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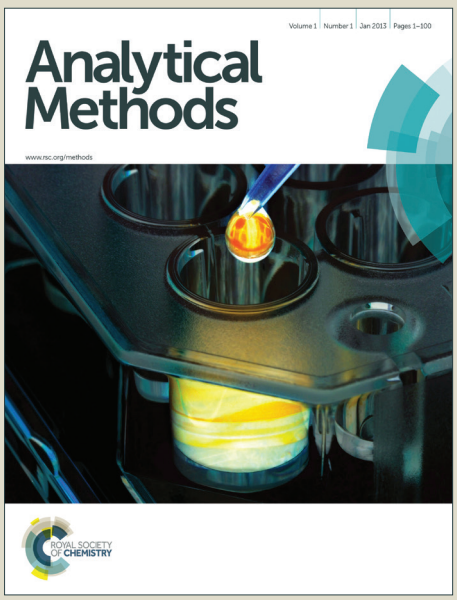

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A sensitive LC-MS/MS method employing a THF/water solvent system for the determination of chloramphenicol, thiamphenicol and florfenicol in bovine muscle

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A THF/water solvent system was used in the development of a method for the LC-MS/MS determination of chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FFC) in bovine muscle. The tetrahydrofuran (THF)/water solvent system was recently demonstrated to possess superior figures of merit compared to either the methanol/water or acetonitrile/water solvent systems which are almost exclusively used in LC-MS for the determination of fenicols in food producing animals. The figures of merit included ease of de-solvation when electrospray ionization (ESI) is employed in the negative mode thus leading to more intense mass spectral signals. This phenomenon was shown to be due to greater association of the methyl groups in methanol or acetonitrile with the amide nitrogen common in all the three fenicols. This association is absent in a THF/water solvent system since it's devoid of methyl groups that can easily interact with the amide nitrogen. As a result of the use of THF/water solvent system, the method detection limits (MDLs) were 0.047, 2.1 and 4.3 µg/kg for CAP, TAP and FFC respectively while the limits of quantitation were 0.141, 6.3 and 12.9 µg/kg for CAP, TAP and FFC respectively. The decision limits i.e. CC_{α} values according to Commission Decision 2002/657/EC were 0.36, 50 and 111 µg/kg for CAP, TAP and FFC respectively. Linearities were also within acceptable values i.e. 0.9983, 0.9916 and 0.9996 for CAP, TAP and FFC respectively.

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Introduction

Chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FFC), classified as fenicols are broad-spectrum antibiotics. They have been extensively used as veterinary drugs for the treatment of a wide variety of infections. Due to their haemopoietic system toxicity[1], and other deleterious effects hinging on bacterial resistance, their applications are regulated in many countries.[2] The chemical structures of CAP, TAP and FFC are shown in Figure 1 below.

INSERT FIGURE 1

The strict regulation of their use in many countries raises the need to develop and update existing methods of detection with lowering detection limits, reducing the time and the cost of analysis as part of the goals.

In a recent study in our laboratory and for the first time, we demonstrated figures of merit for using tetrahydrofuran (THF)/water as a solvent system in contrast to methanol/water or acetonitrile/water solvent systems for electrospray ionization (ESI) and liquid chromatography (LC) separation of CAP, TAP and FFC followed by subsequent detection by mass spectrometry (MS).[3] Some of the figures of merit were the ease of solvent de-clusterization in the tube lens region after employing electrospray ionization (ESI). This was shown to be due to lack of methylation of the analytes which occurs when methanol/water and/or acetonitrile/water are used as solvents systems. The study also revealed that using the THF/water solvent system and carefully optimizing parameters such as the tube lens voltage (TLV), enhanced signals as much as 94 % in some cases for ions of the same *m/z* ratio were obtained compared with methanol/water or acetonitrile/water solvent systems. Adduct formation was also observed where $[M + Cl]^-$ type of ions were obtained as base peaks at certain TLVs. This subsequently motivated the development of a method for the determination of the three fenicols based on a THF/water solvent system instead of the methanol/water or acetonitrile/water solvent systems. As a precaution, it has been recommended that THF should be used in combination with water in order to forestall fire hazards[3] and as well as reduce its reactivity with polymeric materials. FFC has also been reported to have excellent solubility characteristics in THF compared to methanol[4] and due to the structural relationship between the three analytes, we assumed relatively good solubility for the other two analytes i.e. TAP and CAP.

