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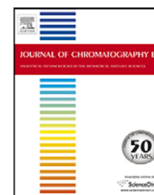

Comprehensive fast multiresidue screening of 150 veterinary drugs in milk by ultra-performance liquid chromatography coupled to time of flight mass spectrometry☆

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ABSTRACT

This paper shows the use of ultra-performance liquid chromatography (UPLC) coupled to orthogonal acceleration time of flight mass spectrometry (TOF MS) for the comprehensive screening of 150 veterinary drugs residues in raw milk. An easy sample preparation based on protein precipitation associated with ultrafiltration was hyphenated to fast chromatography. This method enabled the screening for more than 50 samples per day and searched for 150 drugs and metabolites including avermectines, benzimidazoles, beta-agonists, beta-lactams, corticoides, macrolides, nitroimidazoles, quinolones, sulfonamides, tetracyclines and some others. Identification of contaminants is based on accurate mass measurement. UPLC–TOF also showed very good performances for quantitation and allowed the determination of majority of compounds below MRL. An in-house validation procedure was conducted based on European directive 2002/657/EC with measurement of response function, accuracy, repeatability, limits of detection (LOD), decision limit (CCα) and detection capability (CCβ).

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1. Introduction

The widespread use of veterinary drugs in dairy cattle management may induce the presence of drugs residues in milk. Antibiotic residues are currently the most frequent inhibitory substances found in milk, having undesirable effects on milk quality, milk technological properties, dairy products quality, and last but not least human health problems. Indeed, contaminated milk can cause allergic reactions or indirect problems in clinical treatment due to the development of bacterial resistance. To protect consumer health and to ensure high quality of produced milk, the European Union (EU) as well as the Swiss regulation authorities have established maximum residue limits (MRLs) to set allowed maximum levels for drugs residues in milk [1,2]. The importance of continuous control of antibiotic residues in milk is emphasized with respect to the role of milk and dairy products in human nutrition. Main veterinary drugs used today include β-lactams, sulfonamides, tetracyclines, aminoglycosides, chloramphenicol, macrolides and quinolones. Since 1970s, methods for detecting residues were primarily inhibition tests (e.g. Delvotest) by means of test cultures using various microorganisms like *B. subtilis*, *Sarcina lutea*, *Strep thermophilus* and *Strep lactis*. Today, rapid test kits based on immune receptor (Twin-Sensor, β-Screen, Charm II assay, β-star, etc.) for the detection of common antibiotic residues in milk are increasingly used. While such rapid screening tests are commonly used to detect the presence of antibiotics in milk, several problems often occurred: lack of selectivity with ambiguous substance identification, false negative or positive results, and approximate quantitative results when it's possible to quantify. Furthermore, one test kit is required for each family of antibiotics and kits are only available for most common antibiotics used. Sensitivity of immune test kits depends on cross reactivity of each compound and leads to very different limits of detection within a same drugs family. In case of positive results with use of rapid test kits, more accurate chromatographic methods are usually required by government regulatory agencies to confirm the identity and quantity of antibiotic present. As regulations became more stringent with respect to drug allowed concentrations (MRLs), the need for developing qualitative methods as well as confirmation and identification techniques becomes of greater interest in order to minimize false positives. Recently, new approaches using the potential of liquid chromatography coupled with tandem mass spectrometry (MS–MS) or time of flight mass spectrometry (TOF MS) have been developed to carry out mutliclass residues screening. In food safety area, ultra-performance liquid chromatography (UPLC) hyphenated to TOF MS has already been used for pesticides [3–9], veterinary drugs [8,10–14], toxins [15,16] or illegal dyes determinations [17,18]. The advantages are to conduct very rapidly a single analysis in order to simultaneously identify few hundred

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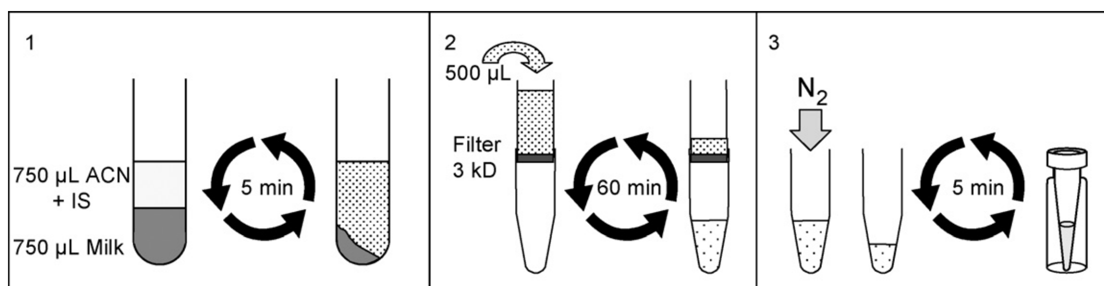

Fig. 1. Scheme of generic milk sample preparation. (1) Protein precipitation with ACN containing IS. Vortex 1 min and centrifuge for 5 min. (2) 500 μL of supernatant is ultracentrifuged for 60 min with cut-off at 3 kD. (3) Evaporation of ACN, centrifugation and supernatant collection.

contaminants without ambiguity and allows to obtain preliminary quantitative results in order to identify a possible not compliant sample. A similar application was published by Stolker et al. [12] for screening and quantification of veterinary drugs by LC–TOF. At the beginning, this work was initiated by van Rhijn using an ultrafiltration device at 30 kDa for the UPLC–TOF analysis of milk and published as a Waters application note [19]. This sample preparation approach was finally replaced before publication by using a generic solid phase extraction (SPE) on polymeric sorbent. The generic SPE approach compared to ultrafiltration seemed to give lower matrix effect even if Stolker et al. did not give much information on this topic. However, for the screening of high number of samples with relatively few positive cases, ultrafiltration generic sample preparation was found to be faster and very efficient. Therefore, the first idea of van Rhijn was reevaluated using narrower ultrafiltration device (3 kDa) to decrease matrix effect. The present paper describes the use of UPLC coupled to orthogonal acceleration TOF MS for comprehensive screening in raw milk of 150 veterinary drugs and metabolites included avermectines, benzimidazoles, beta-agonists, beta-lactams, corticoides, macrolides, nitroimidazoles, quinolones, sulfonamides, tetracyclines and some other veterinary medicinal products. To ensure rapid screening, an easy and very fast sample preparation was developed based on protein precipitation associated with ultrafiltration allowing the analysis of more than 50 samples per day. UPLC separation was used to perform fast analyses while keeping good efficiency and resolution. Separation was coupled to orthogonal acceleration TOF MS in order to combine efficiency of separation with a high sensitivity and selectivity of detection. TOF MS produces accurate mass spectra which is interesting data for selective and sensitive detection, it's certain why an increasing number of publications use this technology for simultaneous qualitative and quantitative measurement at low levels.

2. Experimental

2.1. Reagents and chemicals

All veterinary drugs reference standards were purchased from Riedel-de-Haën (Buchs, Switzerland) or from Dr. Ehrenstorfer GmbH (Augsburg, Germany) as powder or standard solution. 1 mg/mL stock solutions of each were prepared by dissolving 20 mg of the pure analytical standard in 20 mL of appropriate solvent. For each family of compounds, a composite standard solution was prepared by combining aliquots of each stock solution and diluting to obtain a final concentration of 10, 1 and 0.1 μg/mL. These composite solutions were used to prepare calibration samples and quality control (QC) samples. QC samples were spiked by adding appropriate volume of one of these composite solution to 5 mL of blank raw milk making sure that the spiking volume did not exceed than 5% of sample volume. QC samples were left standing for at least one hour at room temperature before starting extraction. Carbendazim-D4 (CBZ-D4) used as internal standard was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) as a 100 ng/μL solution in acetone. Precipitation solution was prepared by adding 500 μL of formic acid and 1 mL of CBZ-D4 to 500 mL of acetonitrile. Organic solvents and formic acid were of LC–MS grade and obtained from Riedel-de-Haën (Buchs, Switzerland). Water was purified with an Elix 3 and MilliQ apparatus from Millipore (Molsheim, France). Other chemicals were of HPLC or analytical grade and were used without any further purification. When not in use, all standard solutions were stored in the dark at −20 °C.

2.2. Sample preparation

A scheme of sample preparation is shown in Fig. 1. Milk samples (at least 50 mL) were mixed to a homogeneous mixture before considering a test portion. 750 μL of milk were introduced into a 2 mL microtube and 750 μL of precipitation solution were added. Tubes were closed and thoroughly vortexed during 1 min. Tubes were then centrifuged at 13,300 rpm (17,000 × *g*) for 5 min using a Heraeus pico17 centrifuge (Thermo Fisher Scientific Inc., Waltham, USA). 500 μL of supernatant were collected and transferred into a microcon-3 device (Millipore, Molsheim, France) with a cut-off membrane at 3 kD. Up to 24 microcon devices were simultaneously centrifuged for 60 min at 17,000 × *g*. All collection microtubes containing the resulting protein free solution were transferred into the Cyclone evaporator (Prolab, Reinach, Switzerland). Evaporation parameters (15 min, 50 °C, 50 rpm and 300 mbar under nitrogen flow) were chosen to evaporate only the acetonitrile fraction to avoid samples dryness. Variation of the final extract volume from sample to sample was verified by checking response of the internal standard CBZ-D4. The microtubes were centrifuged for 5 min and then the final supernatant extract introduced into a microvial for UPLC–TOF analysis.

