with ammonium acetate or acetic acid. But, the high pH limited the choice of column. Three reverse phase columns were, however,

Table 2. Recovery and repeatability for AHD, AMOZ, AOZ, and SEM from spiked samples of 3 g swine meat.

		AHD (%)		AMOZ (%)		AOZ (%)		SEM (%)	
n[a]	Amount of drug (ng/g)	SD[b]	RC[c]	SD	RC	SD	RC	SD	RC
6	0.6	—	—	1.2	50	2.2	49.3	3.7	76.8
6	1.0	3.5	91.5	1.0	50.7	2.2	44.3	1.8	87.1
6	5.0	0.8	98.9	1.5	58.3	3.5	50.8	1.4	73.9

[a]Number of samples.
[b]Standard deviation.
[c]Recovery.

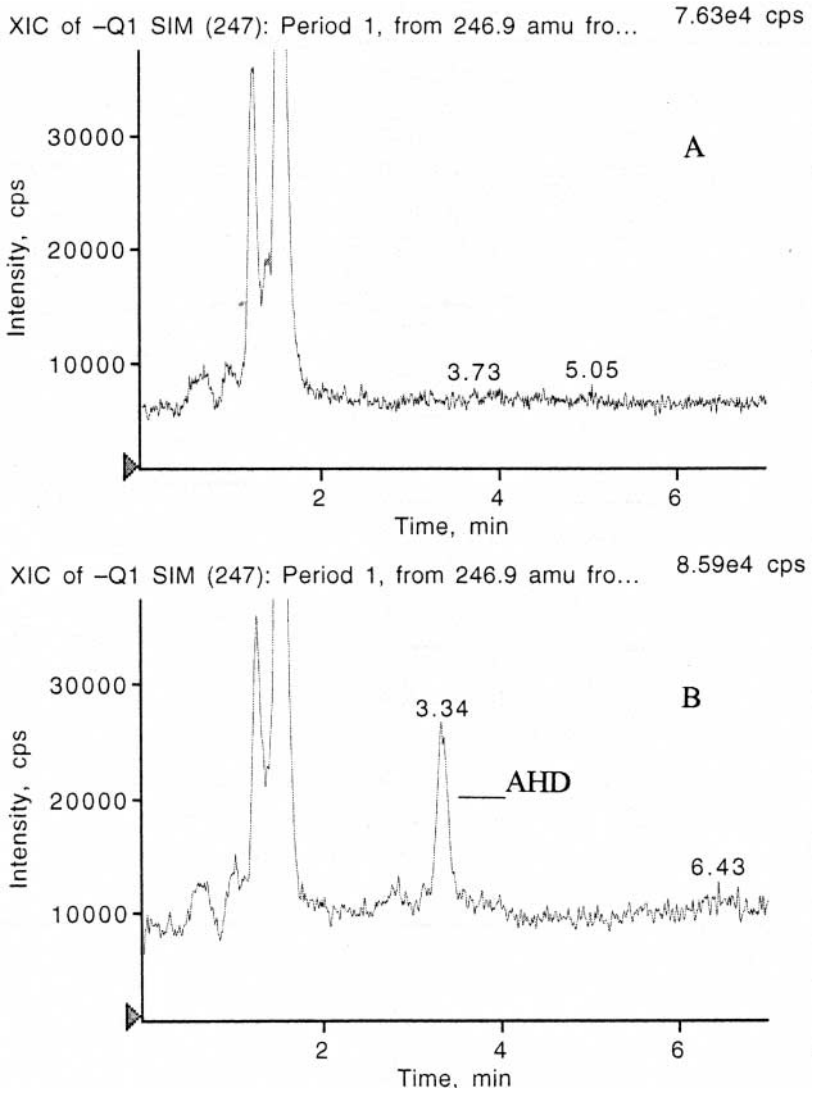

Figure 1. Chromatograms of extract from swine meat. A, AHD-free meat; B, meat spiked with AHD (2 ng/g).

