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A simple, fast and cheap non-SPE screening method for antibacterial residue analysis in milk and liver using liquid chromatography–tandem mass spectrometry

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ABSTRACT

In routine laboratory work, screening methods for multiclass analysis can process a large number of samples in a short time. The main challenge is to develop a methodology to detect as many different classes of residues as possible, combined with speed and low cost. An efficient technique for the analysis of multiclass antibacterial residues (fluoroquinolones, tetracyclines, sulfonamides and trimethoprim) was developed based on simple, environment-friendly extraction for bovine milk, cattle and poultry liver. Acidified ethanol was used as an extracting solvent for milk samples. Liver samples were treated using EDTA-washed sand for cell disruption, methanol:water and acidified acetonitrile as extracting solvent. A total of 24 antibacterial residues were detected and confirmed using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), at levels between 10, 25 and 50% of the maximum residue limit (MRL). For liver samples a metabolite (sulfaquinoxaline-OH) was also monitored. A validation procedure was conducted for screening purposes in accordance with European Union requirements (2002/657/EC). The detection capability (CCβ) false compliant rate was less than 5% at the lowest level for each residue. Specificity and ruggedness were also discussed. Incurred and routine samples were analyzed and the method was successfully applied. The results proved that this method can be an important tool in routine analysis, since it is very fast and reliable.

1. Introduction

Pharmaceuticals are widely used in veterinary medicine, including mainly antibacterials, applied for both therapeutic and prophylactic purposes. When a veterinary drug is administered, it is necessary to observe the withdrawal period to avoid the presence of drug residues in tissues, eggs, milk and other products of animal origin. Antibacterial residues in food are a risk to human health and could be an important vehicle for the development of bacterial resistance, besides toxicological, immunological and allergic problems in susceptible individuals [1–4].

Maximum residue limits (MRLs) for veterinary drugs in food were set by regulatory agencies and government authorities worldwide [5–7]. In Brazil, the National Residue Control Plan (NRCP) defines which residues must be monitored and their MRLs, aiming mainly to monitor the incidence of residues and prevent potential risk to a population exposed to those products [8,9].

Different tissues can be considered for residue analysis, depending on the animal species, drug pharmacokinetics and their physical–chemical properties. Milk is a universally consumed food, especially during childhood, and its safety is a permanent concern. Liver is organ responsible for enzymatic drug metabolism processes, and it is an appropriate matrix for monitoring veterinary drug residue. Liver can be considered the most complex matrix [10,11] and milk is a biological fluid that can be considered as a matrix, rich in proteins, lipids, carbohydrates, salts and minerals [12].

Tetracyclines (TCs), sulfonamides (SAs), quinolones (Qs), fluoroquinolones (FQs) and trimethoprim (TMP) are antibacterials widely used in veterinary practice because of their advantages, including low price and broad spectrum. Residue monitoring programs require improved methods able to detect non-compliant samples with residues above the MRL in order to ensure food safety. A high throughput screening method is also a very useful tool for routine

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analysis laboratories since a few samples are normally expected to be non-compliant. Screening methods are the best strategy in order to analyze a large number of samples in a short time, at the lowest possible cost. This is especially important in residue analyses in which costs are very important [13].

Microbiological and immune assays, as FAST, Premi®, Delvotest®, Charm® among other bioactivity-based methods have been most commonly used in antibacterial residue analysis, since they are quickly performed, but lack selectivity and specificity [14]. Furthermore, a high number of false negative or positive results was reported for this class of methods and it became necessary to use another method for confirmation [15,16].

Multiclass screening methods using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) are very useful and allow the development of simple, cheap and fast methods [14,17,18]. LC–MS/MS is currently a widely used analytical tool for multiclass veterinary drug residues in food since it is possible to detect a large number of analytes from different classes, with high selectivity and sensitivity and a decreasing rate of false negative and positive results, especially when the multiple reaction monitoring mode (MRM) is adopted. Another important work, recently published, covering sample preparation for the determination of drug residues in products of animal origin for analysis by LC–MS was described by Berendsen et al. In this work generic procedures for sample preparation were described and are quite useful for methods that include different classes of analytes [19].

Although several reports were published about antibacterial residues in milk analysis and other matrices, there were only a few reports on analysis of the liver [20–29]. Generally, even for screening purposes, almost all methods need a purification step using solid phase extraction (SPE) or SPE-like techniques. For instance, a recent report by Lehotay et al. describes a method for screening 9 aminoglycosides in liver using a SPE-like method using weak cation exchange adsorbent inside pipette tips [30]. Zhan et al. reported a screening method for 33 antibiotics in muscle using conventional SPE cartridges [31]. In other work, Freitas et al. applied liquid–liquid extraction and low temperature purification followed by dispersive SPE to analyze antibiotics in milk [32].

Generally, drug residue analysis methods were validated according to regulatory protocols or guidelines [7]. In Brazil, the National Residue Control Plan recommends the use of an internal validation manual which is in strict agreement with the 2002/657/EC Commission Decision [33]. However, these protocols have not yet been well established to validate screening methods. Several discussions about this specific validation mode have been published in recent years [34–37].

In a previous work, we report the development of a screening method for veterinary drug residue analysis in meat samples, using muscle as the target tissue [21]. Here, we present the development and validation of two screening methods that can analyze 24 antibacterial and one metabolite residue in milk and liver (poultry and cattle) using a non-SPE sample preparation for antibacterial residue analysis. The validation procedure was conducted for screening purposes, in accordance with the 2002/657/EC Commission Decision and the Brazilian Analytical Quality Assurance Manual [7,33]. Parameters such as detection capability (CCβ), stability, specificity, ruggedness and applicability were evaluated and determined.

2. Materials and methods

2.1. Chemicals

Analytical standards (all with purity ≥ 95%) of sulfadimethoxine (SDMX), sulfaquinoxaline (SQX), sulfadiazine (SDZ), sulfachlorpyridazine (SCP), sulfathiazole (STZ), sulfamerazine (SMR), sulfamethoxazole (SMA), sulfamethazine (SMZ), sulfadoxine (SDX), sulfisoxazole (SFX), chlortetracycline (CTC), tetracycline (TC), oxytetracycline (OTC), doxycyclin (DOX), oxolinic acid (OXO), nalidixic acid (NALI), flumequine (FLU), ciprofloxacin (CIPRO), difloxacin (DIFLO), enrofloxacin (ENRO), norfloxacin (NOR), sarafloxacin (SARA), danofloxacin (DANO) and trimethoprim (TMP) were purchased from Riedel–de-Haen (Buchs, Switzerland) or from Sigma-Aldrich (St. Louis, MO, US). Sulfapyridine (SPY), Demeclocycline (DEMO) and Enrofloxacin_D_5 (ENRO_D_5) were used as internal standards, one for each class.