2.3. Liquid chromatography

An Acquity UPLC system coupled to LCT Premier XE (Waters Corp., MA, USA) was employed for all experiments. The chromatography was carried out on a Waters Acquity UPLC BEH C18, 1.7 μm 100 × 2.1 mm column protected with a precolumn VanGuard Acquity UPLC BEH C18, 1.7 μm 5 × 2.1 mm. The mobile phase

consisted of 0.1% of formic acid in water (A) and 0.1% of formic acid in MeCN (B). The chromatographic separation was performed in a gradient mode (0 min: A/B 95/5, v/v; 0.25 min: A/B 95/5; 6 min: A/B 5/95; 7 min: A/B 5/95; 7.2 min: A/B 95/5; 9.0 min: A/B 95/5) at a flow of 400 μL/min for a total run time of 9 min. The column and autosampler were maintained at 40 °C and 10 °C, respectively. 5 μL of the extract were injected. A diverter valve led the effluent into the waste between 0 and 0.5 min and from 7.5 to 9 min.

2.4. Time of flight mass spectrometry detection

Mass spectrometry was performed using a LCT Premier XE (Waters, Manchester, UK) equipped with a dual ESI source (lockspray). The system was tuned for optimum sensitivity and resolution using leucine-enkephalin solution at 0.5 ng/μL infused at 5 μL/min in positive electrospray ionization mode. The TOF was calibrated daily using sodium formate solution. The system was operated in V mode (resolution ∼7000 FWHM) with acquisition from 50 to 1150 *m*/*z* with a scan time of 0.2 s in order to reach the best sensitivity. The capillary voltage was set at 3 kV and the source temperature at 120 °C. The desolvation temperature was fixed at 380 °C with nitrogen flow rates of 20 and 750 L/h, respectively, for the cone gas and desolvation gas. The dynamic range enhancement (DRE) mode was not selected during screening. Indeed, the DRE mode would extend the dynamic range and reduce shifts of exact masses caused by peak saturation but would reduce drastically sensitivity. During confirmation analyses DRE mode was activated to ensure a wider dynamic range and more accurate quantification. Cone and aperture voltages were fixed at 40 V and 5 V, respectively, for both analyte and reference spray. MassLynx software, version 4.1, was used for instrument control and data acquisition. Data were centroided during acquisition using the leucine-enkephalin reference solution infused via the reference probe interface (lockspray). Veterinary drugs were detected using reconstructed ion chromatogram with 0.02 Da mass window. A mass spectrum of each compound was recorded by injecting a standard solution at 100 ng/mL and employed to choose best ions for detection. As a general use, the protonated ion (MH+) was used to detect veterinary drugs. The exact theoretical mass based on formula was calculated using the molecular weight calculator tools of MassLynx software. In some cases, practical measurement showed a very weak signal for the protonated ion and another ion such as sodium adduct or fragment have been chosen. All formula, *m*/*z* values and types of ion are summarised in Table 1 for the 150 analytes. Automated integration was carried out with Targetlynx software using the apex track methods. Peak areas were used as response without dividing by internal standard area as it was shown that matrix effects are compounds dependent and could be an additional source of error.

2.5. Validation procedure

Based on European directive 2002/657/EC [20], an in-house validation procedure was conducted to determine method performances. For quantitative screening purposes, validation shall determines decision limits, precision, selectivity and specificity as well as applicability. Only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate of <5% (β-error) at the level of interest shall be used for screening purposes in conformity with Directive 96/23/EC. In the case of a suspected non-compliant result, this result shall be confirmed by a suitable confirmatory method. Even if the described method was mainly dedicated to screening, the in-house was carried out to determine repeatability and accuracy, detection capability (CCβ) and decision limit (CCα) and finally the method specificity. Two different types of standards were prepared: calibration samples (standard solutions) and quality control samples corresponding to fortified samples. Nine calibration samples (0.5, 1, 2, 5, 10, 25, 50, 100 and 200 μg/L) were prepared in triplicate. Peak areas were plotted against concentration to determine the response function. A simple regression using the least square method was applied. Nine levels of QC fortified at 0.5, 1, 2, 5, 10, 25, 50, 100 and 200 μg/L were prepared in four replicates using a blank raw milk. For tetracyclines and sulfonamides which have an homogenous MRL at 100 μg/L, the lowest level of validation was 10 μg/L. After extraction, samples were quantified using the external calibration of the calibration samples. The added amount procedure used in confirmation for quantitative determination of positive samples was not validate in this screening validation process. Data treatment allowed to determine CCα and CCβ calculated according to the EU-decision 657/2002/EC [20–22] with the so-called calibration curve procedure considering the MRL or MRPL value of each drug. According to the use of the same levels of fortification for all substances, some concessions have been made for the determination of CCα and CCβ. Calculation was done with levels which were closest to the 0.5, 1 and 1.5× MRL levels which are usually required. The specificity was evaluated by the analysis of 20 blanks of raw milk samples and searching for interfering peaks. Blank samples were coming directly from 20 different Swiss farm sampled for the national control plan for residues monitoring. The same 20 blanks samples were also fortified at a single level of 50 μg/L to determine the variability due to the matrix effect. Accuracy and precision were calculated from these 20 QC samples. Accuracy was expressed as recovery value in percent and repeatability values as relative standard deviation (RSD) in percent. As a final validation test, a blind test on 70 unknown real samples containing blank and positive samples was carried out.

3. Results and discussion

3.1. Sample preparation

The aim of this work was to develop a comprehensive screening in raw milk for the most used veterinary drugs. The procedure described here uses the very good performances of UPLC–TOF to simplify the sample preparation step. A simple, fast and robust procedure based on protein precipitation associated with ultrafiltration was efficient to realise a generic sample preparation for raw milk. Therefore, fastidious clean-up is no more required. The developed method can be easily applied in routine within the day for 50 samples including calibration and stabilisation of the analytical system. The complete sample preparation was carried out using single-use disposable materials in order to resolve problems of contamination from sample to sample. Then, the major drawback of a generic sample preparation is the lack of selectivity which involves inevitable matrix effects. The latter can then reduce or enhance substantially the response signal. Thus, the matrix effects can contribute in highly variable accuracy and makes it more difficult to quantify positive samples. Three different ultrafiltration devices with cut-off membrane at 3, 10 and 30 kDa were tested. Ultrafiltration was achieved faster with 10 and 30 kDa membrane but the resulting extract was less clean and a precipitation was observed after the evaporation step. Therefore, the cut-off membrane at 3 kDa was kept for all further experiments. The efficiency of clean-up procedure was evaluated and compared with a generic SPE clean-up as described by Stolker et al. [12]. Fig. 2 shows the total ion chromatograms (TIC) obtained for three different raw milk samples after ultrafiltration or SPE clean-up. The sample preparation by SPE gives cleaner extracts early in the chromatogram. However, with regard to apolar products at the end of chromatogram, the preparation by ultrafiltration seems more appropriate. It may be noted also that the preparation by ultrafiltration seems more

Table 1
List of analytes: properties and validation results.

