



# Critical assessment of two sample treatment methods for multiresidue determination of veterinary drugs in milk by UHPLC-MS/MS

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## Abstract

In this work, two sample treatment procedures have been evaluated for the determination of veterinary drug residues in milk. In order to cover a wide range of polarities, a total of 66 veterinary drugs with  $\log K_{ow}$  ranging from  $-1$  to  $5$  were selected. Two sample cleanup steps, (i) dispersive solid-phase extraction (dSPE) using enhanced matrix removal lipid as sorbent and (ii) solid-phase extraction (in pass-through mode) using Oasis HLB PRiME cartridges, were critically assessed in terms of sample throughput, recovery, matrix effect, cleanliness of extracts, limit of quantification, and repeatability. The veterinary drugs tested (viz. benzimidazoles, cephalosporins, imidazothiazoles, macrolides, NSAIDs, penicillins, quinolones, steroids, sulfonamides, and  $\beta$ -agonists) were analyzed by ultra-high-performance liquid chromatography tandem mass spectrometry. According to the results, both methods exhibited similar recovery rates between 70 and 120% for most of compounds tested. Matrix effects were satisfactory for both methodologies, although the tolerance to matrix effects was slightly higher with HLB PRiME with nearly negligible matrix effects in most cases. Limits of quantitation were also well below the current maximum residue levels established by the European Union. Notably, sample throughput was higher in the case of HLB PRiME, since this pass-through SPE cleanup approach involved fewer steps than the EMR-Lipid dSPE approach. The results in terms of analysis time, sensitivity, precision, cleanliness of extracts, and matrix effect showed the suitability of both procedures for the monitoring of veterinary drugs residues in milk samples in a single run.

**Keywords** Sample treatment · Veterinary drugs · Milk · HLB PRiME · EMR-Lipid · UHPLC-MS/MS

## Introduction

Dairy products can be regarded as one of the main markets in terms of global economic importance, due to its high consumption and the fact that milk is one of the most complete animal origin food to maintain a healthy and balanced diet [1, 2]. On the other hand, the use of veterinary drugs in livestock farming and bovine milk production has contributed to

improve animal health and well-being [3]. However, a misuse of these compounds in veterinary practice may yield residues in foodstuffs that may originate adverse effects in humans, as allergic reactions or antibiotic resistance [4]. In fact, the World Health Organization warns that antibiotic resistance is one of the biggest threats to global health and food security nowadays [5]. According to this, the European Union has set maximum residue limits (MRLs) of antibiotics in foodstuffs of animal origin by means of Commission Regulation 37/2010. These MRLs are lower than  $100 \mu\text{g kg}^{-1}$  in the case of milk [6].

Consequently, the development of sensitive and rugged analytical methods to address the current framework is of utmost importance. In order to fulfill the EU requirements in terms of sensitivity and specificity, reported methods so far make use of high-performance liquid chromatography (HPLC) or ultra-high-performance liquid chromatography (UHPLC) coupled with tandem mass spectrometry (MS/MS) [7–11]. Despite countless advances in detection techniques and separation such as development of new stationary phases

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and more sensitive mass spectrometers, sample treatment step remains a mayor bottleneck in food analysis [12], involving typically more than 70% of the total analysis time.

Several procedures have been proposed for the determination of veterinary drugs in milk involving solid-phase extraction (SPE) [13, 14], protein precipitation [15], simple solvent extraction [16], or QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) [17, 18]. Due to the high concentration of proteins and lipids in milk, procedures should be conceived to diminish their content or even completely remove them from the final extract. The target is, thus, to minimize matrix effects while achieving appropriate recoveries without time-consuming steps. To accomplish this, the coextracted lipids (e.g., fatty acids and triglycerides) obtained when applying the mentioned solvent-based generic sample treatment methods (e.g., QuEChERS) in fatty matrices should be subjected to an additional cleanup step. A new generation of sample preparation sorbents has been recently developed [19–25], including HLB PRiME (process, robustness, improvements, matrix effects, ease of use), used in pass-through SPE mode, and the so-called Enhanced Matrix Removal-Lipid (EMR-Lipid) sorbent, used as dispersive SPE (dSPE) agent. EMR-Lipid has been proposed for the determination pesticides in fatty vegetable matrices [19, 20]. Meanwhile, HLB PRiME has been successfully applied for the determination of antibiotics in milk samples [21, 22]. Satisfactory extraction has been reported for the multiresidue determination of veterinary drugs with HLB PRiME [23] and EMR-Lipid [24] in milk and bovine liver, respectively. It should be noted that, Wittenberg et al. developed a method for the determination of 40 veterinary drugs in various milk-based powders using HLB PRiME [25]. In this work, the use of EMR-Lipid was also tested. Unfortunately, an in-depth comparison of both procedures has not been addressed hitherto.