Methanol and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Acetic acid, ethanol and formic acid were of HPLC grade J.T.Baker (Phillipsburg, NJ, USA). Ultrapure deionized water was produced by a Milli-Q apparatus (Millipore, Bedford, MA, US). Disodium ethylenediaminetetracetate (Na_2EDTA) was obtained from Sigma. Hydrochloric acid was purchased from Synth (SP, Brazil).

Stock standard solutions of each compound were prepared dissolving 10 mg of analytical standard in 10 mL of appropriate solvent. Methanol for tetracyclines, quinolones and sulfonamides (except for sulfachlorpyridazine, which was previously dissolved with some milliliters of acetone and methanol and a few drops of NaOH 1 M or acetic acid for fluorquinolones). Aliquots of each stock solution were diluted to obtain adequate final concentration.

EDTA 150 mM solution was prepared using 5.58 g of Na_2EDTA in 100 mL of ultrapure water. Sand was purified in house using ordinary sea sand washed twice with hydrochloric acid: water (1:2) followed by a washing step with EDTA 150 mM. Deproteinization solution for milk analysis was composed by ethanol with 4% acetic acid.

Blank samples were obtained from previously analyzed samples, purchased in local markets or collected by the Federal Inspection Service (FIS).

2.2. Instrumentation

LC-ESI–MS/MS measurements were carried out using an Agilent 1100 Series chromatographic system coupled to an AB Sciex API 5000 triple quadrupole mass spectrometer with an electrospray source in positive ionization mode. Compound-dependent parameter optimization was achieved through infusion of standard solutions of target compounds using a syringe with a flow injection of 10 μL min^{-1}. Each standard solution was prepared separately in methanol with formic acid 0.1% at 200 ng mL^{-1}. Ion source conditions were obtained through flow injection analysis (FIA) evaluation of a condition that presented good results for as many compounds as possible. Acquisition was performed in multiple reaction monitoring (MRM) mode.

Instrument control and data processing were carried out by means of Analyst 1.6.1 software. Separation was achieved in a Symmetry C18 LC column (75 mm × 4.6 mm; 3.5 μm particle diameter) from Waters and a C18 column (4.0 mm × 3.0 mm) was used as guard column (Phenomenex). Liquid chromatography parameters were also optimized. The flow rate adopted was 300 μL min^{-1}. Mobile phase is composed by solvent A (aqueous solution 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient elution program used was initially 98% of A decreasing to 2% in the course of 15 min (0–15 min). The A:B (2:98) was maintained for 2 min (15–17 min) and after that returned to initial composition A:B (98:2) (17–20 min). The total run time was 20 min and column equilibration time between each run was 3 min.

2.3. Samples

Commercial milk samples were purchased in local markets (Porto Alegre, RS, Brazil). Raw milk was obtained from different

producers from the South and Southwest regions of Brazil. Milk blank samples were obtained from previously analyzed samples for which no detectable amount of antibiotics was found. Samples were received frozen and were kept frozen (−20 °C). An aliquot of 0.5 mL of milk was used for analysis.

Liver samples of cattle and poultry were purchased from local markets or collected by the Federal Inspection Service (FIS). Liver blank samples were obtained from previously analyzed samples for which no detectable amount of antibiotics was found. Before analysis, they were homogenized using a food blender and stored at −18 °C until analysis. Samples were kept at room temperature until defrosted and an aliquot of 3 g was transferred into a 50 mL polypropylene centrifuge tube.

Spiked samples were prepared by adding the proper amount of working solution containing all analytes. After the spiking procedure, samples were stirred and allowed to stand for 10 min before extraction.

2.3.1. Sample extraction procedure—milk

Milk samples (0.5 mL) were placed in a 2 mL centrifuge microtube. Spiked samples were prepared by adding the proper amount (25.0 μL) of a working solution containing all the analytes at a concentration corresponding to 0.5 × MRL and 25.0 μL internal standard solution. Then, 20 μL of a 150 mM EDTA solution were added to prevent tetracyclines chelation (see Section 4). Mixing was performed in a vortex (10 s), and equilibration for 10 min protected from light. Deproteinization solution (0.6 mL of ethanol: acetic acid, 96:4 v:v) was added. Samples were mixed in a vortex for a few seconds, frozen (−18 °C) for 30 min and, then, centrifuged at 12,000*g* for 30 minutes. Then an aliquot (0.75 mL) of the supernatant was transferred to an HPLC vial. A solution composed by formic acid (0.1% in water) and formic acid 0.1% in acetonitrile (98:2) was added in order to complete 1.0 mL. An aliquot of 10 μL was injected into the LC–MS/MS system.

2.3.2. Sample extraction procedure—liver

Before the extraction procedure, cell disruption was achieved by adding 2 ± 0.05 g of previously treated sand, mixing with a glass stick. To this mixture, 500 μL of EDTA 150 mM was added, to avoid tetracyclines chelation. Sample was homogenized in vortex and rested for 10 min. Antibacterials were extracted using 1.0 mL of extraction solvent (methanol:water, 70:30 v:v, with formic acid 0.1%), mixed in a vortex and frozen in a freezer (−18 °C) for 30 min. Then, samples were centrifuged for 15 min at 3000*g* (5 °C), and an aliquot of supernatant (600 μL) was transferred to a microtube containing 400 μL of formic acid 0.1% in acetonitrile, mixed in a vortex and centrifuged for 30 min at 12,000*g* (5 °C). Another aliquot (600 μL) of the supernatant was transferred to a microtube containing 600 μL of initial mobile phase (formic acid 0.1% in water: formic acid 0.1% in acetonitrile) (98:2) and centrifuged for 20 min at 12,000*g* (5 °C). The final supernatant was transferred to an HPLC vial and analyzed by LC–MS/MS.