Compound	CAS	Formula	RT (min)	*m*/*z*	Ion	LD (μg/L)	MRL [a,b] (μg/L)	CCα (μg/L)	CCβ (μg/L)	Acc. (%)	RSD (%)
Avermectins (**5**)											
Abamectin	71751-41-2	$C_{48}H_{72}O_{14}$	6.53	895.4820	MNa+	25	–	51	87	3	61
Doramectin	117704-25-3	$C_{50}H_{74}O_{14}$	6.84	921.4977	MNa+	25	–	–	–	6	86
Emamectin B1a	137335-79-6	$C_{49}H_{75}NO_{13}$	5.11	886.5316	MH+	25	–	43	73	10	64
Eprinomectin B1a	133305-88-1	$C_{50}H_{75}NO_{14}$	6.18	914.5266	MH+	10	20	32	55	1	86
Ivermectin B1a	70288-86-7	$C_{48}H_{74}O_{14}$	7.36	897.4977	MNa+	25	–	–	–	3	122
Benzimidazoles (**16**)											
Albendazole	54965-21-8	$C_{12}H_{15}N_3O_2S$	3.36	266.0963	MH+	1	100	113	131	112	10
Albendazole sulfone	75184-71-3	$C_{12}H_{15}N_3O_4S$	2.87	298.0861	MH+	0.5	100	106	113	115	9
Albendazole sulfoxide	54029-12-8	$C_{12}H_{15}N_3O_3S$	2.32	282,0912	MH+	0.5	100	107	115	108	10
Cyclobendazole	31431-43-3	$C_{13}H_{13}N_3O_3$	2.72	260.1035	MH+	1	–	0.6	1	120	7
Febantel	58306-30-2	$C_{20}H_{22}N_4O_6S$	4.90	447.1338	MH+	2	10	11.7	14.2	30	15
Fenbendazole	43210-67-9	$C_{15}H_{13}N_3O_2S$	3.86	300.0806	MH+	2	10	11.8	14.8	134	13
Fenbendazole sulfone	54029-20-8	$C_{15}H_{13}N_3O_4S$	3.29	332.0705	MH+	2	10	10.9	12	92	9
Flubendazole	31430-15-6	$C_{16}H_{12}FN_3O_3$	3.58	314.0941	MH+	10	10	11.4	13.8	37	8
Flubendazole-amine	82050-13-3	$C_{14}H_{10}FN_3O$	2.69	256.0886	MH+	2	10	11.8	14.3	443	6
Mebendazole	31431-39-7	$C_{16}H_{13}N_3O_3$	3.33	296.1035	MH+	2	10[a]	11	12.3	103	9
Mebendazole-5-hydroxy	60254-95-7	$C_{16}H_{15}N_3O_3$	2.62	298.1191	MH+	0.5	10[a]	10.6	11.3	141	9
Mebendazole-amine	52329-60-9	$C_{14}H_{11}N_3O$	2.57	238.0980	MH+	1	10[a]	10.8	11.7	436	7
Oxfendazole	53716-50-0	$C_{15}H_{13}N_3O_3S$	2.78	316.0756	MH+	0.5	10	10.7	11.4	102	9
Oxibendazole	20559-55-1	$C_{12}H_{15}N_3O_3$	2.82	250.1191	MH+	0.5	10	11.4	13.4	103	11
Thiabendazole	148-79-8	$C_{10}H_7N_3S$	1.90	202.0439	MH+	0.5	100	107	112	78	6
Thiabendazole-5-hydroxy	948-71-0	$C_{10}H_7N_3OS$	1.70	218.0388	MH+	2	100	104	108	104	4
Beta-agonists (**6**)											
Brombuterol	41937-02-4	$C_{12}H_{18}Br_2N_2O$	2.64	366.9844	MH+ (Br^{81})	0.5	–	0.2	0.4	143	6
Cimbuterol	54239-39-3	$C_{13}H_{19}N_3O$	1.79	234.1606	MH+	0.5	–	0.2	0.4	120	7
Clenbuterol	37148-27-9	$C_{12}H_{18}Cl_2N_2O$	2.46	277.0874	MH+	0.5	0.05	0.3	0.5	160	8
Clenproperol	38339-11-6	$C_{11}H_{16}Cl_2N_2O$	2.26	263.0718	MH+	0.5	–	0.1	0.2	123	7
Salbutamol	18559-94-9	$C_{13}H_{21}NO_3$	1.48	240.1599	MH+	0.5	–	0.3	0.5	236	10
Tulobuterol	41570-61-0	$C_{12}H_{18}ClNO$	2.45	228.1155	MH+	0.5	–	0.2	0.3	123	6
Beta-lactams (**25**)											
Amoxicillin	26787-78-0	$C_{16}H_{19}N_3O_5S$	1.50	366.1123	MH+	10	4	–	–	73	8
Ampicillin	69-53-4	$C_{16}H_{19}N_3O_4S$	2.00	350.1174	MH+	0.5	4	4.2	4.4	93	6
Carbenicillin	4697-36-3	$C_{17}H_{18}N_2O_6S$	2.96	379.0964	MH+	2	–	2.8	4.2	86	8
Cefaclor	70356-03-5	$C_{15}H_{14}ClN_3O_4S$	1.89	368.0472	MH+	0.5	–	0.3	0.5	65	11
Cefadroxil	66592-87-8	$C_{16}H_{17}N_3O_5S$	1.62	364.0967	MH+	0.5	–	0.2	0.4	72	7
Cefalexin	15686-71-2	$C_{16}H_{17}N_3O_4S$	2.05	348.1018	MH+	0.5	100[b]	106	111	97	7
Cefamandole	34444-01-4	$C_{18}H_{18}N_6O_5S_2$	2.84	463.0858	MH+	1	–	0.9	1.6	96	10
Cefazolin	27164-46-1	$C_{14}H_{14}N_8O_4S_3$	2.34	455.0378	MH+	2	–	0.9	1.6	118	6
Cefoperazone	62893-20-3	$C_{25}H_{27}N_9O_8S_2$	2.62	646.1502	MH+	2	50	1.4	2.4	69	10
Cefotaxime	63527-52-6	$C_{16}H_{17}N_5O_7S_2$	2.18	456.0647	MH+	10	–	8.3	14.2	135	10
Cefoxitin	35607-66-0	$C_{16}H_{17}N_3O_7S_2$	2.67	450.0406	MH+	2	–	0.7	1.2	177	6
Cefquinom	118443-89-3	$C_{23}H_{24}N_6O_5S_2$	1.90	529.1328	MH+	1	20	22.2	24.7	661	7
Cefsulodin	62587-73-9	$C_{22}H_{20}N_4O_8S_2$	1.45	533.0801	MH+	10	–	3.8	6.4	92	11
Ceftiofur	104010-37-9	$C_{19}H_{17}N_5O_7S_3$	2.98	524.0368	MH+	0.5	100	108	117	87	8
Cefuroxime	56238-63-2	$C_{16}H_{16}N_4O_8S$	2.51	447.0587	MNa+	2	–	1.8	3.1	122	13
Cephacetrile	10206-21-0	$C_{13}H_{13}N_3O_6S$	2.08	362.0423	MNa+	10	125	148	189	69	18
Cephalothin	153-61-7	$C_{16}H_{16}N_2O_6S_2$	3.17	337.0313	Fragment	25	–	18.7	31.8	91	17
Cephapirin	24356-60-3	$C_{17}H_{17}N_3O_6S_2$	1.72	424.0637	MH+	0.5	10[a] 60[b]	13.4	16.9	99	9
Cephradin	38821-53-3	$C_{16}H_{19}N_3O_4S$	2.15	350.1174	MH+	0.5	–	0.3	0.6	93	6
Cloxacillin	61-72-3	$C_{19}H_{18}ClN_3O_5S$	4.03	436.0734	MH+	5	30	34	39	76	7
Dicloxacillin	3116-76-5	$C_{19}H_{17}Cl_2N_3O_5S$	4.31	470.0344	MH+	2	30	35	42	72	8
Nafcillin	985-16-0	$C_{21}H_{22}N_2O_5S$	4.13	415.1327	MH+	1	30	34	39	74	9
Oxacillin	66-79-5	$C_{19}H_{19}N_3O_5S$	3.79	402.1124	MH+	2	30	34	40	94	8