With this in mind, a thorough comparison between these two sample treatment approaches has been accomplished in this work in terms of sample throughput, matrix effect, recovery, and precision. A set of 66 veterinary drugs, which covered a wide ranges of polarities, were selected and determined by UHPLC-MS/MS, in order to critically assess these two cleanup procedures in milk samples.

## Experimental

### Chemicals, reagents, and apparatus

HPLC grade methanol (MeOH), acetonitrile (MeCN), and formic acid were supplied from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was obtained from a Milli-QPlus system (Millipore, Milford, MA, USA).

Sixty-six veterinary drugs including benzimidazoles, cephalosporins, imidazothiazoles, macrolides, NSAIDs, penicillins, quinolones, steroids, sulfonamides, and  $\beta$ -agonist were selected so that a wide range of polarities between  $\log K_{ow} - 1$  and  $+ 5$  was covered (see Electronic Supplementary Material (ESM) Table S1). Notably, the list of selected compounds studied in this work is essentially different and more thorough than those included in related studies [19, 21]. Analytical standards of veterinary drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA). Each veterinary drug solution ( $500 \text{ mg L}^{-1}$ ) was prepared in MeCN and stored at  $-18 \text{ }^\circ\text{C}$ . After that, a working solution which contained a mixture of all the standards ( $10 \text{ mg L}^{-1}$ ) was prepared in  $\text{H}_2\text{O}:\text{MeOH}$  (80:20) and also stored at  $-18 \text{ }^\circ\text{C}$ .

For sample treatment procedures, centrifuge tubes obtained from Deltalab (Barcelona, Spain) were required. Oasis HLB PRiME 3-cc cartridges (Waters, Milford, MA, USA) were used for the first sample treatment. For the second one, EMR-Lipid dSPE tubes and EMR-Lipid Polish tubes were supplied by Agilent Technologies (Santa Clara, CA, USA).

A centrifuge (Sartorius 2-16P from Sigma, St. Louis, MO, USA), an evaporator (TurboVap LV, Caliper Life Sciences, Waltham, MA, USA), and a vortex (Vortex-Genie 2 from Scientific Industries, NY, USA) were also used.

### Sample treatment

#### Procedure I: Solvent extraction followed by pass-through SPE cleanup using HLB PRiME

The proposed method was adapted from a previous study [23] with slight changes. The first step for milk is often a liquid-liquid extraction with an organic solvent with few percent of formic acid to prompt the removal of proteins by its precipitation. It was made by adding 1 g of cow milk to a 15-mL centrifuge tube; then, 4 mL of MeCN (2% formic acid) was added to the same tube and the mixture was vortexed for 30 s. After centrifugation at 6460g for 5 min, 4 mL of the organic layer were pipetted and passed through the HLB PRiME cartridge, which was previously conditioned with 3 mL of MeCN (2% formic acid). One hundred microliters of the extract eluted from the cartridge was diluted with 100  $\mu\text{L}$  of MeCN and 800  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , so that the final composition was 80:20 (v/v) aqueous:organic, the same organic content of the initial mobile phase.

#### Procedure II: Solvent extraction followed by EMR-Lipid dSPE cleanup

The procedure was adapted from a previous method for the analysis of veterinary drugs in bovine liver [24]. The procedure is as follows: 2 g of cow milk was pipetted into a 50-mL centrifuge tube and 10 mL of MeCN (5% formic acid) for the