From literature[5-11], all the methods that have been developed to date traditionally used a combination of either methanol/water or acetonitrile/water for liquid chromatography and recently in combination with mass spectrometry (MS) for the analysis of CAP, TAP and FFC. In some cases gas chromatography-mass spectrometry (GC-MS) has been used for the determination of the fenicols after derivatizing the analytes.[12]

We report here a method for the LC-MS determination of fenicols in bovine muscle taking advantage of the figures of merit described herein using the THF/water solvent system. Method validation was accomplished by using the Commission Decision 2002/657/EC criteria. A comparison of some analytical characteristics from literature of some methods based on methanol/water or acetonitrile/water solvent systems was also done.

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Experimental

Reagents and Materials

CAP was purchased from Sigma-Aldrich, Germany. FFC, d_5-CAP, TAP, Isooctane and chlorobutane were donated by the International Agency for Atomic Energy (IAEA) through the National Veterinary Laboratory in Gaborone, Botswana. The purity of the standards was 99.9% Vetranal® a registered trademark of Sigma-Aldrich Biotechnology LP and Sigma Aldrich Company. HPLC grade methanol, ethyl acetate and THF were purchased from Merck, (Modderfontein, South Africa). Analytical grade acetic acid was purchased from Sigma Aldrich (St. Louis, MO, USA). Ultra high purity water was processed through a MilliQ Ultrapure Ionex Gradient A10 purification system (Millipore Cop, Bedford, MA, USA). 0.45µm PVDF Whatman syringe filters were purchased from Whatman international Ltd (Maidstone, England). Ultramark, caffeine and L-methionyl-arginyl-phenyl-anaineacetate.H_2O (MRFA) for tuning and calibrating the mass spectrometer were purchased from Thermo Fisher Scientific (Edenvale , South Africa).

Instrumentation

An Agilent HP 1100 series high performance liquid chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler, automatic degasser, a quaternary pump, a column thermostat and a diode array detector (DAD) was used for all separations. The HPLC is interfaced to a Thermo Scientific Finnigan LCQ DECA Quadrupole Ion Trap Mass Spectrometer with an ESI ion source (Thermo Scientific, San Jose, CA, USA). XCalibur version 2.0. acquisition software (Thermo Scientific, San Jose, CA, USA) was used to operate the LC-MS system. Ultrahigh purity nitrogen (Aflox, Germiston, South Africa) was used as a sheath and auxiliary gas. An XTerra column MS C_{18} 100mm x 2.1mm i.d. 5µm purchased from Waters (Milford MA, USA) was used throughout for separating the fenicols. A Sartorious Super- micro balance (Sartorious GmbH, Goettingen, Germany) was used to weigh analytical standards. The samples were centrifuged in a Thermo Scientific Medilite 12 Benchtop Centrifuge. Turbo VapII Zymark Automatic concentrator (Zymark Corp, Hopkinton, MA, USA) was used for evaporating the solvents during sample preparation.

Standard Solutions

Stock solutions of each compound were prepared separately in tetrahydrofuran at concentration of 1000 ppm. The stock solutions were prepared by weighing 5 mg of the compound and dissolving in 5 mL of solvent. The solutions were stored at -10 ^{0}C. Intermediate solutions in THF were prepared by diluting the stock solutions. Fresh final working cocktail solutions of all compounds for LC-MS/MS were prepared by diluting and mixing aliquots of the standard stock solutions with the 70:30 THF/water solvent.

Sample collection and storage

Bovine muscle was chosen as a matrix because the EU prescribes residue monitoring of fenicols in urine, milk and muscle and also because of ease of access. Pre-screened samples which were found to contain no fenicols were donated by the Botswana National Veterinary Laboratory. The samples were kept in a freezer at - 40°C to preserve them before preparation.