3. Validation

The validation process was carried out in accordance with 2002/657/EC. For screening purposes, criteria include detection capability (CCβ), recovery and matrix effect, specificity/selectivity, robustness and applicability. The stability of the standard solution was evaluated in a diluted solution, containing all analytes at 100 ng ml^{-1} prepared in the mobile phase (water with 0.1% formic acid:acetonitrile 0.1% formic acid) (98:2). The storage conditions were described in a previous work and results demonstrated that these solutions were stable, at least, for 2 months, when stored at −20 °C and for 1 month at 4 °C [21,34]. This procedure is being repeated in all routine analyses, demonstrating that the working solutions have remained stable in the period described above, corroborating the stability of the stock solutions prepared for each analyte. For matrix effect, the equation presented by Gosetti et al. was used [38]. The experiment was performed using 3 samples prepared in solvent at the MRL concentration (*S*) and 3 samples spiked after extraction procedure at the MRL concentration (*TS*). For matrix effect, results above 100% indicate signal enhancement and below 100% indicate signal suppression [38]. The equation is described below

$$\text{Matrix Effect}(\%) = TS/S*100$$

3.1. Detection capability (CCβ)

Detection capability (CCβ) is the concentration at which the method is able to detect undoubtedly contaminated samples with a statistical certainty of 1-β (false compliant results were ≤5%). Batches of 21 samples were analyzed, using different levels for the matrices, including quality control samples (blank, *R*, *TS* and *S* samples). In which *R* is a spiked sample, *TS* is a *tissue standard* sample spiked after the extraction procedure and *S* is a sample prepared in mobile phase. In a batch composed by 21 samples, this means that a minimum of 20 samples must present analyte detection and only one (5%) cannot be detected.

For milk samples, levels of 5, 10, 15, 25 and 50 ng mL^{-1} were tested and for liver samples, levels of 25, 50 and 100 ng mL^{-1} were tested. These values were chosen taking into account that a real sample with any of the studied analytes at the 0.5 × MRL would be correctly detected.

3.2. Selectivity and specificity

LC–MS/MS using MRM mode with 2 *m/z* transitions for each analyte is a specific technique per se and the parameters are demonstrated in Table 1. However, additional specificity tests were performed in milk by analysis of blank samples (n=35) obtained from local markets and collected by the Federal Inspection Service (FIS). These milks were: raw milk (n=5), UHT whole milk (n=10), UHT skim milk (n=9), pasteurized milk (n=9), powdered whole milk (n=1) and powdered skim milk (n=1). Liver samples were obtained from local markets and collected by the Federal Inspection Service (FIS), totaling 20 samples for each species (cattle and poultry). The samples analyzed were spiked with the three internal standards, one of each class. For liver samples, especially for bovine liver samples, sulfaquinoxaline-OH was also monitored.

3.3. Ruggedness

Ruggedness was analyzed using a Youden experimental plan proposed by 2002/657/EC. Experiments were carried out using blank milk and liver samples spiked at the CCβ level for each compound. The chosen and changed factors (n=5) are summarized in Table 2 for milk samples and liver samples. Four replicates were analyzed for each experiment. The values obtained with chosen factors were adopted as 100%.

3.4. Applicability

Incurred and routine samples were used to evaluate applicability of the present multiresidue method. For milk samples, raw milk samples (n=339) collected directly on small milk production farms in South and Southwest of Brazil were also analyzed by this methodology. For liver, including poultry and bovine liver samples, a total of 15 samples collected by FIS inspectors have been analyzed so far. A poultry liver sample previously analyzed through a method proposed by Hoff et al. (2009) [24] that was incurred with SMZ was also used for applicability evaluation (n=1).

Table 1
Mass spectrometry and retention times for each analyte.

	$[M+H]^+$	Fragment	Typical retention time ($_{Min}$)	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Exit cell potential (V)
Sulfonamides and thrimetoprim							
STZ	256.0	156.1	10.32	71	10	21	12
	256.0	108.2		71	10	37	8
SMZ	279.1	108.0	11.28	31	10	41	24
	279.1	92.10		31	10	41	14
SMA	254.0	156.0	12.77	71	10	23	22
	254.0	92.00		71	10	35	14
SDZ	251,1	156.0	10.16	31	10	21	16
	251,1	108.0		31	10	33	16
SMR	265.0	156.0	10.80	100	10	25	14
	265.0	108.0		100	10	25	14
SDX	311.2	245.1	12.49	31	10	27	14
	311.2	139.9		31	10	35	8
SDMX	311.2	156.1	13.45	31	10	27	8
	311.2	108.2		31	10	35	10
SFX	268.1	112.8	13.04	31	10	20	10
	268.1	156.0		31	10	20	10
SQX	301.2	156.0	13.36	106	10	25	20
	301.2	108.0		106	10	37	16
SQX-OH	317.0	156.0	12.54	106	10	25	20
	317.0	108.0		106	10	37	16
SCP	285.0	108.0	12.39	31	10	35	14
	285.0	156.0		31	10	35	14
TMP	290.8	230.2	8.28	45	10	31	34
	290.8	275.0		45	10	35	30
SPY (IS)	250.1	156.0	10.47	26	10	23	10
	250.1	108.0		26	10	33	46
Fluoroquinolones							
CIPRO	332.1	288.2	9.20	211	10	25	32
	332.1	245.2		211	10	33	34
ENRO	360.2	316.3	9.42	51	10	27	36
	360.2	245.2		51	10	37	26
NOR	320.2	276.3	9.11	51	10	25	30
	320.2	233.2		51	10	33	26
NALID	233.2	215.2	14.44	100	10	21	30
	233.2	187.0		100	10	35	26
SARA	400.1	356.0	9.80	21	10	31	24
	400.1	299.3		21	10	39	30
DIFLO	386.2	342.1	9.72	41	10	29	24
	386.2	299.0		36	10	37	30
FLU	262.3	244.2	14.57	126	10	21	36
	262.3	202.3		126	10	43	28
OXO	262.1	244.2	12.92	66	10	23	24
	262.1	216.0		66	10	39	14
DANO	358.4	96.20	9.29	100	10	30	15
	358.4	340.3		100	10	30	15
ENRO D5 (IS)	365.2	321.3	9.45	51	10	27	36
	365.2	245.0		51	10	37	26
Tetracyclines							
TETRA	445.1	427.0	9.49	126	10	29	16
	445.1	410.0		126	10	31	48
	445.1	154.0		126	10	25	30
OTC	461.1	426.3	9.32	76	10	29	16
	461.1	444.3		76	10	23	16
CTC	479.2	444.2	10.32	101	10	31	50
	479.2	462.2		101	10	29	18
DOXI	445.0	428.0	10.48	131	10	15	54
	445.0	154.0		126	10	27	18
DEMO (IS)	465.4	448.3	9.90	101	10	23	48
	465,4	430.1		101	10	31	16