initial extraction step. This tube was vortexed for 2 min and centrifuged at 2650g for 5 min. Prior to its use, the EMR sorbent must be activated/conditioned. For this, 5 mL of ammonium acetate buffer solution (5 mM) was added to an EMR-Lipid dSPE tube. After that, 5 mL of the organic layer—resulting from the centrifugation—were added to the EMR-Lipid dSPE tube, and it was manually shaken for 1 min and vortexed for another minute. The tube was centrifuged at 2650g for 3 min; at this stage, no phase separation was observed because H<sub>2</sub>O and MeCN are miscible solvents. Then, 5 mL of the upper layer of the tube was transferred to an EMR-Lipid Polish tube (containing 1.6 g of MgSO<sub>4</sub> + 0.4 g NaCl). The mixture was then vigorously shaken for 2 min. After this, the tube was centrifuged at 2650g for 3 min, and subsequently two different liquid phases are generated, being the organic phase extract collected. A 1:10 final dilution was made using a 100- $\mu$ L aliquot of the extract, yielding a final composition of 80:20 (H<sub>2</sub>O/MeCN, v/v).

### LC-MS analysis and equipment

Separation was performed on a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC) instrument (Thermo Fisher Scientific, Waltham, MA, USA), using a Zorbax Rapid Resolution High Definition (RRHD) Eclipse Plus C18 column (2.1 mm  $\times$  50 mm, 1.8- $\mu$ m particle size) supplied by Agilent Technologies (Santa Clara, MA, USA). The UHPLC system was coupled to a triple quadrupole analyzer (TSQ Quantiva, Thermo Fisher Scientific, San José, CA, USA) using a heated electrospray ionization source ((HESI-II, Thermo Fisher Scientific, San José, CA, USA). Mobile phases were water (solvent A) and MeCN (solvent B) both of them with 1% formic acid. The following gradient program was used: an initial composition of 10% B was kept constant for 4 min; it increased to 30% B in 1 min and then a linear gradient to 95% B for 3 min, being then held for 2.5 additional minutes. Finally, the initial mobile composition was reached after 0.5 min followed by a re-equilibration time of 4 min. The injection volume was 10  $\mu$ L and the flow rate was 0.4 mL min<sup>-1</sup> during all the analysis. Column temperature was kept constant at 25 °C throughout the analysis. Veterinary drugs were detected in the positive ionization mode using multiple reaction monitoring (MRM) MS acquisition mode. Ion source parameters were as follows: spray voltage, 3500 V; sheath gas, 35 arbitrary units; aux gas, 15 arbitrary units; sweep gas, 1 arbitrary unit; ion transfer tube temp, 250 °C; vaporizer temp, 300 °C; collision gas (CID), 2 mTorr.

The cleanliness of extracts was assessed using UHPLC-TOFMS analysis with full-scan acquisition so that all the ionizable species present in the extract were accounted. It was performed with an UHPLC system (Agilent Series 1290 Infinity, Agilent Technologies, Santa Clara, CA, USA), coupled to a time-of-flight (TOF) mass spectrometer

(Agilent Technologies, Santa Clara, CA, USA) using electrospray ionization with default parameters (capillary voltage, 4000 V; fragmentor voltage, 190 V; drying gas, 9 L/min; drying gas temperature, 325 °C). The full-scan acquisition was made in the range from *m/z* 50 to 1000 in the positive ionization mode and with the same optimized chromatographic conditions (e.g., column, mobile phases, flow rate, injected volume) described previously for UHPLC-MS/MS analysis.

## Results and discussion

### Optimization of UHPLC-MS/MS method

The mass spectrometer parameters were optimized to obtain the best response for the quantification of each individual veterinary drug using an MRM MS/MS method. Two products ions were selected, the most abundant was used for the quantification and the second one was used as confirmatory MS/MS transition. MRM transitions, optimized collision energy, and lens voltage of each veterinary drugs are shown in the ESM (Table S2). These two precursor-product ion transitions together with the corresponding retention time of each compound were used for the identification of each targeted analyte. Precursor ion was in all cases the protonated molecule [M-H]<sup>+</sup>.

Two C18 columns were tested: Accure aQ 2.1 mm  $\times$  100 mm, 2.6  $\mu$ m (Thermo Fisher Scientific, Waltham, MA, USA) and Zorbax RRHD Eclipse Plus C18 column (2.1 mm  $\times$  50 mm, 1.8  $\mu$ m). Better overall results in terms of or selectivity, ruggedness, and reproducibility were obtained using Zorbax RRHD Eclipse Plus C18 column. Different organic solvents such as MeCN and methanol as organic mobile phases were then studied. The use of MeCN improved analyte peak shape and separation in terms of peak resolution for all analytes. In order to enhance analyte signal, the concentration of formic acid was tested between 0.1 and 1.5%. The ionization efficiency was improved for several compounds with 1% of formic acid and no ammonium formate was used in both phases. Finally, other parameters of the UHPLC-ESI method including mobile phase flow rate, column temperature, injection volume, spray voltage, and vaporizer temperature were also optimized, being the optimum values detailed in the “[Experimental](#)” section.