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Sample Preparation

Liquid extraction

A slight modification of the extraction employed by Agilent Technologies was used.[13] The bovine muscle samples were ground using a food mini chopper for 5 minutes before weighing 3 g of each into 50 mL Falcon tubes. 0.1 M sodium acetate buffer solution adjusted to pH 5 with glacial acetic acid was added to the sample and the mixture was homogenized using an Ultra-Turrax homogenizer at 9000 rpm for 2 minutes. The fenicols were extracted with 12 mL of ethyl acetate by first thoroughly shaking the mixture for 15 minutes in a reciprocal shaker, followed by centrifugation at 4000 rpm at 5 ^{0}C for 5 minutes. The samples were at this time placed in a -70 ^{0}C freezer for 2 hours followed by decanting the upper organic layer into a glass test tube. The ethyl acetate was evaporated to dryness under a stream of nitrogen at 40 ^{0}C in a Turbo VapII Zymark Automatic concentrator. The residue was reconstituted with 1mL water before de-fattening with 2 mL of a 1:1 isooctane: chlorobutane mixture. The aqueous phase was collected and filtered through a 0.45 µm Whatman PVDF syringe filter directly into an HPLC vial and was ready for injection into the LC-MS system.

Mass Spectrometry

All mass spectrometric acquisitions were processed using the XCalibur version 2.0. acquisition software. Calibration and tuning of the mass spectrometer was accomplished using a solution of caffeine, MRFA and Ultramark. Acquisitions were done in the mass spectrometry/mass spectrometry (MS/MS) mode and the ESI source was operated in the negative mode implying that the ions observed throughout were deprotonated molecular ions of the type $[M-H]^-$. In the MS/MS mode, fragmentation of precursor ions was done using optimized collision energies, isolation windows and TLVs for each of the analytes. To obtain optimum MS/MS conditions for each analyte, individual pure standards were infused into the ESI source of the mass spectrometer and parameters of interest varied while noting those that gave the most intense signals. The collision energy, isolation window and TLV for the MS/MS parameters were varied for each standard to obtain the optimum values for each as seen in Table 1. The XCalibur acquisition software allows for segmentation of the analysis times to accommodate differences in retention times and the optimum conditions for each of the analytes which were different for the three analytes. To this effect, three retention time segments were created in the software according to the retention times of the eluting compounds and internal standard during the LC-MS/MS analysis. The voltages on the heated capillary, the ion optics and the electron multiplier were automatically set for each standard using the LCQ auto-tune function in the acquisition software and these saved as individual analyte tune files. These individual tune files were loaded in the scan segments that were created according to individual analyte retention times.

INSERT TABLE 1

The optimum isolation window for each standard was selected based on the *m/z* ratio which gave the highest ion current. The collision energy was optimized by varying the energies until the precursor ion was fragmented to 10 % of its original intensity. To avoid space charge and enhance sensitivity, the automatic gain control (AGC) was used in all the acquisitions. Helium gas was used as a buffer gas in the quadrupole ion trap (QIT). The sheath gas flow rate was optimized and set at 90 arbitrary units, the capillary temperature was set at 300 ^{o}C and the spray needle voltage was optimized and set at 5.00 kV.

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High Performance Liquid Chromatography

Working standard solutions (10 mg/L) were run using isocratic elution on an XTerra® column MS C_{18} 100mm x 2.1mm i.d. 5µm at a flow rate of 0.4 ml/min individually, to establish their retention times. After this, the standard solutions were mixed to form a working cocktail that was used for optimizing separation conditions. Optimization of the flow rate and gradient elution was done while focusing on improving the resolution and analysis time. An optimized gradient elution program shown in the Table 2 was used in all the separations.

INSERT TABLE 2

Analysis time was 10 minutes with a post run time of 3 minutes before the next run using the THF/ water solvent system as the mobile phase.

Quantitation

The product ion with the highest intensity was used in quantitative analysis procedures, to increase analytical sensitivity in the LC-MS/MS experiments. Quantitation was based on the peak areas of the standards as ratios of the internal standard for the construction of the calibration curves. The product ions with the highest intensities were used to get the peak areas in the reconstructed ion chromatograms (RICs). The RICs were obtained using the XCalibur software by auto-filtering the total ion chromatograms (TIC) using selected ions as scan filters.