4. Results and discussion

4.1. LC–Ms/Ms

Acetonitrile and water with formic acid proved adequate for chromatographic separation as described in a previous work [21]. A high water proportion in the beginning (98%) becomes an important tool in dirty matrices like liver, promoting the elution of hydrophilic interfering substances in the first minutes of the chromatographic run, avoiding co-elution with any analyte. A high percentage of acetonitrile at the end of the gradient provides satisfactory cleaning in the column avoiding carry-over phenomena. Results obtained from a typical MRM chromatogram of blank and spiked milk and liver samples at CCβ level are shown in Fig. 1. Individual extracted ion chromatograms for each class of analyte are shown in Figs. 2, 3 and 4.

Table 2
Chosen and changed factors for milk and liver samples used in Youden design.

Number	Factor	Chosen condition	Changed condition
MILK			
1	Additive in mobile Phase	Formic acid 0.1%	Acetic acid 0.1%
2	Solvent for protein precipitation	Ethanol	Methanol
3	Matrix	Bovine liver	Poultry liver
4	Low temperature for protein precipitation	Freezer	No
5	Filtration prior to analysis	Yes	No
LIVER			
1	Additive in mobile phase	Formic acid 0.1%	Acetic acid 0.1%
2	EDTA Sand-washed	2 g	No
3	Matrix	Bovine Liver	Poultry liver
4	Low temperature for protein precipitation	Freezer	No
5	Agitation	No	20 min

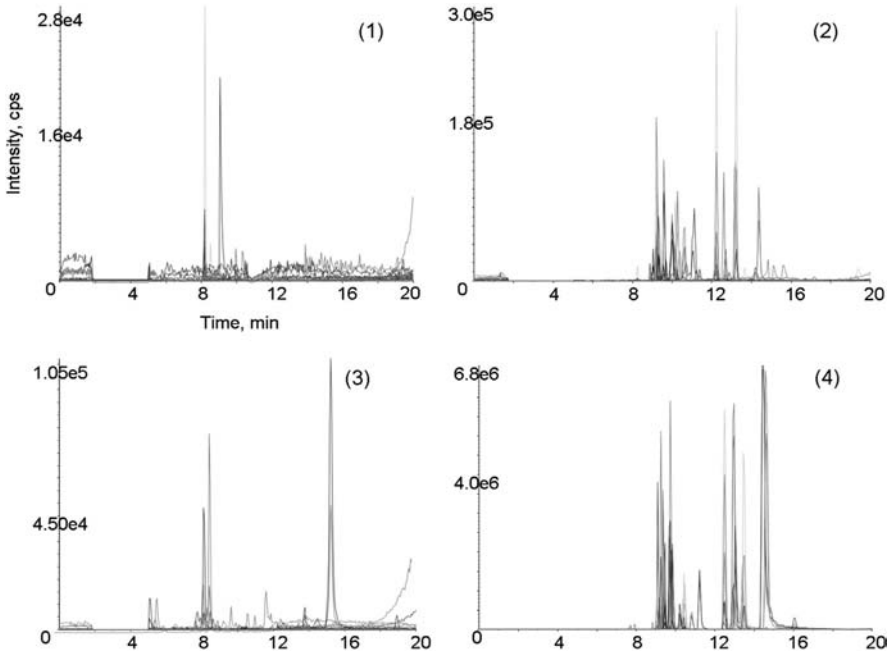

Fig. 1. Multiple Reaction Monitoring chromatograms for 24 antibacterial at milk blank sample (1), blank sample spiked with 24 analytes at CCβ level in milk (2), liver blank sample (3) and blank sample spiked with 24 analytes at CCβ level in liver (4).

Despite the fact that our purpose was not quantitative, the monitoring of two MRM transitions for each analyte provides a high degree of specificity, increasing the method confidence level. Beside the fragmentation pattern, retention time and ion ratio could be used to confirm the identity of the compounds.

4.2. Sample extraction

4.2.1. Milk

The extraction procedure was developed for easy, fast, cheap and environment-friendly performance. For this purpose, a single deproteinization procedure was tested. ACN, methanol and ethanol (acidified or not) were evaluated. Similar results were found for all solvents, when these were acidified. However, methanol shows poor recoveries for tetracyclines. Besides, methanol provides a turbid final extract, probably because of the presence of fat. Acetonitrile presents greater selectivity compared to ethanol since milk fat was less soluble in ACN than ethanol. However, as this lipid solubility is not a very critical point, ethanol was selected because it showed the lowest hazardous characteristics. Since the final extract is diluted before injection, matrix interfering compounds co-extracted using ethanol do not have a significant impact on the chromatogram or selectivity. The matrix effect observed in milk samples was signal enhancement (results above 100%) for ENRO, DANO and OTC. The signal suppression (results below 100%) were observed for OXO, CTC, DOXI, TMP and more pronounced in sulfonamides. For SARA, NOR, DIFLO, FLU, NALI, CIPRO and TC the values were around 100%.

Organic acids are used as additives in order to improve the analyte extraction yield which is more evident especially for fluoroquinolones. Formic and acetic acid were tested and acetic acid showed the best results.

Considering that tetracyclines react with multivalent cations forming chelation complexes, addition of EDTA was used to prevent chelate formation through tetracyclines interaction with Ca^{+2} present in milk [39].