### Evaluation of the sample treatment procedures

The determination of veterinary drugs in milk samples is challenging due to the inherent complexity of the matrix. Milk contains about 4–5% sugar (lactose), 2–5% fat, and 4–5% proteins (caseins and whey proteins), as well as calcium, potassium, and B vitamins [26]. Milk fat is composed primarily of triglycerides (95% of the total milk fat). Other milk lipids

**Table 1** Recoveries and precision obtained for each veterinary drug in milk using HLB PRiME and EMR-Lipid methods

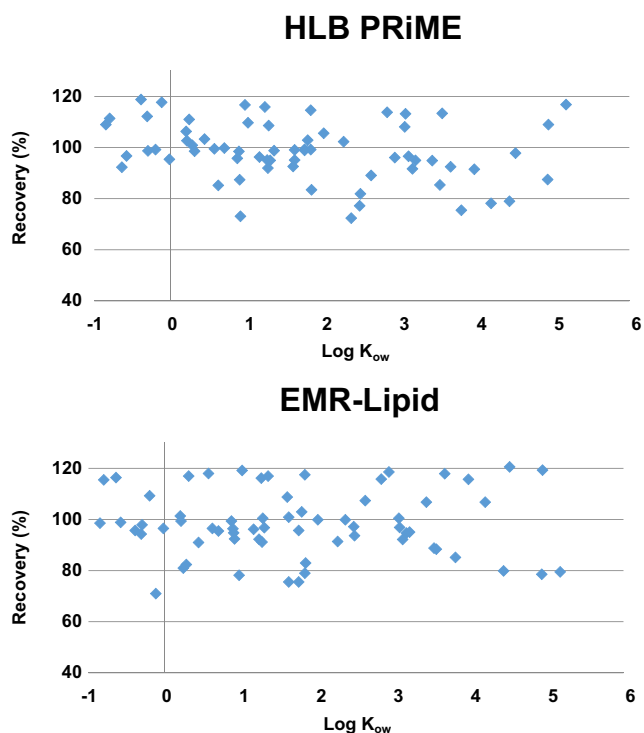
Veterinary drug	HLB PRiME			EMR-Lipid		
	Recovery % (RSD%) (n = 30)	Intraday RSD% (n = 15)	Interday RSD% (n = 15)	Recovery % (RSD%) (n = 30)	Intraday RSD% (n = 15)	Interday RSD% (n = 15)
<b>Benzimidazoles</b>						
Albendazole	113.2 (8.5)	1.3	8.4	116.4 (5.9)	3.5	6.9
Albendazole sulfone	95.8 (11.2)	3.7	6.0	98.9 (18.1)	3.9	6.3
Albendazole sulfoxide	92.0 (5.9)	5.3	8.6	95.7 (6.2)	4.6	7.8
Febantel	72.4 (8.6)	4.4	6.9	92.3 (7.3)	5.9	7.2
Fenbendazole	113.4 (6.6)	4.7	8.1	116.2 (7.3)	5.8	9.2
Mebendazole	113.8 (12.9)	3.8	3.7	117.5 (8.2)	2.9	4.9
Oxfendazole	99.1 (9.3)	4.8	9.8	99.9 (10.8)	2.7	6.1
Oxibendazole	102.3 (7.7)	5.7	9.4	97.2 (9.6)	5.2	8.0
Thiabendazole	77.2 (9.3)	2.7	9.8	120.6 (12.4)	5.1	10.3
<b>Cephalosporins</b>						
Cefaclor	102.7 (10.8)	16.1	17.2	71.0 (12.5)	11.2	16.1
Cefazolin	96.7 (11.7)	14.8	18.2	96.5 (14.7)	10.5	13.9