Method validation

Method validation was carried out according to the criteria set by CD 2002/657/EC. The parameters taken into consideration were: response, linearity, trueness, precision, within laboratory reproducibility, decision limit (CC_α) and detection capability (CC_β). CC_α is the concentration above which a decision with a statistical certainty of 1-α can be made, that if a signal is detected, the identified analyte is truly present or above the maximum residue limit (MRL) for substances with an established level. α = 5% for compounds with established MRLs and 1% for compounds not authorized for use in food producing animals. CC_β is the concentration of analyte at which the method is able to detect violations with a statistical certainty of 1-β, where β = 5% and is analogous to "detection limit". The decision limit, CC_α was calculated using Equation 1 for authorised drugs i.e. TAP and FFC. For CAP which is prohibited it was calculated according to Equation 2 below. The detection capability, CC_β, was calculated using Equation 3 for TAP, FFC and CAP.[14-16]

$$CC_\alpha = \text{MRL} + (1.64 \times \text{in-house reproducibility at the MRL})\ \alpha = 5\ \% \qquad (1)$$

$$CC_\alpha = \text{MRL} + (2.33 \times \text{in-house reproducibility at the MRL})\ \alpha = 1\ \% \qquad (2)$$

$$CC_\beta = CC_\alpha + (1.64 \times \text{in-house reproducibility at the MRL})\ \alpha = 5\ \% \qquad (3)$$

All parameters in the equation are as defined previously except for the in-house reproducibility at the MRL which is the within laboratory standard deviation of seven replicates spiked at the MRL for each analyte. Precision batches were prepared as described herein. Three controls for each analytical batch were prepared. Each spiking level was represented by 7 replicates.[17-18] The samples that were used to prepare the validation batch were those which had previously been analysed and were found to be negative. Samples were spiked at half MRL (7replicates), at the MRL (7 replicates) and at twice the MRL (7 replicates) for TAP and FFC. For CAP which is

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unauthorized, they were spiked at the MRPL (7 replicates), at twice the MRPL (7 replicates) and at three times the MRPL (7 replicates). The spike levels were done equidistant according to Commission Decision 2002/657/EC. The resulting blank matrices were 0.3, 0.6 and 0.9 µg/kg for CAP, 25, 50 and 100 µg/kg for TAP and 50, 100 and 150 for FFC. Precision was calculated from the analysis of batches of fortified replicates and this was repeated on two occasions

Single analyst repeatability was calculated from the data. Single factor analysis of variance (ANOVA) was performed on the 14 resulting measured concentrations (7 replicates x 2) at each spiking level. The total repeatability was calculated as the within-batch variance. In house repeatability was calculated as total variance i.e. root sum of squares of within batch variance and between batch variance.

Results and Discussion

Accuracy and precision obtained using liquid extraction with ethyl acetate

Accuracy and precision were estimated by calculating the percent recoveries of the 3 analytes in spiked bovine muscle using 7 replicates on 2 validation days according to Equation 4 below.

$$\text{Mean \% Recovery} = \frac{\text{Mean concentration of PrEMS}}{\text{Concentration of PoEMS}} \times 100 \qquad (4)$$

Where PrEMS is an acronym for pre extraction matrix spikes i.e. spiked blanks and PoEMS is an acronym for post extraction matrix spikes i.e. spiked after extraction just before measurement.

INSERT TABLE 3

Percent recoveries and accuracies for the recovery of the three fenicols are shown in Table 3 along with the coefficients of variation. The mean recoveries for the three analytes ranged from 90 to 112 %. The within batch and inter-day variation, expressed as coefficients of variation (COVs) ranged between 5 and 15 %.

Decision limits (CC_α) and detection capabilities (CC_β)

Analyst inter day reproducibility, decision limits (CC_α) and detection capabilities (CC_β) were calculated and were as shown in Table 4.

INSERT TABLE 4

The calculated CC_α and CC_β values were within acceptable levels according to the criteria set by Commission Decision 2002/657/EC. In this decision CC_β for banned substances such as CAP must be as low as possible and less or equal to the MRLs for compounds with established MRLs such as TAP and FFC. The CC_β value for TAP in this case was equal to the MRL and nearly equal to the MRL for FFC. The lower precision of the method for all the fenicols resulted in correspondingly lower decision limits (CC_α). The implication of CC_β i.e. in CAP is that using the method, a measured concentration of 0.43µg/kg would have to be detected in a sample in order to demonstrate statistical confidence of MRL violation.