Penicillin G	61-33-6	$C_{16}H_{18}N_2O_4S$	3.41	335.1065	MH+	2	4	5.6	8	95	7
Penicillin V	87-08-1	$C_{16}H_{18}N_2O_5S$	3.67	414.1099	M/ACN/Na+	2	–	1.8	3.1	76	10
Corticoides (**5**)											
6a-Methylprednisolone	83-43-2	$C_{22}H_{30}O_5$	3.48	375.2171	MH+	2	–	0.8	1.3	88	8
Betamethasone	378-44-9	$C_{22}H_{29}FO_5$	3.55	393.2077	MH+	0.5	0.3[b]	0.5	0.8	64	10
Dexamethasone	50-02-2	$C_{22}H_{29}FO_5$	3.58	393.2077	MH+	1	0.3	0.5	0.8	54	7
Hydrocortisone	50-23-7	$C_{21}H_{30}O_5$	3.21	363.2171	MH+	0.5	–	0.3	0.5	96	12
Prednisolone	50-24-8	$C_{21}H_{28}O_5$	3.17	361.2015	MH+	1	6[b]	6.4	6.9	97	6
Macrolides (**10**)											
Erythromycin	114-07-8	$C_{37}H_{67}NO_{13}$	3.19	734.4690	MH+	1	40	44	49	15	21
Josamycin	16846-24-5	$C_{42}H_{69}NO_{15}$	3.80	828.4745	MH+	5	–	10.4	17.6	12	28
Leucomycin A1	1392-21-8	$C_{40}H_{67}NO_{14}$	3.67	786.4640	MH+	25	–	11	18.8	76	25
Natamycin	7681-93-8	$C_{33}H_{47}NO_{13}$	3.22	666.3125	MH+	1	–	0.3	0.5	57	12
Roxithromycin	80214-83-1	$C_{41}H_{76}N_2O_{15}$	3.66	837.5324	MH+	10	–	9.5	16.2	18	18
Spiramycin I	8025-81-8	$C_{43}H_{74}N_2O_{14}$	2.51	843.5218	MH+	10	200	222	245	30	31
Tilmicosin	108050-54-0	$C_{46}H_{80}N_2O_{13}$	2.86	869.5738	MH+	10	50[b]	57	67	14	30
Troleandomycin	2751-09-9	$C_{41}H_{67}NO_{15}$	3.64	814.4589	MH+	5	–	6.2	10.6	10	33
Tylosin	1401-69-0	$C_{46}H_{77}NO_{17}$	3.30	916.5270	MH+	10	50	67	98	15	29
Virginiamycin M1	21411-53-0	$C_{28}H_{35}N_3O_7$	3.82	548.2373	MNa+	0.5	–	0.3	0.5	45	12
Nitroimidazoles (**8**)											
Dimetridazole	551-92-8	$C_5H_7N_3O_2$	1.88	142.0616	MH+	0.5	–	0.2	0.3	62	8
Dimetridazole-hydroxy	936-05-0	$C_5H_7N_3O_3$	1.68	158.0565	MH+	1	–	0.3	0.6	114	4
Ipronidazole	14885-29-1	$C_7H_{11}N_3O_2$	2.98	170.0929	MH+	0.5	–	0.2	0.3	102	7
Ipronidazole-hydroxy	35175-14-5	$C_7H_{11}N_3O_3$	2.44	186.0878	MH+	0.5	–	0.2	0.4	111	14
Metronidazole	443-48-1	$C_6H_9N_3O_3$	1.67	172.0722	MH+	0.5	–	0.2	0.3	118	5
Metronidazole-hydroxy	4812-40-2	$C_6H_9N_3O_4$	1.48	188.0671	MH+	0.5	–	0.2	0.4	114	7
Ronidazole	7681-76-7	$C_6H_8N_4O_4$	1.88	201.0624	MH+	5	–	1.9	3.3	57	11
Ternidazole	1077-93-6	$C_7H_{11}N_3O_3$	1.93	186.0878	MH+	0.5	–	0.2	0.3	73	13
Quinolones (**14**)											
Cinoxacin	28657-80-9	$C_{12}H_{10}N_2O_5$	2.79	263.0668	MH+	10	–	4.4	7.6	217	8
Ciprofloxacin	85721-33-1	$C_{17}H_{18}FN_3O_3$	2.17	332.1410	MH+	0.5	100	106	112	489	7
Danofloxacin	112398-08-0	$C_{19}H_{20}FN_3O_3$	2.19	358.1567	MH+	0.5	30	32	34	807	9
Difloxacin	98106-17-3	$C_{21}H_{19}F_2N_3O_3$	2.47	400.1472	MH+	1	–	0.2	0.4	139	8
Enoxacin	74011-58-8	$C_{15}H_{17}FN_4O_3$	2.02	321.1363	MH+	1	–	0.3	0.5	259	11
Enrofloxacin	93106-60-6	$C_{19}H_{22}FN_3O_3$	2.24	360.1723	MH+	0.5	100	104	108	353	7
Fleroxacin	79660-72-3	$C_{17}H_{18}F_3N_3O_3$	2.10	370.1378	MH+	0.5	–	0.2	0.3	140	5
Flumequine	42835-26-6	$C_{14}H_{12}FNO_3$	3.64	262.0879	MH+	0.5	50	54	58	133	5
Marbofloxacin	115550-35-1	$C_{17}H_{19}FN_4O_4$	2.02	363.1468	MH+	1	75	78	82	98	9
Nalidixic acid	389-08-2	$C_{12}H_{12}N_2O_3$	3.54	233.0926	MH+	0.5	–	0.4	0.6	125	6
Norfloxacin	70458-96-7	$C_{16}H_{18}FN_3O_3$	2.07	320.1410	MH+	5	–	1.7	2.9	704	7
Oxolinic acid	14698-29-4	$C_{13}H_{11}NO_5$	2.99	262.0715	MH+	0.5	10[a]	12	15	141	5
Pefloxacin	70458-92-3	$C_{17}H_{20}FN_3O_3$	2.10	334.1567	MH+	0.5	–	0.2	0.4	385	7
Sarafloxacin	98105-99-8	$C_{20}H_{17}F_2N_3O_3$	2.44	386.1316	MH+	2	–	0.4	0.6	164	13
Sulfonamides (**25**)											
Dapsone	80-08-0	$C_{12}H_{12}N_2O_2S$	2.60	249.0697	MH+	10	100	112	128	91	8
Sulfabenzamide	127-71-9	$C_{13}H_{12}N_2O_3S$	3.13	277.0647	MH+	10	100	141	239	57	23
Sulfacetamide	144-80-9	$C_8H_{10}N_2O_3S$	1.77	215.0490	MH+	10	100	106	112	79	7
Sulfachlorpyrazine	1672-91-9	$C_{10}H_9ClN_4O_2S$	3.15	285.0213	MH+	10	100	132	189	72	17
Sulfachlorpyridazine	80-32-0	$C_{10}H_9ClN_4O_2S$	2.69	285.0213	MH+	10	100	115	135	93	7
Sulfadiazine	68-35-9	$C_{10}H_{10}N_4O_2S$	1.89	251.0603	MH+	10	100	114	134	37	7
Sulfadimethoxine	122-11-2	$C_{12}H_{14}N_4O_4S$	3.18	311.0814	MH+	10	100	117	140	90	13
Sulfadoxine	2447-57-6	$C_{12}H_{14}N_4O_4S$	2.82	311.0814	MH+	10	100	111	124	94	9
Sulfaethidol	94-19-9	$C_{10}H_{12}N_4O_2S_2$	2.83	285,0480	MH+	10	100	120	147	99	9
Sulfaguanidine	57-67-0	$C_7H_{10}N_4O_2S$	0.95	215.0603	MH+	10	100	108	117	47	8
Sulfamerazine	127-79-7	$C_{11}H_{12}N_4O_2S$	2.17	265.0759	MH+	10	100	107	115	75	10

Table 1 (*Continued*)