Ceftiofur	108.6 (14.1)	8.5	10.5	101.4 (9.1)	7.1	10.9
<b>Imidazoles</b>						
Metronidazole	95.4 (5.3)	4.5	6.7	79.0 (4.9)	5.0	6.7
<b>Imidazothiazoles</b>						
Levamisole	83.5 (9.3)	6.5	10.3	100.9 (13.9)	4.6	8.8
<b>Lincosamides</b>						
Lincomycin	106.3 (7.7)	5.7	6.5	75.6 (9.6)	5.6	8.9
<b>Macrolides</b>						
Erythromycin	108.1 (9.9)	6.7	9.4	119.2 (10.4)	7.2	18.2
Josamycin	96.6 (14.3)	5.2	11.9	117.0 (8.1)	7.9	7.3
Roxithromycin	96.1 (4.2)	5.4	9.2	95.1 (4.7)	5.3	10.1
Tilmicosin	75.5 (5.7)	5.8	6.8	78.6 (6.2)	5.0	6.1
<b>Nitroimidazoles</b>						
Dimetridazole	98.6 (10.2)	4.1	6.0	96.4 (14.3)	6.7	10.7
<b>NSAID</b>						
Diclofenac	97.8 (17.3)	5.3	7.7	96.5 (6.5)	6.3	9.1
Phenylbutazone	91.7 (15.3)	4.2	9.0	115.8 (7.8)	5.7	11.8
Sulindac	94.9 (10.8)	6.4	9.8	106.8 (8.3)	5.9	9.1
Tolfenamic acid	116.8 (9.3)	6.7	12.2	119.3 (12.9)	6.7	12.2
<b>Penicillins</b>						
Ampicillin	98.8 (5.4)	7.7	4.3	97.9 (3.6)	6.1	6.7
Cloxacillin	81.9 (15.8)	4.2	6.3	82.4 (5.4)	4.5	6.9
Penicillin G	99.2 (12.4)	9.0	12.9	107.4 (7.7)	7.2	5.6
<b>Quinolones</b>						
Ciprofloxacin	100.9 (10.7)	5.8	9.6	99.4 (8.8)	5.5	8.3
Danofloxacin	103.3 (11.6)	5.4	16.4	91.0 (11.1)	5.9	8.7
Difloxacin	98.5 (7.5)	4.0	13.2	95.5 (8.7)	2.5	5.1
Enoxacin	99.2 (11.2)	5.4	9.4	94.8 (9.7)	5.6	8.8
Enrofloxacin	99.8 (10.2)	4.7	9.2	92.4 (10.6)	6.0	11.1
Fleroxacin	111.0 (9.7)	4.8	9.1	100.5 (10.7)	6.0	10.8
Lomefloxacin	98.7 (5.8)	5.4	7.4	95.7 (9.8)	5.1	9.9