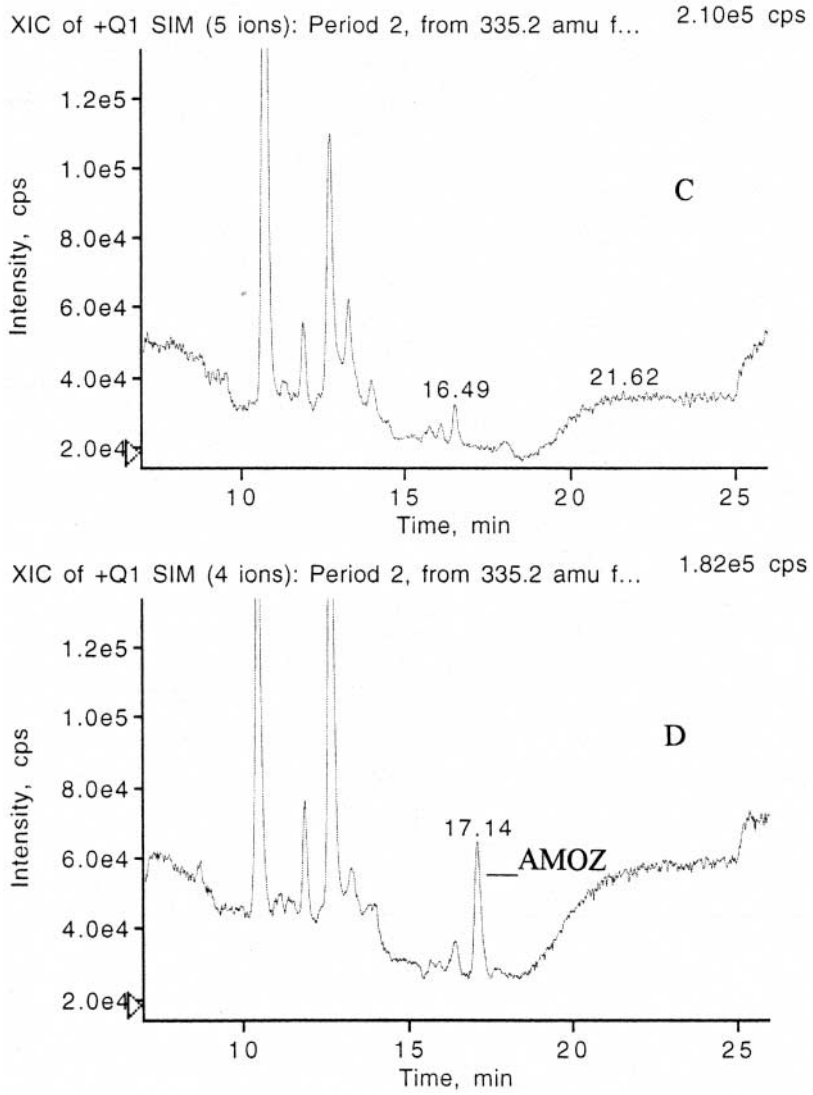

Figure 2. Chromatograms of extract from swine meat. C, AMOZ-free meat; D, meat spiked with AMOZ (2 ng/g).