Compound	CAS	Formula	RT (min)	*m*/*z*	Ion	LD (µg/L)	MRL [a,b] (µg/L)	CCα (µg/L)	CCβ (µg/L)	Acc. (%)	RSD (%)
Sulfameter	651-06-9	$C_{11}H_{12}N_4O_3S$	2.41	281.0708	MH+	10	100	108	116	106	6
Sulfamethazine	57-68-1	$C_{12}H_{14}N_4O_2S$	2.37	279.0915	MH+	10	100	112	128	79	10
Sulfamethizole	144-82-1	$C_9H_{10}N_4O_2S_2$	2.37	271.0323	MH+	10	100	109	120	90	9
Sulfamethoxazole	723-46-6	$C_{10}H_{11}N_3O_3S$	2.83	254.0599	MH+	10	100	117	142	80	11
Sulfamethoxypyridazine	80-35-3	$C_{11}H_{12}N_4O_3S$	2.41	281.0708	MH+	10	100	107	115	106	6
Sulfamonomethoxine	1220-83-3	$C_{11}H_{12}N_4O_3S$	2.59	281.0708	MH+	10	100	108	116	113	6
Sulfamoxole	729-99-7	$C_{11}H_{13}N_3O_3S$	2.28	268.0756	MH+	10	100	139	246	82	10
Sulfanitran	122-16-7	$C_{14}H_{13}N_3O_5S$	3.63	336.0654	MH+	10	100	119	145	68	11
Sulfapyridine	144-83-2	$C_{11}H_{11}N_3O_2S$	2.08	250.0650	MH+	10	100	108	116	64	8
Sulfaquinoxaline	59-40-5	$C_{14}H_{12}N_4O_2S$	3.19	301.0759	MH+	10	100	113	129	87	10
Sulfathiazole	72-14-0	$C_9H_9N_3O_2S_2$	2.00	256.0214	MH+	10	100	106	113	54	7
Sulfatroxazole	23256-23-7	$C_{11}H_{13}N_3O_3S$	2.88	268.0756	MH+	10	100	114	134	96	10
Sulfisomidine	515-64-0	$C_{12}H_{14}N_4O_2S$	1.75	279.0915	MH+	10	100	109	119	87	11
Sulfisoxazole	127-69-5	$C_{11}H_{13}N_3O_3S$	2.94	268.0756	MH+	10	100	125	164	83	14
Tetracyclines (**6**)											
Chlortetracycline	57-62-5	$C_{22}H_{23}ClN_2O_8$	2.67	479.1221	MH+	10	100	113	130	188	9
Demeclocyclin	127-33-3	$C_{21}H_{21}ClN_2O_8$	2.45	465.1064	MH+	10	100	110	121	141	9
Doxycyclin	564-25-0	$C_{22}H_{24}N_2O_8$	2.78	445.1611	MH+	10	100[a]	106	114	242	7
Minocyclin	10118-90-8	$C_{23}H_{27}N_3O_7$	2.00	458.1927	MH+	10	100	116	133	258	20
Oxytetracyclin	79-57-2	$C_{22}H_{24}N_2O_9$	2.14	461.1560	MH+	10	100	106	111	229	12
Tetracyclin	60-54-8	$C_{22}H_{24}N_2O_8$	2.35	445.1611	MH+	10	100	108	118	170	8
Various (**30**)											
Benzocaine	94-09-7	$C_9H_{11}NO_2$	3.34	166.0868	MH+	0.5	–	0.4	0.6	98	13
Bromhexine	3572-43-8	$C_{14}H_{20}Br_2N_2$	3.41	377.0052	MH+ (Br^{81})	2	–	2.5	4.3	47	14
Carazolol	57775-29-8	$C_{18}H_{22}N_2O_2$	2.76	299.1759	MH+	0.5	1	0.3	0.6	196	10
Carbadox	6804-07-5	$C_{11}H_{10}N_4O_4$	2.12	263.0780	MH+	5	–	2.2	3.7	25	14
Clopidol	2971-90-6	$C_7H_7Cl_2NO$	1.82	191.9983	MH+	0.5	–	0.1	0.2	24	13
Diaveridine	5355-16-8	$C_{13}H_{16}N_4O_2$	1.93	261.1351	MH+	0.5	–	0.2	0.3	337	5
Diethylcarbamazine	90-89-1	$C_{10}H_{21}N_3O$	1.60	200.1763	MH+	0.5	–	0.2	0.3	76	6
Flunixin	38677-85-9	$C_{14}H_{11}F_3N_2O_2$	4.31	297.0851	MH+	0.5	40[b]	45	52	55	6
Ketoprofen	22071-15-4	$C_{16}H_{14}O_3$	4.11	255.1021	MH+	1	–	0.4	0.6	49	18
Levamisole	5036-02-2	$C_{11}H_{12}N_2S$	1.92	205.0799	MH+	0.5	10[a]	11.2	12.7	294	15
Lincomycin	154-21-2	$C_{18}H_{34}N_2O_6S$	1.88	407.2216	MH+	0.5	150	159	168	99	14
Meloxicam	71125-38-7	$C_{14}H_{13}N_3O_4S_2$	4.22	352.0425	MH+	0.5	15	16	18	58	18
Morantel	20574-50-9	$C_{12}H_{16}N_2S$	2.52	221.1112	MH+	0.5	100[a] 50[b]	107	116	383	7
Naproxen	22204-53-1	$C_{14}H_{14}O_3$	4.13	231.1021	MH+	5	–	7.5	12.8	37	27
Novobiocin	303-81-1	$C_{31}H_{36}N_2O_{11}$	5.06	613.2397	MH+	2	50[b]	54	59	18	20
Olaquindox	23696-28-8	$C_{12}H_{13}N_3O_4$	1.55	264.0984	MH+	2	–	0.5	0.8	56	8
Praziquantel	55268-74-1	$C_{19}H_{24}N_2O_2$	4.17	313.1916	MH+	2	–	1.8	3.1	102	44
Procaine	59-46-1	$C_{13}H_{20}N_2O_2$	1.70	237.1603	MH+	0.5	–	0.1	0.2	146	13
Promethazine	60-87-7	$C_{17}H_{20}N_2S$	3.32	285.1425	MH+	5	–	1.3	2.2	611	8
Pyrantel	15686-83-6	$C_{11}H_{14}N_2S$	2.16	207.0956	MH+	0.5	–	0.2	0.4	343	13
Pyrimethamine	58-14-0	$C_{12}H_{13}ClN_4$	2.77	249.0907	MH+	1	–	0.6	1	282	11
Rifaximin	80621-81-4	$C_{43}H_{51}N_3O_{11}$	4.53	786.3602	MH+	5	60[b]	6.2	10.6	39	22
Roxarsone	121-19-7	$C_6H_6AsNO_6$	1.57	263.9489	MH+	2	–	0.4	0.7	88	12
Tiamulin	55297-95-5	$C_{28}H_{47}NO_4S$	3.49	494.3304	MH+	0.5	–	0.4	0.6	169	17
Ticlopidine	53885-35-1	$C_{14}H_{14}ClNS$	2.71	264.0613	MH+	0.5	–	0.5	0.9	114	24
Tolfenamic acid	13710-19-5	$C_{14}H_{12}ClNO_2$	5.35	262.0635	MH+	10	50	5.8	9.9	12	21
Triflupromazine	146-54-3	$C_{18}H_{19}F_3N_2S$	3.88	353.1299	MH+	2	–	0.8	1.4	545	33
Trimethoprim	738-70-5	$C_{14}H_{18}N_4O_3$	2.05	291.1457	MH+	0.5	50	57	68	171	14
Xylazine	7361-61-7	$C_{12}H_{16}N_2S$	2.45	221.1112	MH+	1	10	10.7	11.5	189	14
Zeranol	26538-44-3	$C_{18}H_{26}O_5$	4.01	323.18582	MH+	5	–	2.1	3.5	45	8

[a] MRL according to Swiss regulation OSEC 817.021.23.

[b] MRL according to EU regulation 2377/90/EC.

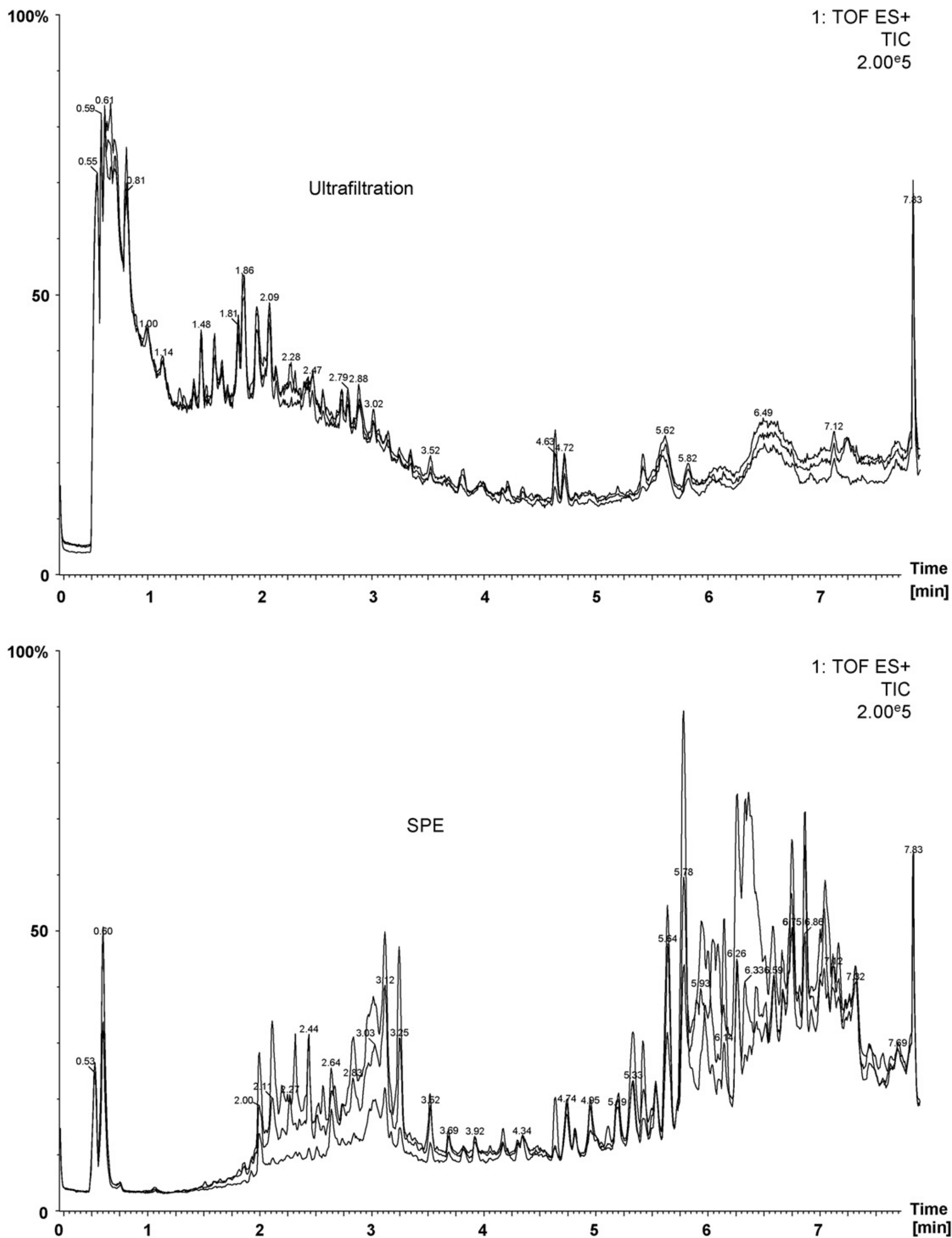

Fig. 2. Total ion chromatograms obtained after ultrafiltration or SPE cleanup on three different raw milk samples.

repeatable and therefore the resulting matrix effects should also be more repeatable. Regarding preparation time, the ultrafiltration approach is much faster than the SPE. This improves the samples throughput and was more appropriate for our laboratory considering irregular arrivals from 5 to 50 samples per day. The evaporation step could be a critical part of the sample preparation process. The use of cyclone evaporation system with electronically controlled pressure, nitrogen flow, temperature and shaking result in a very repeatable evaporation step compared to classical needle nitrogen evaporation. According to programmed parameters, only acetonitrile is evaporated thus avoiding the problem of loss by adsorption. Occasionally, unclear extracts due to precipitation were obtained after the evaporation step. In this case, a centrifugation of these extracts was conducted prior to injection.