**Table 1** (continued)

Veterinary drug	HLB PRiME			EMR-Lipid		
	Recovery % (RSD%) (n = 30)	Intraday RSD% (n = 15)	Interday RSD% (n = 15)	Recovery % (RSD%) (n = 30)	Intraday RSD% (n = 15)	Interday RSD% (n = 15)
Marbofloxacin	112.2 (11.3)	8.3	12.2	103.0 (11.0)	6.2	9.6
Ofloxacin	118.8 (10.6)	4.9	9.9	99.9 (10.5)	3.4	5.8
Orbifloxacin	96.3 (7.0)	5.0	7.1	91.4 (5.8)	4.3	3.3
Sarafloxacin	117.7 (8.3)	5.3	5.3	106.8 (8.6)	3.4	3.9
Rifamycins						
Rifaximin	92.5 (8.5)	6.0	7.6	94.7 (9.6)	5.1	7.1
Steroids						
Betamethasone	114.6 (11.0)	7.8	11.1	109.3 (6.0)	7.1	4.0
Nandrolone	89.1 (14.9)	14.3	18.6	83.0 (15.2)	15.4	19.4
Prednisolone	95.1 (11.1)	9.0	10.9	100.5 (10.7)	7.8	8.4
Sulfonamides						
Sulfabenzamide	115.9 (9.8)	4.5	9.4	117.9 (9.4)	3.6	6.3
Sulfaguanidine	111.4 (9.1)	4.5	8.1	85.2 (8.2)	5.8	7.9
Sulfisoxazol	109.7 (9.9)	4.3	6.3	115.7 (7.2)	4.5	7.5
$\beta$ -Agonists						
Clenbuterol	105.6 (11.8)	4.6	7.8	81.4 (6.5)	5.6	9.2
Ractopamine1	99.3 (8.0)	6.3	10.2	96.9 (14.9)	6.1	7.2
Ractopamine2	98.9 (9.0)	5.3	9.2	92.2 (11.0)	5.7	8.2
Terbutaline	87.4 (8.3)	5.7	9.4	79.9 (5.9)	5.0	8.5
Others						
Acephate	109.0 (10.5)	6.3	17.7	98.6 (6.2)	5.2	8.7
Acetamiprid	92.6 (14.7)	3.4	10.2	115.5 (8.9)	2.5	9.5
Altrenogest	85.4 (16.5)	4.2	13.5	94.3 (8.1)	2.6	9.7
Colchicine	95.0 (9.8)	5.9	10.5	117.0 (4.3)	6.7	9.4
Dapsone	116.7 (12.8)	4.8	8.5	118.3 (15.2)	6.1	9.8
Digoxin	95.1 (12.0)	15.9	16.5	99.4 (10.7)	16.7	18.3
Eprinomectin	87.5 (13.8)	4.5	9.3	78.2 (9.6)	5.6	10.2
Famotidine	92.3 (10.2)	4.3	7.1	96.2 (11.4)	13.5	14.9
Fenthion	91.5 (9.4)	6.8	9.8	91.2 (11.6)	8.6	10.7
Imidacloprid	99.5 (5.7)	4.0	9.6	96.8 (6.7)	4.5	9.6
Leucomalachite green	109.0 (7.6)	7.7	14.3	108.8 (12.5)	7.1	11.2
Malachite green	85.2 (14.5)	5.9	9.6	75.6 (10.3)	5.0	7.7
Paclobutrazol	95.0 (5.4)	4.5	10.9	93.7 (9.6)	7.3	12.2
Phenylthiouracil	102.9 (10.2)	8.9	7.6	118.6 (11.6)	7.3	10.2
Spynosin A	79.0 (4.3)	4.6	9.7	88.8 (5.3)	2.8	9.6
Spynosin D	78.2 (5.3)	4.8	10.1	88.4 (9.0)	3.2	8.7
Trimethoprim	73.1 (6.2)	5.6	9.2	79.5 (7.1)	5.5	9.7

are diacylglycerides (0.25–0.48%), monoacylglycerides (0.02–0.04%), phospholipids (0.6–1.0%), cholesterol (0.2–0.4%), glycolipids (0.006%), and free fatty acids (0.1–0.4%) [27]. In particular, the high concentration of proteins and lipids in milk adds on complexity to the sample treatment leading to final extracts rich in potentially interfering

substances. Thus, it is central to reduce the number of coeluting interfering species to obtain clean extracts, keeping also recovery rates and precision at appropriate levels. All these aspects together with sample throughput were considered for the comparison between HLB PRiME SPE pass-through and EMR-Lipid dSPE cleanup procedures.