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Sensitivity, selectivity and linearities

The sensitivity and selectivity were examined by estimating the limits of detection and limits of quantitation (LOQs). These were calculated from the mean signal of 10 muscle blank sample. The LODs were calculated using Equation 5 as the mean of the blank concentrations plus three times the standard deviation of the concentrations of the 10 blanks. The LOQs were calculated using Equation 6 as means of blank concentrations times ten times the standard deviation of concentrations of the 10 blank signals.

$$\mathrm{LOD} = \mathrm{Mean}_{\mathrm{blank}} + 3s_b \quad (5)$$

$$\mathrm{LOQ} = \mathrm{Mean}_{\mathrm{blank}} + 10s_b \quad (6)$$

Where s_b is the standard deviation of the concentrations of the 10 blanks. The results are shown in Table 5 which also shows good linearities which were all greater than 0.990. No interferences were observed in the reconstructed ion chromatograms (RICs) after performing MS/MS of the sample extracts as shown in Figure 2.

INSERT TABLE 5

Variability of means at different fortification levels

One way ANOVA excel output i.e. single factor analysis showed no variations in the means of the batches even at low fortification levels. For instance, CAP at a fortification level of 0.3 µg/kg at p = 0.01, the ANOVA single factor output was F = 0.445476, p = 0.517121 and F_{crit} = 4.747225. For TAP and fortification level 25 µg/kg at p = 0.01, the single factor ANOVA output was F = 0.197713, p = 0.664487 and F_{crit} = 4.747225. For FFC and fortification level of 100 µg/kg at p = 0.01, the ANOVA single factor output was F = 2.853038, p = 0.116993 and F_{crit} = 4.747225. Similar ANOVA outputs at fortification levels of 0.6 and 0.9 µg/kg for CAP, 50 and 100 µg/kg TAP and 50 and 150 µ/g for FFC were obtained. In all instances, the means obtained at different fortifications for each analyte were not significantly different at p = 0.01.

MS/MS chromatogram of sample extracts using optimum conditions

MS/MS reconstructed ion chromatograms (RICs) of spiked bovine sample extracts showing TAP, FFC and CAP peaks using optimum separation and mass spectrometer conditions is shown in Figure 2. **The r**econstructed ion chromatograms (RICs) were obtained using a spiked bovine sample extract containing TAP spiked at 25 µg/kg, FFC spiked at 50 µg/kg, d_5-CAP spiked at 0.3 µg/kg and CAP spiked at 0.3 µg/kg. The scan filters used for each analyte were *m/z* 227 for TAP, *m/z* 336 for FFC, *m/z* 326 for d_5-CAP spiked and *m/z* 194 for CAP.

INSERT FIGURE 2

Figure 3 shows the MS/MS reconstructed ion chromatograms (RICs) of a negative control i.e. blank sample using the same ions as in Figure 2 for scan filtration. Figure 3 was included here to demonstrate that the spiked blanks did not contain any of the three analytes.

INSERT FIGURE 3

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Comparison with analytical performance characteristics reported in literature

Table 6 shows analytical performance characteristics that have been reported in literature before as a contrast to what is reported in this study. The comparison was made only for methods employing liquid extraction. From Table 6, the use of THF/water as a solvent system for LC-MS for the determination of the three fenicols was clearly superior in many instances.

Conclusions

Deliberate efforts were made to develop a method that uses the THF/water solvent system for the determination of CAP, TAP and FFC in bovine muscle and validate it according to Commission Decision 2002/657/EC. This was done after excellent figures of merit in comparison with either a MeOH/water or CAN/water system were observed using ESI in LC-MS. Validation parameters were comparable and in some cases even better than those that have been reported in the literature before. THF proved to be very unstable when used in the neat form in addition to being a fire hazard but performed extremely well in mixtures with water which also lessens the fire hazards.