A central composite design (2^2) was carried out to optimize milk extraction [40]. Control variables were ethanol acidification

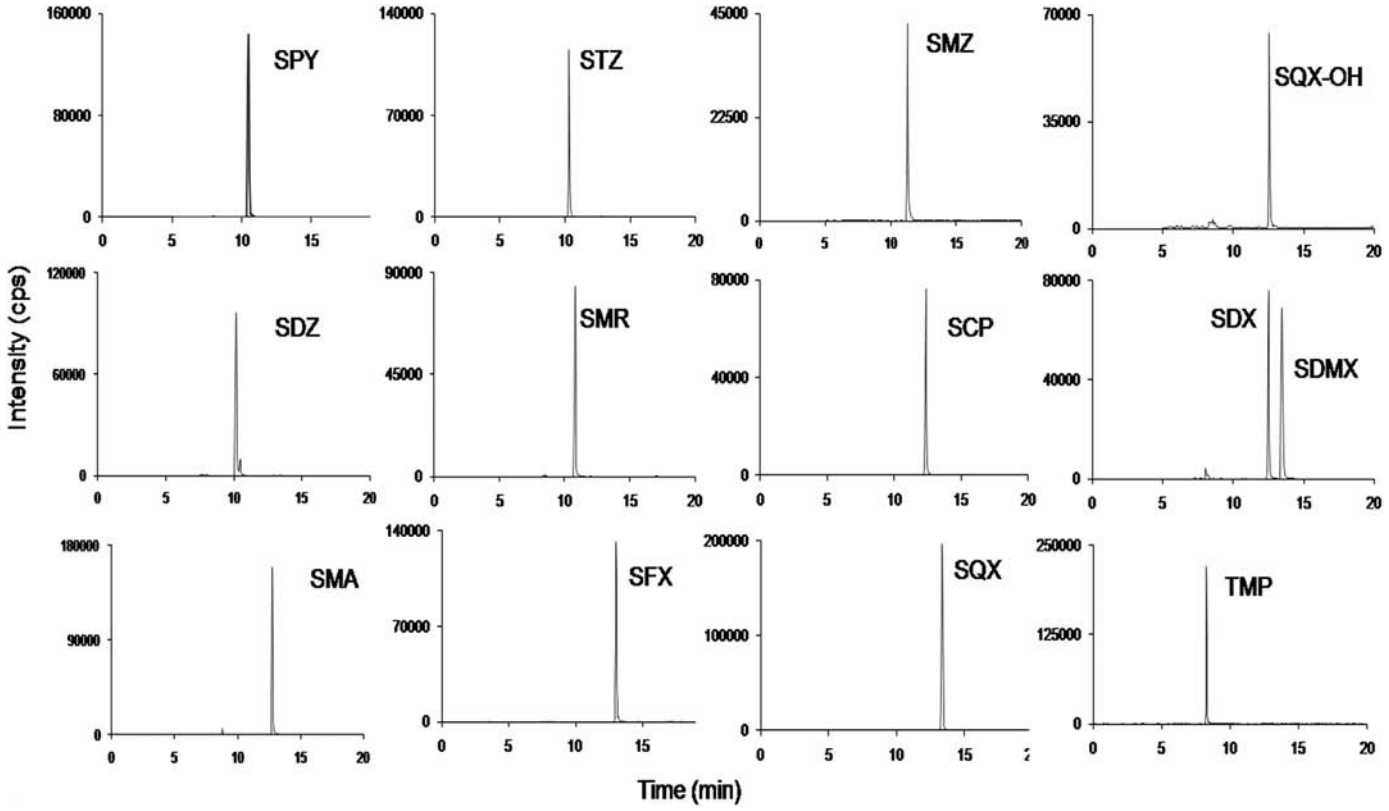

Fig. 2. Extracted ion chromatogram for milk liver samples spiked in CCβ concentration level for sulfonamides and thrimetoprim.

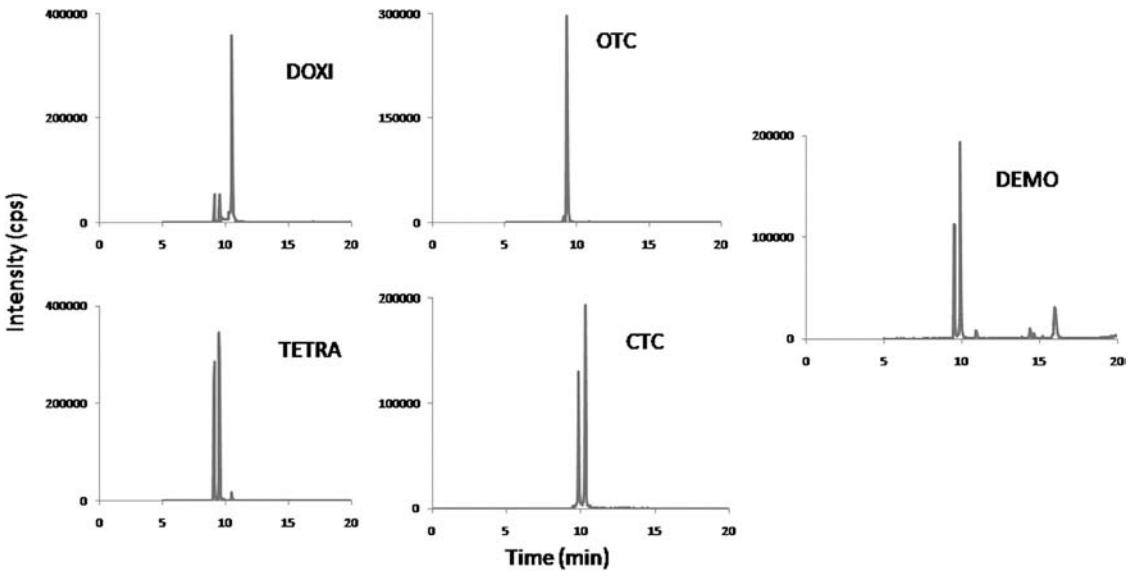

Fig. 3. Extracted ion chromatogram for milk liver samples spiked in CCβ concentration level for tetracyclines.

(%) and EDTA concentration (mM). The response variable evaluated was peak area. This experiment was performed including 4 axial points and 3 replicates for the center point. Center point conditions were established as the initial extraction levels obtained in the solvent selection stage. Data analysis and mathematical models construction were processed using Minitab 16 statistical software (Minitab, State College, PA, USA). Raw data were tabled and regression analysis was performed. Mathematical models were validated using ANOVA. The results obtained with the statistical analysis from the data of the CCD 2^2 were plotted in contour plots and/or surface response graphics. Interestingly, all analytes presented better responses using high values of EDTA, not just tetracyclines, as expected. High values of EDTA or high acetic acid concentration, each factor isolated from the other, produced low responses. Combination of high values for both reagents produces elevated peak areas for all investigated compounds. Despite the fact that contour plots indicate that more extreme conditions provide higher response values, the evaluated concentrations of 150 mM of EDTA and 4% of acetic acid in ethanol were adopted for extraction.