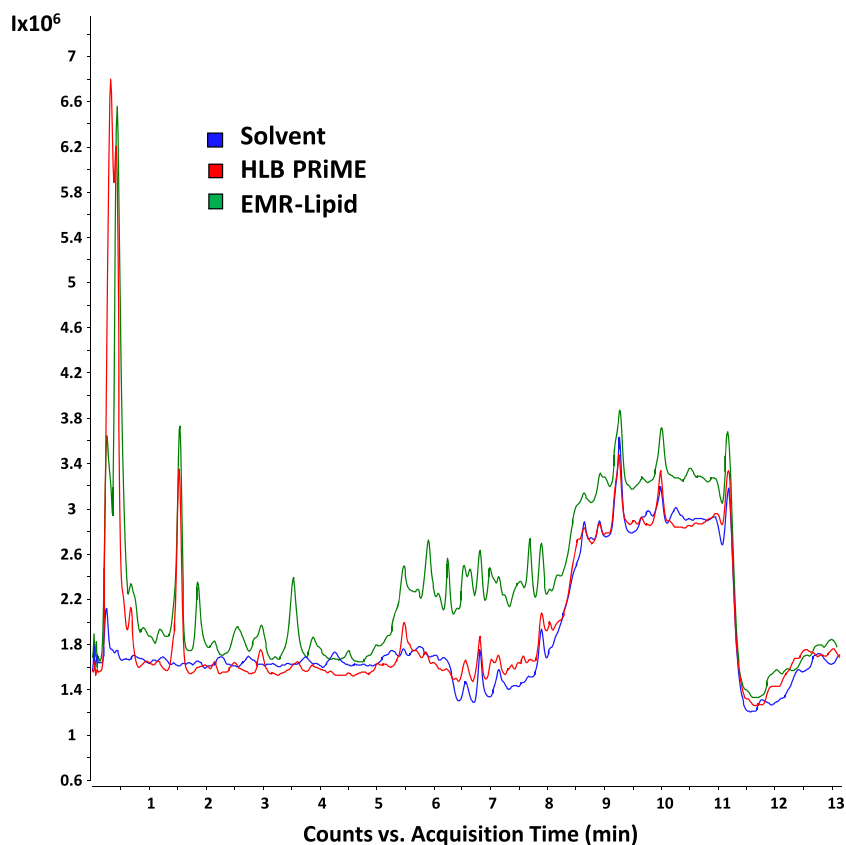


**Fig. 1** 2D plot of the average recovery rates ( $n=30$ )/ $\log K_{ow}$  in milk using EMR-Lipid and HLB PRiME

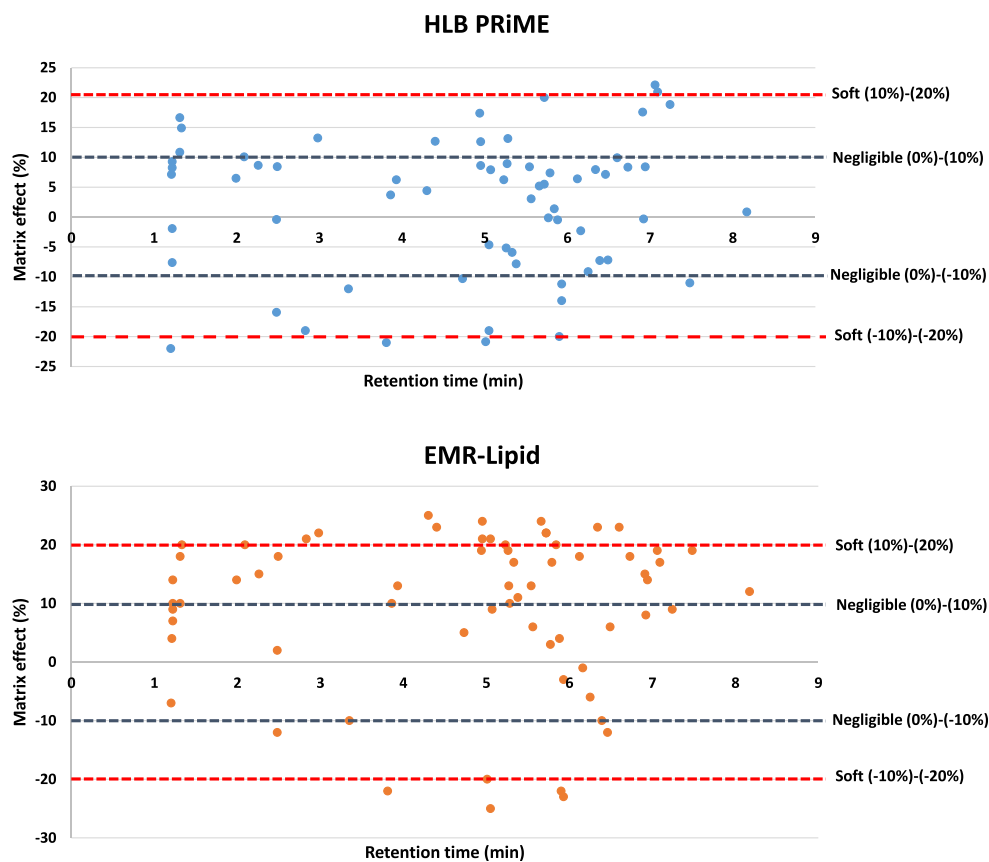
**Extraction efficiency** Ten blank samples of whole cow milk were spiked with the mixture of analytes at  $50 \mu\text{g kg}^{-1}$  and subjected to both HLB PRiME and EMR-Lipid procedures, being subsequently injected by triplicate in the UHPLC-MS/MS ( $n=30$ ). Recovery rates were calculated for each compound by means of interpolation of the areas integrated in the quantification peaks with the matrix-matched calibration curves. These average recovery rates for each compound (Table 1) are plotted against the corresponding octanol/water partition coefficient ( $\log K_{ow}$ ) in Fig. 1. Notably, both sample treatments led to outstanding recovery rates between 70 and 120%, thus yielding a similar extraction efficiency. In addition, no significant patterns were noticed for increasing low  $K_{ow}$  values, nor for family classes. The repeatability was also satisfactory ( $RSD \leq 19.4\%$ ), thus fulfilling the current regulatory requirements [28].