Acknowledgements

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Table 1. Optimized MS/MS parameters used in this study

Analyte	Precursor ion (*m/z*)	Isolation window (*m/z*)	Collision Energy Q (%)	Tube Lens Voltage (V)	Product ions
CAP	321	3	35	40	194, 257, 249, 237
TAP	354	2	34	40	282, 270, 240, 227
FFC	356	7	29	50	336

Table 2. Optimized separation parameters used in this study

Time (min)	Flow rate (ml/min)	% THF	% Water
0	0.4	30	70
2	0.4	30	70
10	0.4	90	10

Table 3. Accuracy values

	CAP	TAP	FFC
Fortification level(µg/kg)	0.3	25	100
Mean Recovery (%)	99	90	112
Maximum recovery (%)	119	114	143
Minimum recovery (%)	70	74	100
COV (%)	15	9	5

COV = Coefficient of Variation

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Table 4. Summary results for precision studies, CC_α and CC_β values

	CAP	TAP	FFC
MRL (µg/kg)	0.3	50	100
Mean concentration at MRL/MRPL(µg/kg)	0.3	35.2	100
Inter day reproducibility(µg/kg)	0.04	4.52	4.36
CCα(µg/kg)	0.36	50	111.0
CCβ(µg/kg)	0.43	69.2	118.0

*CC_α is the concentration above which a decision with a statistical certainty of 1-α can be made, that if a signal is detected, the identified analyte is truly present or above the maximum residue limit (MRL) for substances with an established level. α = 5% for compounds with established MRLs and 1% for compounds not authorized for use in food producing animals.

* CC_β is the concentration of analyte at which the method is able to detect violations with a statistical certainty of 1-β, where β = 5% and is analogous to "detection limit".

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Table 5. MDLs, LOQs and r^2 values estimated in the study

	CAP	TAP	FFC	Acceptable range
LOD (µg/kg)	0.047	2.1	4.31	< MRL/MRPL
LOQ (µg/kg)	0.141	6.3	12.9	< MRL/MRPL
Linearity	0.9983	0.9919	0.9996	> 0.990
Regression equation	y =2.416x-0.0786	y=0.309x+5.111	y= 0.366x-4.68	
Linear range	0.3-0.9 µg/L	25-100 µg/L	50-150 µg/L	

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Table 6. Comparison of validation parameters with other solvent systems reported in literature

Analyte	Solvent system	Matrix	Analytical Method	LOD (ng/g)	LOQ (ng/g)	CC_{α} (ng/g)	CC_{β} (ng/g)	Reference
CAP	THF/Water	Bovine muscle	LC-MS/MS	0.047	0.141	0.36	0.43	This method
TAP	THF/Water	Bovine muscle	LC-MS/MS	2.1	6.3	50.0	69.2	This method
FFC	THF/Water	Bovine muscle	LC-MS/MS	13.8	41.3	111	118	This method
CAP	ACN/water	Chicken muscle	LC-MS/MS	0.1	0.2	0.37	0.43	19
TAP	ACN/water	Chicken muscle	LC-MS/MS	1	3	57.4	65.8	19
FFC	ACN/water	Chicken muscle	LC-MS/MS	0.2	0.5	113.12	126	19

Liquid extraction of analytes from samples was used in all cases for sample preparation.

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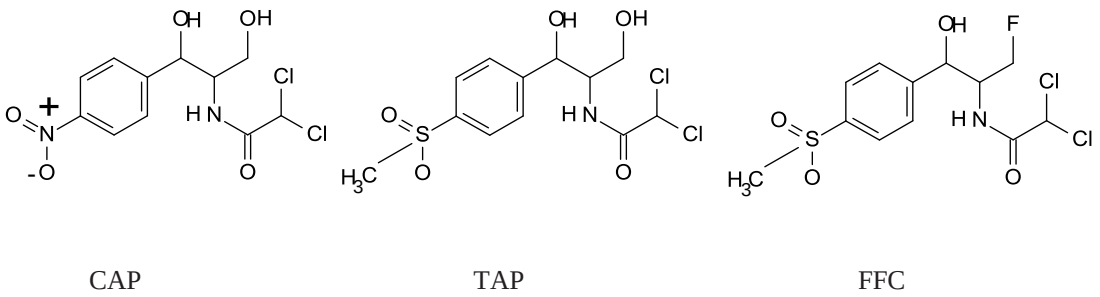