For additional sample deproteinization and clean up, two centrifugation steps were included, without any additional

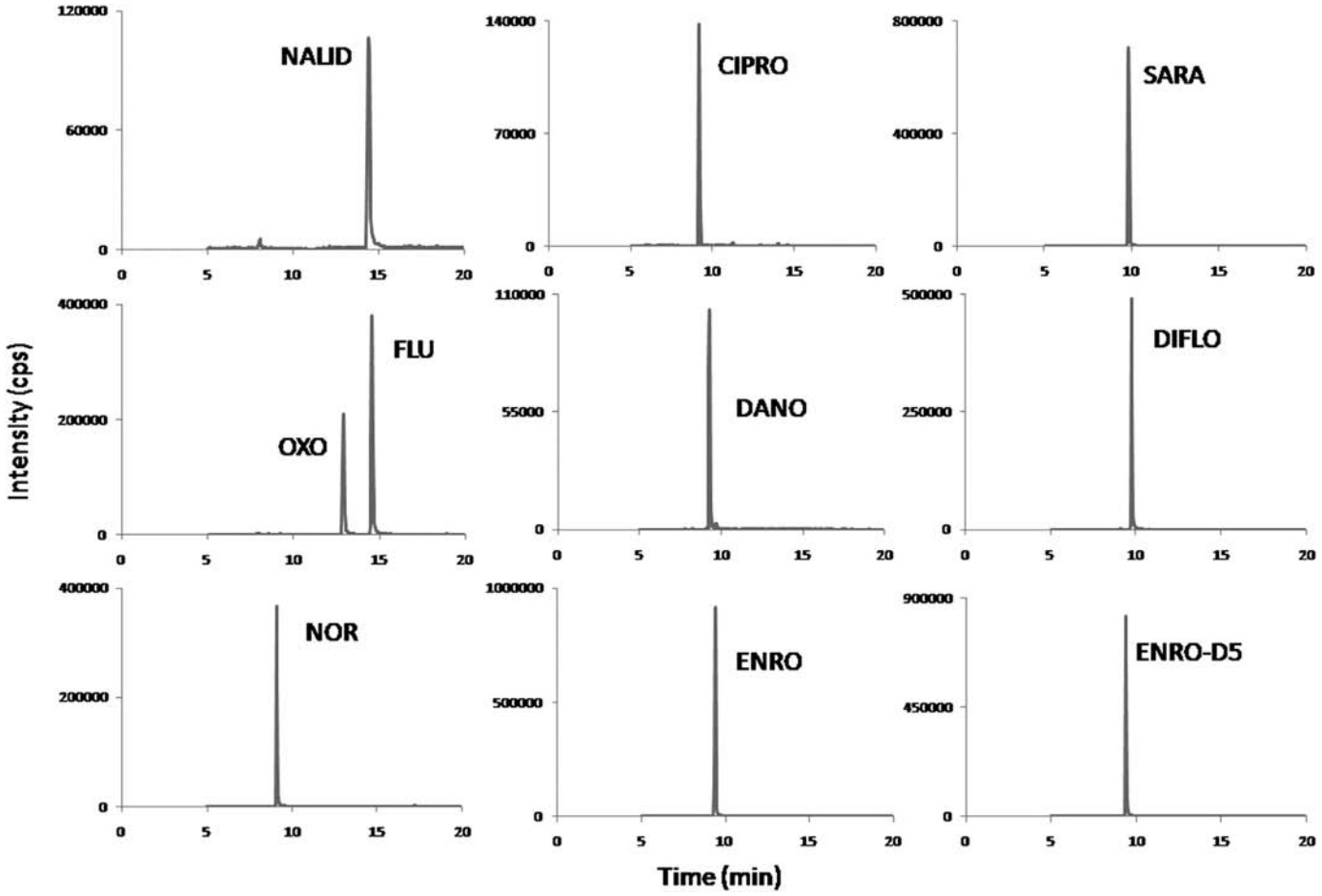

Fig. 4. Extracted ion chromatogram for milk liver samples spiked in CCβ concentration level for fluoroquinolones.

procedure. Extract refrigeration (− 18 °C for 30 min) preceding the second centrifugation appears to be very effective in promoting complete matrix proteins and co-extracted compound precipitation. These techniques were shown to be applicable for multi-class analysis of veterinary drugs in products of animal origin and can be considered a simple and feasible clean-up procedure.

4.2.2. Liver

The first step of sample preparation was tissue disruption using sand as a solid phase. Sand was home-purified, providing a cheap and virtually endless source of adsorbent. In a previous report published elsewhere, we applied sand in a matrix solid phase dispersion (MSPD) method for muscle extraction, with satisfactory results [21].

Extraction solvent choice was a very critical point. Granelli et al. developed a method that uses methanol for extraction of multi-class antibacterial from muscle and kidney, showing that this solvent allows the extraction of a significant number of compounds with different characteristics [41]. In the present work, ethanol and methanol (both with formic acid 0.1%) were evaluated for target compounds extraction, and methanol proved to be adequate for liver because ethanol use results in highly turbid extracts. The main purpose of sample treatment is remove constituents that may affect the chromatographic system or interfere in the detection, but keeping all analytes [9]. Since methanol extracts excessive matrix interferences, it is important to include additional steps for sample cleanup [19]. It is important to associate simplicity with cost-effectiveness for qualitative screening methods [13]. These characteristics were achieved using freezing, centrifugation and protein precipitation with ACN with formic acid 0.1%, followed by another centrifugation step. Sample dilution with the initial mobile phase composition (water 0.1% formic acid: ACN 0.1% formic acid, 98:2), guaranteed a clean extract with acceptable matrix effect. All analytes presented matrix effect of signal enhancement (results above 100%) for liver samples, especially fluoroquinolones.

The use of EDTA prior to extraction increases tetracyclines recovery, considering the high prevalence of minerals in liver composition. The use of EDTA-treated sand was previously reported for tetracyclines determination in animal tissues and in our previous work published elsewhere [21,42]. The final procedure, using EDTA 150 mM, a universally used solvent like methanol in a small proportion (1 mL) and ACN (400 μL) was considered satisfactory and provided an environment-friendly procedure.

For cattle liver samples, is important to monitor SQX-OH, since SQX is metabolized in SQX-OH in vitro, due to the presence of active metabolic enzyme systems in this matrix that can lead in post-mortem. In poultry liver samples this phenomenon do not occur as demonstrated by Hoff et al. [43]. For this reason, the transition *m/z* 317 was included to detect the presence of SQX in cattle liver samples.