3.2. Matrix effect

The matrix effect on response was evaluated by using a post-column infusion system as described previously by Souverain et al. [23]. Six blank milk and six blank water samples from different origins were prepared following the ultrafiltration (3 kDa) procedure and were injected. A syringe pump system was used for continuous post-column infusion of analyte standard solution at 100 ng/mL and 10 μL/min flow rate between the analytical column and the MS source. Data were recorded and signal intensity with or with-

out matrix was compared. Chromatograms for eight representative compounds are reported in Fig. 3. In the different examples, no matrix effect (Fig. 3a) or significant signal suppression or enhancement (Fig. 3b) was observed. Matrix effect varies from case to case and was mainly compounds dependent. Experiments demonstrated that milk samples from different origins exhibited quite homogenous matrix effect. Only febantel and erythromycin showed significant change in signal response according to milk sample. A signal enhancement up to 1300% was noted for enrofloxacin! In general, almost all quinolones were subjected to signal enhancement explaining high accuracy values measured during validation process. Although the matrix effects are not negligible, but even in case of significant signal suppression, detection limits were for almost all compounds well below the MRLs. The risk of false negative due to a complete signal suppression remains limited. At the end, rather than trying to relieve this matrix effect by a more complex sample preparation, it was decided to accommodate and to react case by case for the low number of positive samples. Confirmation quantitative analysis will be carried out either by added amount quantification, by using labelled standards or even by using a dedicated method with specific sample preparation for the corresponding compounds.

3.3. UPLC–TOF analyses

The use of UPLC column filled with small particles (1.7 μm) leads to significant improvements in terms of efficiency and time reduction compare to classical HPLC separation. With a total run time of 9 min (including equilibration time), a sample throughput of more than 100 samples per day is realistic. Fig. 4 shows a selection of extract ion chromatograms for a milk sample fortified at 10 μg/L. According to narrow peakwith, the scan speed of TOF MS was required to achieve enough acquisition point within the peak. In addition to its speed, TOF has the capability to produce exact mass measurements. This allows the generation of reconstructed ion chromatogram having narrow accurate mass windows, thus providing good selectivity in complex sample matrices. An example of TIC and corresponding extract ion chromatogram for

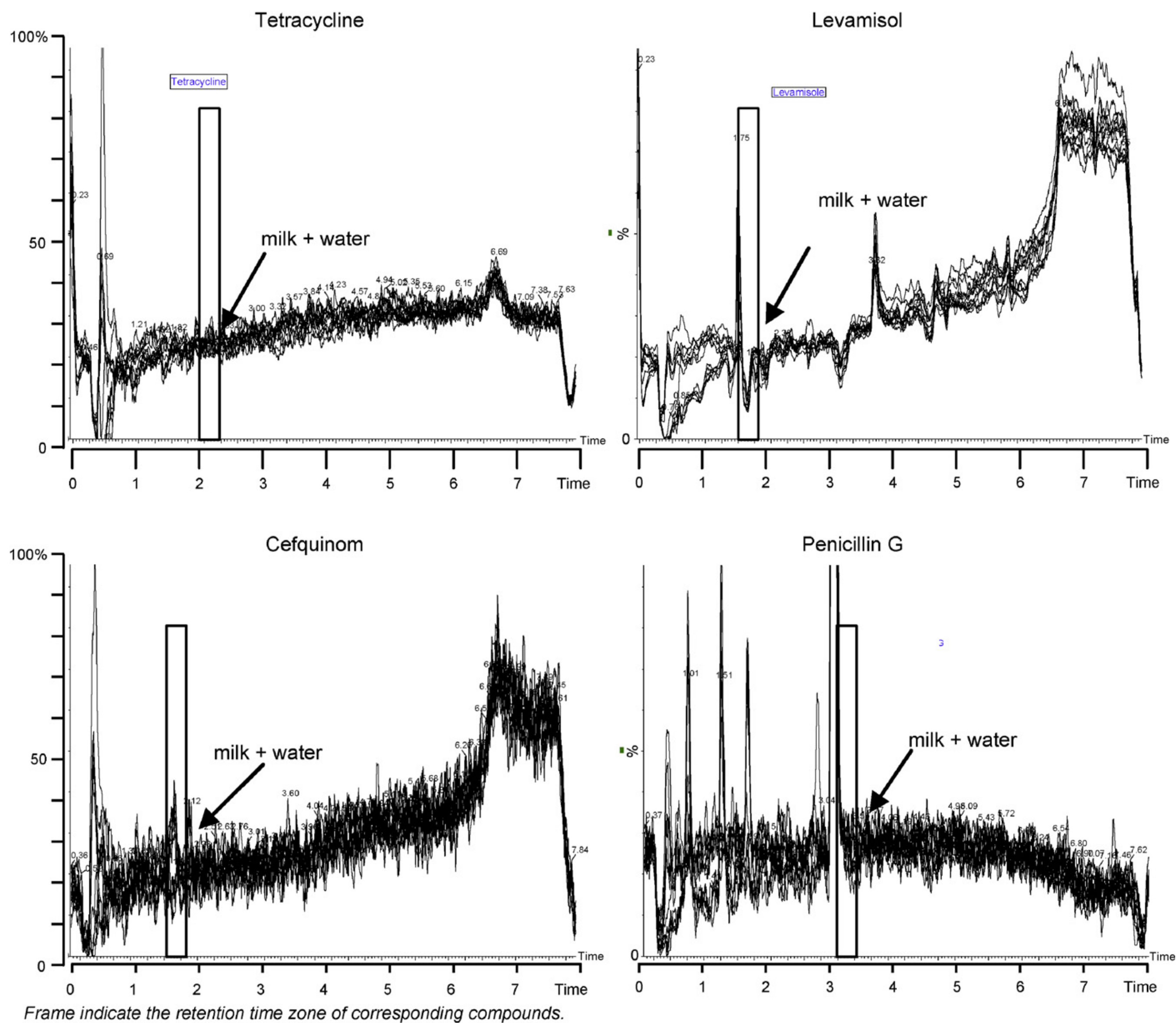

Fig. 3. (a) Matrix effect evaluation: examples of representative compounds without matrix effect. Frame indicate the retention time zone of corresponding compounds. (b) Matrix effect evaluation: examples of representative compounds with significant signal suppression or enhancement due to matrix effect. Frame indicate the retention time zone of corresponding compounds.