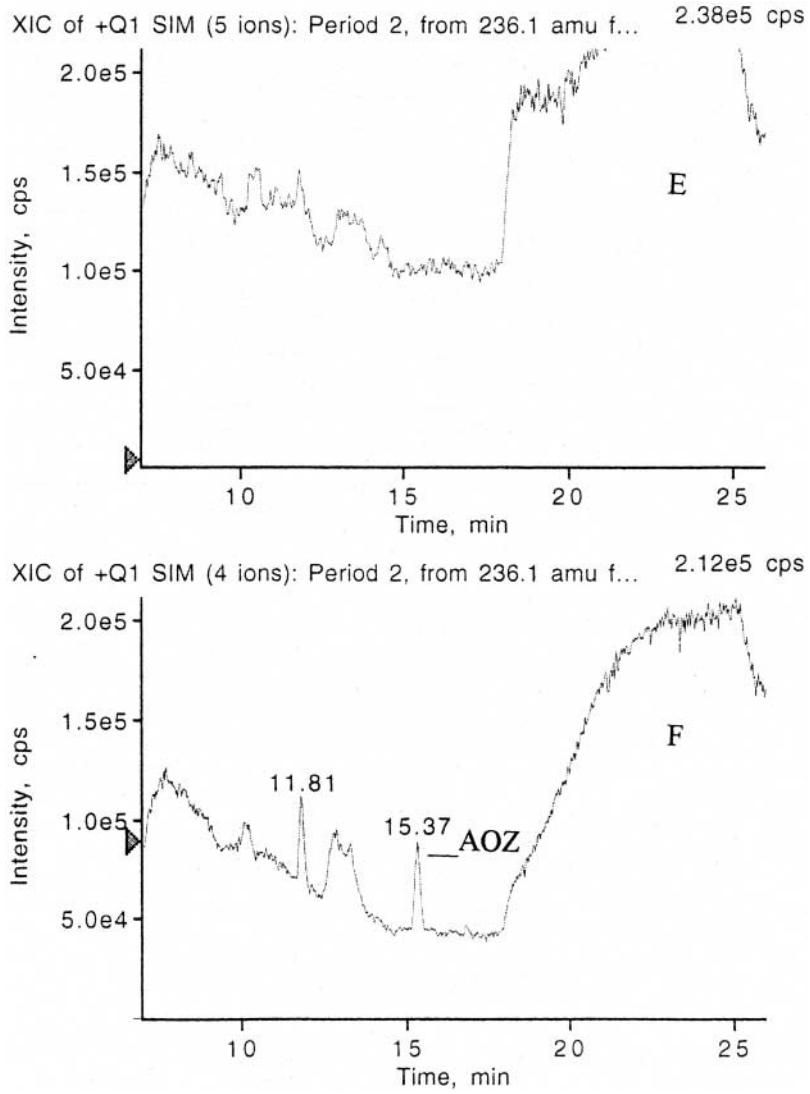

Figure 3. Chromatograms of extract from swine meat. E, AOZ-free meat; F, meat spiked with AOZ (2 ng/g).

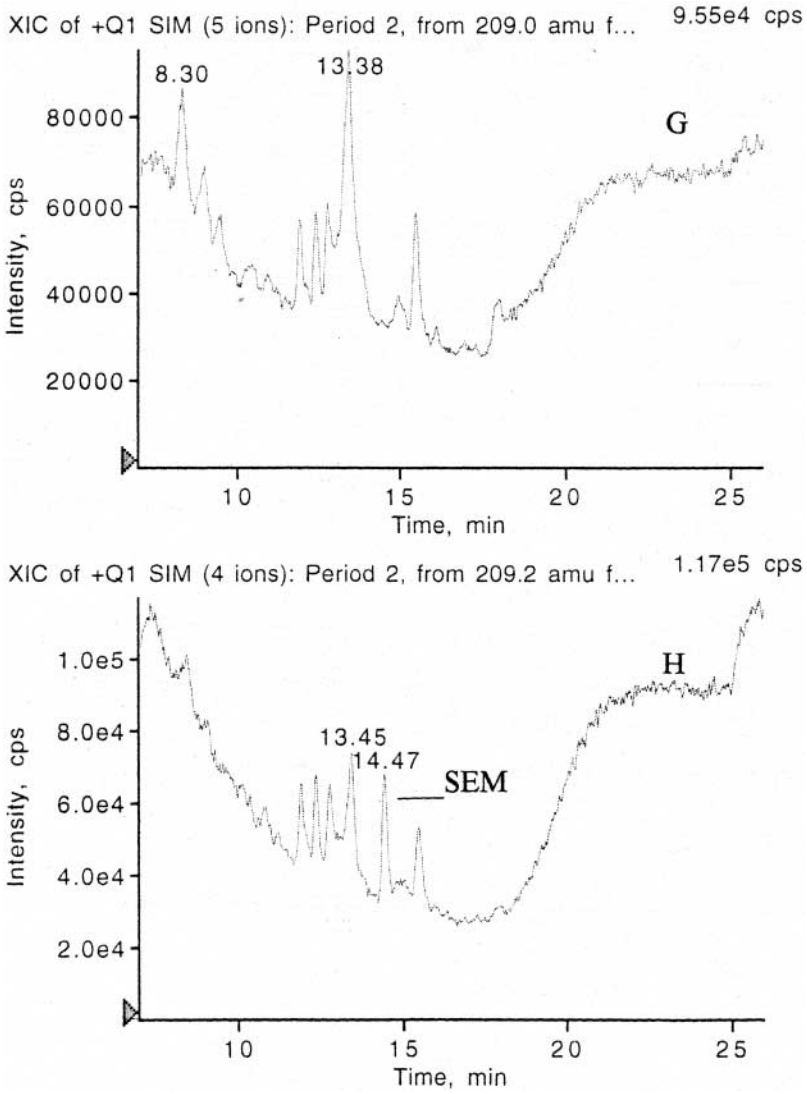

Figure 4. Chromatograms of extract from swine meat. G, SEM-free meat; H, meat spiked with SEM (2 ng/g).

evaluated, PLRP-5 μm (150 × 4.6 mm^2) from Polimer Laboratories (Shropshire, UK), XTerra® RP_{18}, 3.5 μm (3.0 × 50 mm^2), and XTerra® MS C_{18}, 3.5 μm (3.0 × 50 mm^2) from Waters (Milford, MA). The XTerra® MS C_{18} column exhibited the best separation characteristics.

In many laboratories, a stream of nitrogen is used to evaporate samples to dryness. In this study, air produced from a central air compressor was used for evaporation. The use of air is a practical and economically favourable alternative compared with nitrogen, when the analytes of interest are not easily oxidised.

The described method offers a number of significant advantages compared with previously published methods for the detection and quantification of AHD, AMOZ, AOZ, and SEM. The detection limits are below the EU requirement of an MRPL at 1 ng/g for each of the nitrofuran metabolites.

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