Fig. 1 Structures of CAP, TAP and FFC

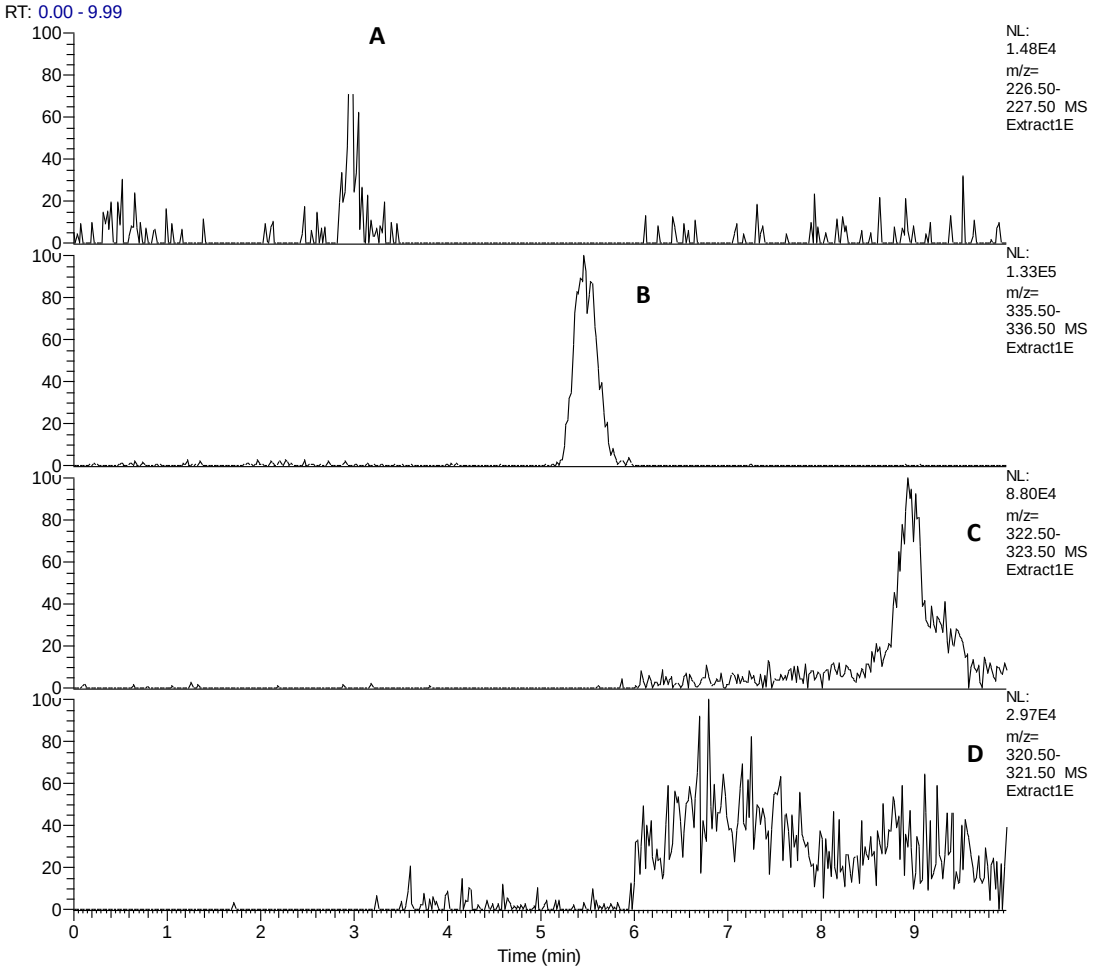

Fig. 2 MS/MS reconstructed ion chromatograms (RICs) of a spiked bovine sample extract (A) *m/z* 227 TAP spiked at 25 μg/kg; (B) *m/z* 336 FFC spiked at 50 μg/kg ;(C) *m/z* 326 d_5-CAP spiked at 0.3 μg/kg ;(D) *m/z* 194 CAP spiked at 0.3 μg/kg