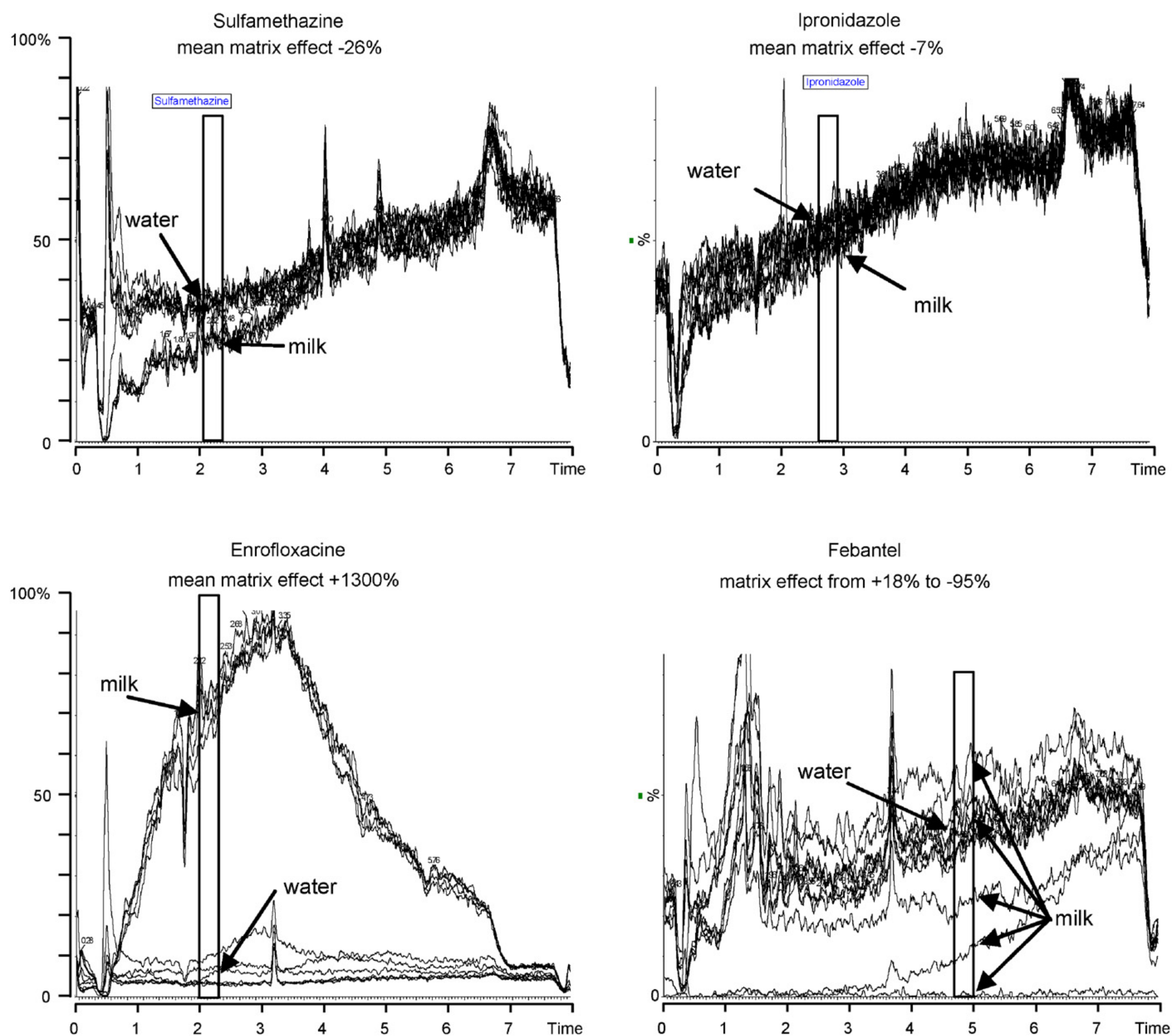

Fig. 3. (*Continued*).

tetracycline obtained on a real positive milk sample is given in Fig. 5A and B. Peaks at 2.21 and 2.35 min correspond to two major epimers of tetracycline. A 0.02 Da mass window was always used in order to minimize the risk to miss the selected analyte in case of mass shift while keeping very good selectivity. On the whole, veterinary drugs were detected with the theoretic m/z value calculated for the protonated ion ($[M+H]^+$). However, there are some exceptions (see Table 1) with the use of more sensitive ions like sodium adducts. Furthermore, according to full mass acquisition, mass spectra of positive samples can be extracted from the peak as shown in example Fig. 5C. The acquired spectra and isotope profile is directly compared to theoretic model. The presence of specific fragments can also be carried out to confirm (or not) the identity of detected peak.

3.4. Data processing and storage

Data processing was realised with TargetLynx software. According to the file size of ~100 MB for each chromatogram, a specific external device of 4 TB was dedicated for resolving the problem of data storage. After data treatment including chromatograms extraction, integration, calibration and quantification, a single file (~2 MB per sample) for the sequence is obtained and more easily stored. Unfortunately, this file does not allow the reprocessing of data for new compounds. Speed of data processing is obviously linked to the speed of the computer and in our case could take up to more than 1 min per sample.

3.5. Method performances

3.5.1. Specificity

The specificity was evaluated using extracted ion chromatogram of 20 blank samples. In many cases, the presence of unknown peak was detected in the 2 min chromatogram windows (see example in Fig. 4) showing the lowest selectivity of TOF MS compared to triple quadrupole detection. However, thanks to the powerful separation of UPLC, all identified interfering peak were baseline resolve with the peak of interest. A fine-tuning of integration windows with a low tolerance on retention time was sufficient to avoid the presence of false positives samples. It should be noted that more selectivity

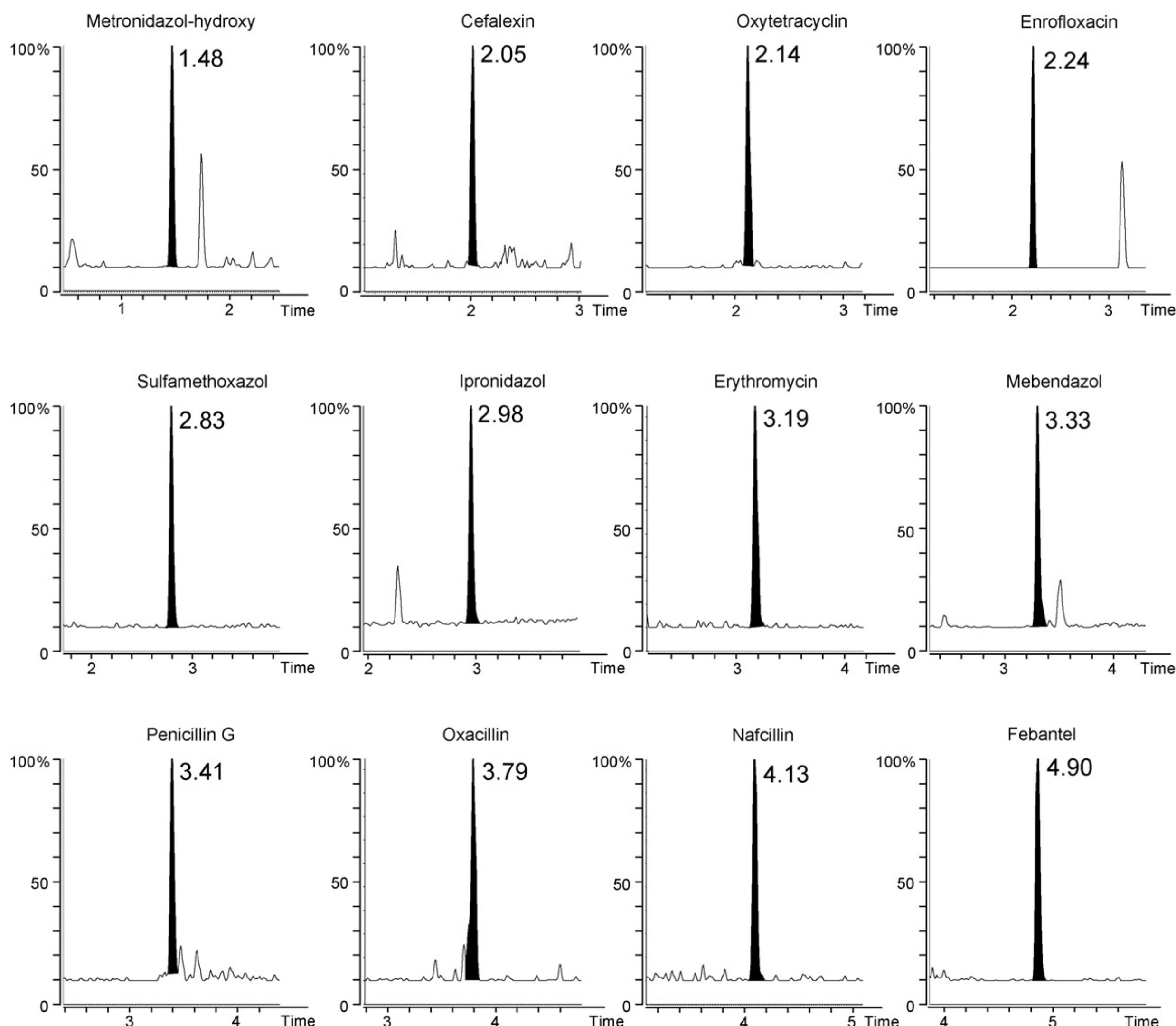

Fig. 4. Selected extracted ion chromatograms for a milk sample fortified at 10 μg/L.

would be achieved if measurements were produced in the W-mode instead of the V-mode. However, this would result in a relevant loss of sensitivity.

3.5.2. Sensitivity

Limit of detection (LD) indicated in Table 1 correspond to the lowest level of QC sample obtain during validation process without any false negative sample. Indeed, it was too difficult to calculate LD according to signal to noise ratio value as the noise is not always present. Except for compounds which have very low MRL such as clenbuterol or corticoides, limits of detection were largely lower than MRL and sensitivity of the proposed method was considered to be ideally suited for official milk control.