4.3. Validation

4.3.1. Detection capability (CCβ)

CCβ was determined using batches composed by 21 spiked samples, each batch at a different level of concentration,

corresponding to 5, 10, 15, 25 and 50 ng mL^{-1} for milk and 25, 50 and 100 for liver, which was based on individual MRL values (Table 3). For milk, levels of 5 ng mL^{-1} (NOR); 10 ng mL^{-1} (OXO, SARA, NALID); 15 ng mL^{-1} (DANO); 25 ng mL^{-1} (FLU, ENRO, CIPRO, TMP) and 50 ng mL^{-1} (DIFLO, SDZ, SQX, STZ, SDMX, SMA, SFX, SMR, SMZ, SDX, SCP OTC, TC, CTC, DOXI) were established as detection capability. For liver, levels of 25 ng mL^{-1} (TMP), 50 ng mL^{-1} for SAs, Qs and FQs and 100 ng mL^{-1} for TCs were established as detection capability. CCβ values were considered satisfactory, taking into account that a real sample with any of the studied analytes at the MRL would be correctly detected. It is important to establish a value of CCβ not very close to MRL, releasing non-compliant samples, but values not so low as to provide many false positive results. The values at 0.5 × MRL are safe enough to avoid rework. Detection capability values were designed to attend to our laboratory quantitation methods, in which limit of quantification usually corresponds to 25% of MRL value for the substance and, therefore, there is no need to detect a substance at a level at it cannot be measured in quantification methods. All CCβ values established are below MRL and considered satisfactory, since for authorized analytes, the screening target concentration is at or below the regulatory limit (MRL) and should preferably be set at one half of the MRL wherever possible [44,45].

4.3.2. Selectivity/specificity

Blank samples obtained in the local market were analyzed for both matrix (milk $n=35$, including raw, pasteurized and powder milk, and liver $n=20$ for cattle and poultry). For all samples analyzed, no interference peaks were observed within the migration time window for any compound and the two transitions chosen were well detected.

4.3.3. Ruggedness

An experiment using the Youden approach was performed to evaluate the method behavior versus minor and major changes (Table 2). Response factor—assigned as peak area—was compared between chosen and changed factors, using precision as parameters. Chosen factors were described as SRW and changed factors were described as SDi. The acceptance criterion was 40%, twice the maximum coefficient of variation (CV%=20) tolerated for quantitative methods, considering concentration ranging from 10 to 100 μg Kg^{-1}. The impact of each change was evaluated for each analyte. For milk samples, the method is not sufficiently robust for proposed changes for SFX, SDX, NOR, OXO, ENRO, TMP, OTC, TC and CTC. Furthermore, DOXI showed deviations above 40%. However, this lack of ruggedness shows a way to improve responses. The main factor of influence for milk was the mobile phase additive. When acetic acid was used to replace formic acid, high responses were obtained for these analytes. Considering that for the majority formic acid was more adequate, we maintained this acid to compose the mobile phase. For liver samples data demonstrate that SDX, SQX, SCP, ENRO, NOR, CIPRO, SARA, DIFLO, CTC, TC and OTC responses were robust for all factors, with CV% values below 40%. For TMP, SFX, SDZ, SDMX, SMR, SMZ, STZ, SMA, OXO, NALI, FLU, DANO and DOXI the most influential factor was the use of agitation for 20 min, suggesting that it can be introduced to the present method. The use of acetic acid as a mobile phase additive promotes signal variations for SMR, SFX, SMA, NALI and FLU. Withdrawing the freezing step before centrifugation demonstrates lack of robustness for SMR, SMA, STZ, OXO, DANO and DOXI. Maceration without sand was a very important factor for SFX and the change in animal species (poultry liver) was important for SDMX and DOXI responses. It is important for liver samples to demonstrate that in these different species we must consider SQX-OH for cattle liver, since this hydroxylation occurs and the SQX decreases its intensity. Method optimization was defined as a compromise between the best responses obtained for the majority of compounds and conditions that sometimes were not the best for isolated cases.

Table 3
Maximum residue limits (MRL), CCβ values and number of compliant samples in each experiment for each analyte.

Analyte	Milk				Cattle and poultry liver			
	MRL (ng mL^{-1})	CCβ (ng mL^{-1})	Detection		MRL (μg Kg^{-1})	CCβ (μg Kg^{-1})	Detection	
			CCβ spiked	Blank samples			CCβ spiked	Blank samples
SDMX	100	25	21/21	35/35	100	50	21/21	20/20
SQX	100	25	21/21	35/35	100	50	21/21	20/20
SDZ	100	25	21/21	35/35	100	50	21/21	20/20
STZ	100	25	21/21	35/35	100	50	21/21	20/20
SMA	100	25	21/21	35/35	100	50	21/21	20/20
SMZ	100	25	21/21	35/35	100	50	21/21	20/20
SCP	100	25	21/21	35/35	100	50	21/21	20/20
SFX	100	25	21/21	35/35	100	50	21/21	20/20
SDX	100	25	21/21	35/35	100	50	21/21	20/20
SMR	100	25	21/21	35/35	100	50	21/21	20/20
CTC	100	50	21/21	35/35	300	100	21/21	20/20
DOXI	-	50	21/21	35/35	300	100	21/21	20/20
TC	100	25	21/21	35/35	300	100	21/21	20/20
OTC	100	25	21/21	35/35	300	100	21/21	20/20
OXO	20	10	21/21	35/35	150	50	21/21	20/20
NALID	20	10	21/21	35/35	-	50	21/21	20/20
FLU	50	25	21/21	35/35	500	50	21/21	20/20
CIPRO	50	25	21/21	35/35	-	50	21/21	20/20
DIFLO	100	25	21/21	35/35	1400	50	21/21	20/20
ENRO	50	25	21/21	35/35	300	50	21/21	20/20
SARA	20	10	21/21	35/35	100	50	21/21	20/20
DANO	30	15	21/21	35/35	400	50	21/21	20/20
NOR	10	5	21/21	35/35	-	50	21/21	20/20
TMP	50	25	21/21	35/35	100	25	21/21	20/20

4.3.4. Applicability

Three hundred and thirty-nine (339) milk samples were analyzed using this screening method. Within these, four were positive for OTC, and one of these was a suspect sample. These samples were confirmed and quantified in triplicate through a class-specific LC–MS/MS method for tetracyclines and presented 27 ng mL^{-1}, 37 ng mL^{-1}, 49 ng mL^{-1} and the non-compliant sample with 981 ng mL^{-1}, almost ten times above the MRL. A positive milk sample is demonstrated in Fig. 5. One sample was positive for DIFLO and one for ENRO, which can be considered positive for ciprofloxacin (metabolite of enrofloxacin), both below the MRL that is 100 ng mL^{-1} for DIFLO and 50 ng mL^{-1} for ENRO and CIPRO.