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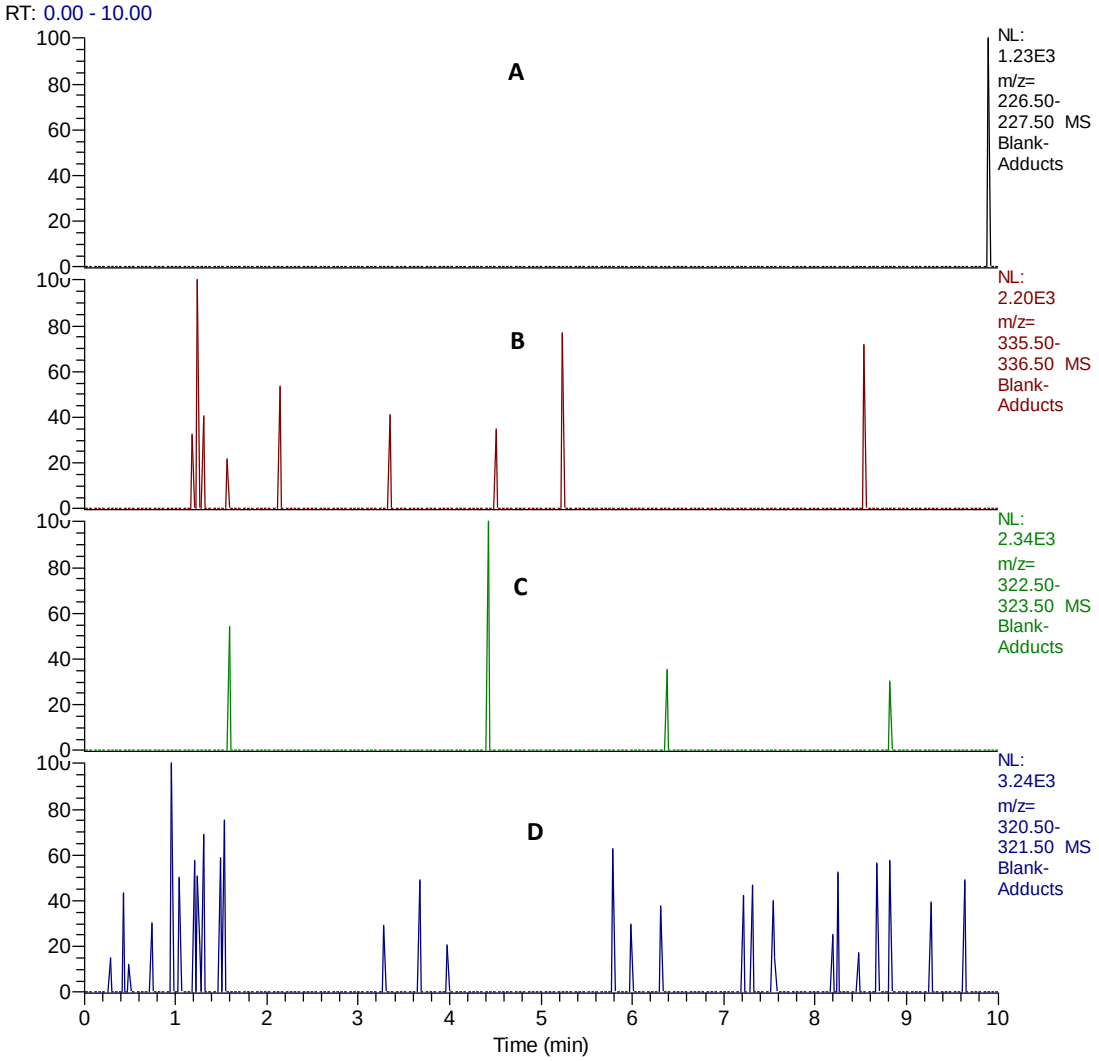

Fig. 3 MS/MS reconstructed ion chromatograms (RICs) of a negative control i.e. blank sample extract (A) *m/z* 227 TAP, (B) *m/z* 336 FFC; (C) *m/z* 326 d_5-CAP and (D) *m/z* 194 CAP

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