3.5.3. Decision limit (CCα) and detection capability (CCβ)

CCα and CCβ were calculated using the so-called calibration procedure [20–22]. This calculation is performed at the level of interest, which means the MRL level when existing and in other case at the level of detection. This two statistical limits allow to evaluate the critical concentrations above which the method reliably distinguish and quantify a substance taking into account the variability of the method and the statistical risk to take a wrong decision. CCα is usually not required for validation of screening method but only for confirmatory analyses [20]. CCα means the limit at and above which it can be concluded with an error probability of 95% (α) that a sample is non-compliant. CCβ means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of 95% (β). In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 5% ($1-\beta$). In the case of substances with an established MRL, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 5% ($1-\beta$). Calculated value of CCα and CCβ are indicated in Table 1. The values are very satisfactory and confirms good method performance quite suitable for the control of milk samples for the majority of compounds.

3.5.4. Accuracy and repeatability

Accuracy and repeatability values were calculated at a single level of 50 ng/mL on 20 different fortified milk samples. These values are related to the recovery rate and method precision but also linked to possible matrix effects which can induce signal suppression or enhancement, decreased or increased sensitivity of analytes over time, imprecision of results, retention time drift and chro-

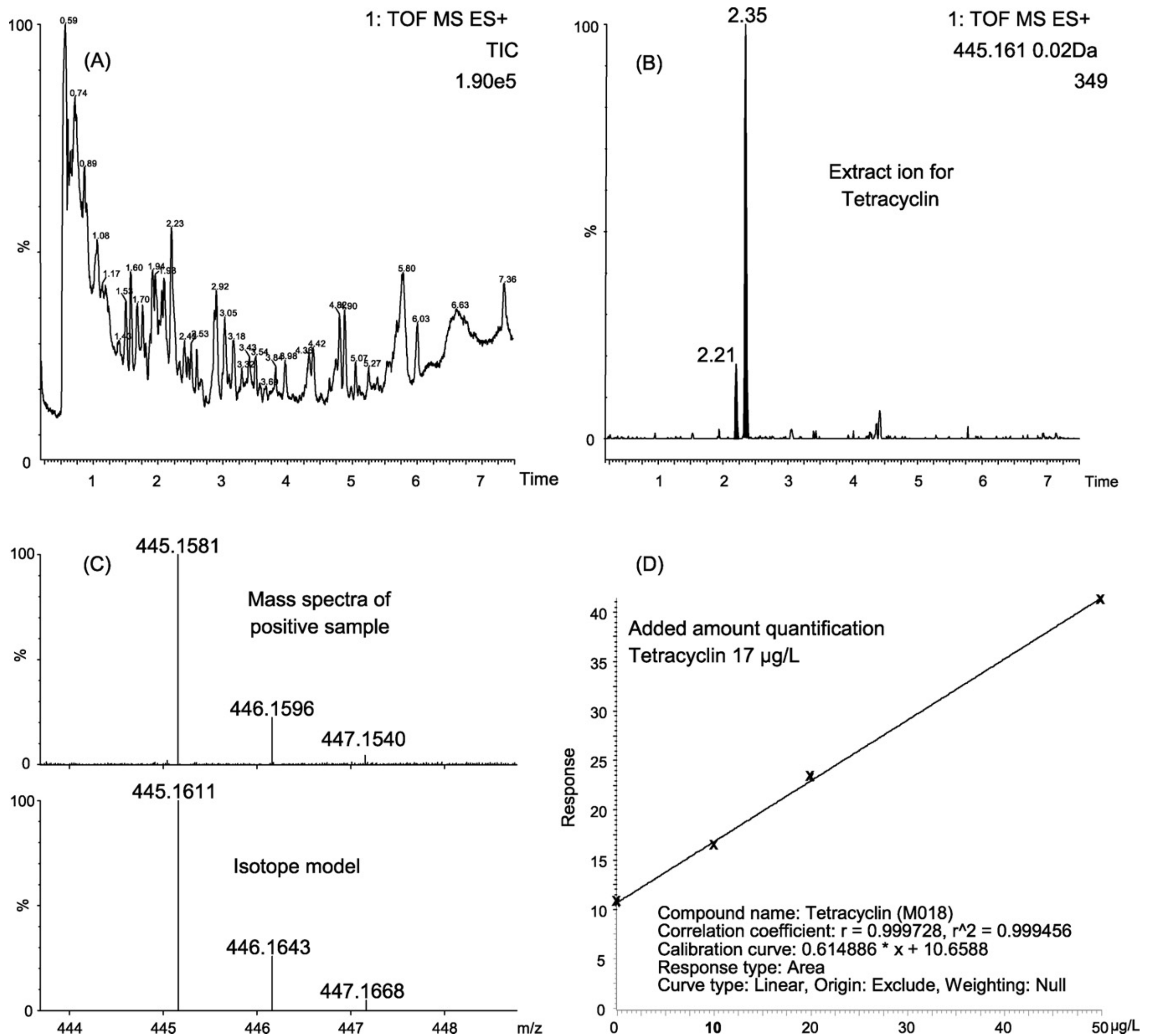

Fig. 5. Real positive milk sample: (A) total ion chromatogram, (B) extract ion chromatogram for tetracycline, (C) practical and theoretical mass spectra of molecular ion with isotope and (D) quantification of tetracycline with added amount calibration.

matographic peak tailing. Surprisingly, results were fairly uniform, looking at the values per family of compound (Table 1). The avermectines results do not fulfill the common admitted criteria of validation. The values of accuracy are below 10% and RSD values are very high. This can be explained by low extraction recovery due to important loss during the ultrafiltration step. Indeed, the avermectines molecules are the most voluminous compounds among the selected analytes even if their molecular weight is well below the cut-off of the membrane (3 kD). Furthermore, important signal suppression has been observed. An experiment was conduct by adding NaCl to the extract which promote sodium adduct and induce a significant increase of the signal reducing the matrix effect. However, it was harmful to the majority of other compounds and this approach was not retained. The avermectines have nevertheless been kept in the screening method and in case of positive sample detected, a specific method for avermectines would be applied for confirmation. Overall, other results are very satisfactory. However, It may be noted that many accuracy values exceed 120%. This is the case for flubendazole-amine, mebendazole-amine and for almost all quinolones and tetracyclines. Indeed, accuracy values up to 807% were measured indicating the presence of very important signal enhancement due to the presence of matrix components. However, the RSD measured on 20 different matrices remains very good. The matrix effect is very important, but repeatable! In this case, the signal enhancement is quite beneficial for the screening as it allows better detection limit. However, for the quantitative aspect, this matrix effect must be controlled to avoid important quantitative mistakes. Due to the low number of positive samples obtained in screening and the impossibility to obtain isotopically labelled internal standard for all substances, the approach chosen for confirmation was the method of added amount. An example of added amount quantification in shown for tetracycline in Fig. 5D. Added amount of approximately one, two and five times of sample concentration is carried out as well as sample without addition. A simple regression using the least square method was applied and the concentration is calculated by dividing the intercept value by the slope value of the calibration curve. In this case, the matrix effect is offset by the presence of standard within the sample.

3.5.5. Applicability

Seventy milk samples (naturally contaminated or spiked) were kindly provided by Quality & Safety Department form Nestlé Research Center. Milk samples were frozen or lyophilised and reconstituted before analysis. All reference Nestlé samples were blindly analysed by introducing systematically few of them each day in routine analyses. Seven samples were negative and others samples were found positive for different substances (five sulfonamides, five quinolones, five beta lactams including penicillin's and cephalosporines, two tetracyclines and one macrolides). No false negative occurred after comparison with Nestlé results. Three possible cases of false positive have been highlighted each time for the presence of enrofloxacin. The results do not match with the negative results obtained by Elisa screening for quinolones. Experiments are underway to determine if this is a false positive from UPLC–TOF method or false negative from Elisa. The method was also applied in routine to samples taken from Swiss farms as a part of the national control plan for residue monitoring. QC samples introduced in each analytical sequence have demonstrated very good stability of system performances within time. For the first half of 2008, four positive samples out of 150 have been identified for the presence of cefalexin, tetracycline, sulfaquinoxaline and penicillin G. The latter was not compliant with a concentration of 30 μg/L largely above the MRL of 4 μg/L. Other cases were far below the allowed values.

4. Conclusions

This paper shows the use of UPLC coupled to orthogonal acceleration TOF MS for the comprehensive screening of 150 veterinary drugs residues in raw milk. An easy sample preparation based on protein precipitation associated with ultrafiltration was hyphenated to fast chromatography. An in-house validation procedure has been carried out and show very good performances for screening. According to the high sensitivity and selectivity of TOF MS detection, limits of detection were between 0.5 and 25 μg/L and largely below MRL for the majority of compounds. Except some problems with avermectines, the method allowed screening and quantification for benzimidazoles, beta-agonists, beta-lactams, corticoides, macrolides, nitroimidazoles, quinolones, sulfonamides, tetracyclines and some others veterinary drugs. Results fulfilled the common criteria of validation and the method was accepted for official control of veterinary drugs residues in milk. The method was successfully applied to 70 quality control unknown samples and in routine for more than 150 raw milk as a part of national control plan for residue monitoring.

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