For liver samples, 15 samples including cattle ($n=8$) and poultry ($n=7$) were analyzed and within these none were incurred with the analytes that are contemplated in the present method. The poultry sample incurred with SMZ was positive, demonstrating that this screening method was capable to detect accordingly this analyte in liver (Fig. 6).

5. Conclusion

A LC–MS/MS method was developed for screening 24 veterinary drug residues in liver and in milk for determination of non-compliant samples, obtaining confirmatory data. Simple and cheap extraction were developed and optimized, rendering the methodology fast and very effective for routine laboratory work, as inspection samples received in our laboratory. Very low solvent consumption was associated with the choice of less hazardous solvents when applicable. The proposed method presented adequate compound separation, simple extraction procedure and detection capability (CCβ), at least, in 0.5 × MRL, considering a false compliant rate of < 5% (β-error). As in the case of a suspected

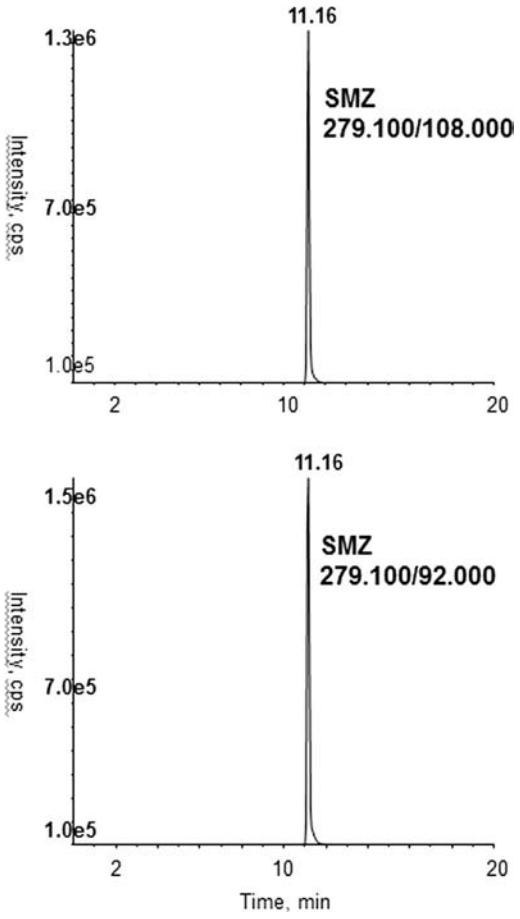

Fig. 6. Incurred poultry liver sample with SMZ.

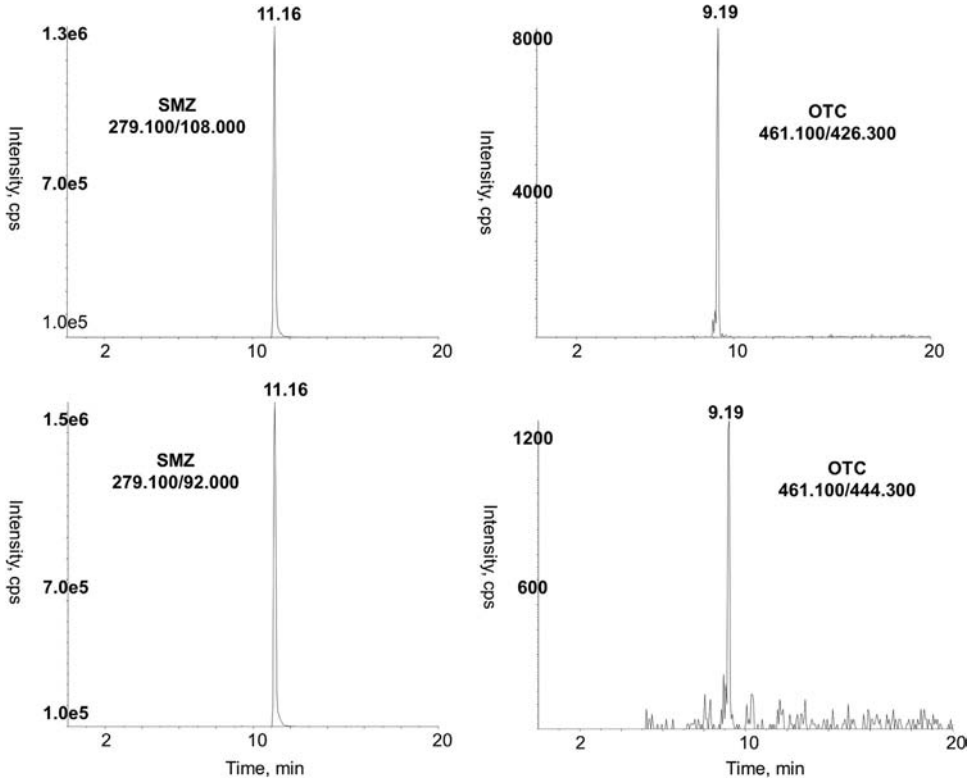

Fig. 5. Incurred milk sample with OTC.

non-compliant result, it must be confirmed by a suitable class-specific quantitative confirmatory method. The method is fast and simple, making it possible to analyze about 50 milk and 40 liver samples per day. A batch composed by 50 milk samples plus 10 quality control samples will consume just 36.0 mL of ethanol, a cheap, safe and low toxic solvent. For liver samples, around 50 mL of extraction solvent and 20 mL of acidified acetonitrile are used. Moreover, for each analysis, aboul 400 mL of mobile phase is consumed. The present method was accredited under ISO 17025 by the National Institute of Metrology, Standardization and Industrial Quality (INMETRO), under CRL 0384-Accreditation Certificate, and currently is part of the Brazil National Residue Control Plan.

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