**Extract cleanliness and matrix effects** In order to estimate the effectiveness of both cleanup procedures, blank matrix extracts of whole milk obtained from the two tested sample treatment approaches and sample solvent (80:20  $\text{H}_2\text{O}/\text{MeCN}$ ) were injected in a UHPLC-TOF-MS instrument operated in high-resolution full-scan (FS) acquisition mode. As shown in the overlapped FS total ion chromatograms (TICs) (Fig. 2), the amount of coextracted species in the case of

**Fig. 2** UHPLC-TOFMS full-scan total ion chromatograms from solvent and milk extracts employing different cleanup procedures



**Fig. 3** 2D plot of the matrix effect of the pesticides/retention time in milk using EMR-Lipid and HLB PRiME.



EMR-Lipid is higher, particularly in the time segment between 5.0 and 8.0 min. To examine whether this fact could have an impact on the analytical performance, matrix effect for all veterinary drugs proposed in this work was assessed for both sample treatment methods. Thus, external (solvent) standard and matrix-matched calibration curves were evaluated at six levels of concentration (0.001, 0.01, 0.1, 1, 10, and 100  $\mu\text{g kg}^{-1}$ ).

Matrix effect is a key factor in LC-ESI-MS analysis. It describes the deviation between the slope of matrix-matched calibration curves and the slope of external standard calibration curves, using the following equation  $[(\text{calibration curve slope in matrix} - \text{calibration curve slope in solvent}) / \text{calibration curve slope in solvent}] \times 100$  [29]. Matrix effect classification was accomplished according to the following criteria: negligible ( $[0\%] - [\pm 10\%]$ ), soft ( $[\pm 10\%] - [\pm 20\%]$ ), and medium ( $[\pm 20\%] - [\pm 50\%]$ ) [30]. The full results are provided in ESM (Table S3) and illustrated as a 2D plot versus retention time for each sorbent in Fig. 3. The best results were obtained with HLB PRiME, achieving negligible matrix effect for the 65% of compounds, whereas EMR-lipid showed that 43% of compounds exhibited soft matrix effect and 23% medium matrix effect. In the case of EMR-Lipid, a relevant matrix effect was observed in the late eluting compounds (e.g., retention times  $> 5.5$  min). Thus, these results in combination with TICs showed that HLB PRiME is more effective to remove coextracted lipids.

According to the literature, HLB PRiME can remove about 90% fat from milk samples, it involves that 45 mg of lipids could have been retained in the cartridge with the procedure applied. This amount can be higher because in other matrix with higher fat concentration, like meat, the cartridge can retain 135 mg of lipids [31], which implies that it could be possible to use more amount of milk sample for the extraction. On the other hand, EMR-Lipid sorbent can remove 56% of matrix co-extractive compounds present in the bovine liver [24], which contains the same percent of fat as whole cow milk. This suggest that in other fatty animal origin matrix with similar percent of lipids, like milk, the removal could be equated. An example of extracted ion chromatograms corresponding to selected compounds (mebendazole and colchicine) at 1  $\mu\text{g L}^{-1}$  in solvent and whole milk sample extracts spiked at 50  $\mu\text{g kg}^{-1}$  is displayed in Fig. 4, showing the absolute absence of matrix effects observed.

#### Linearity, detection, and quantification limits and precision

Appropriate linearity was achieved with both sample treatment methods with regression coefficients ( $R^2$ ) higher than 0.998 in all cases. Limits of quantitation (LOQs) were established using signal-to-noise criterion ( $S/N = 10$ ) and were calculated using the less abundant (confirmatory) MS/MS transition for each compound from the lower level tested in recovery studies. As could be observed in Table S4 (see ESM), these values ranged from 0.02 to 18.25  $\mu\text{g kg}^{-1}$ .

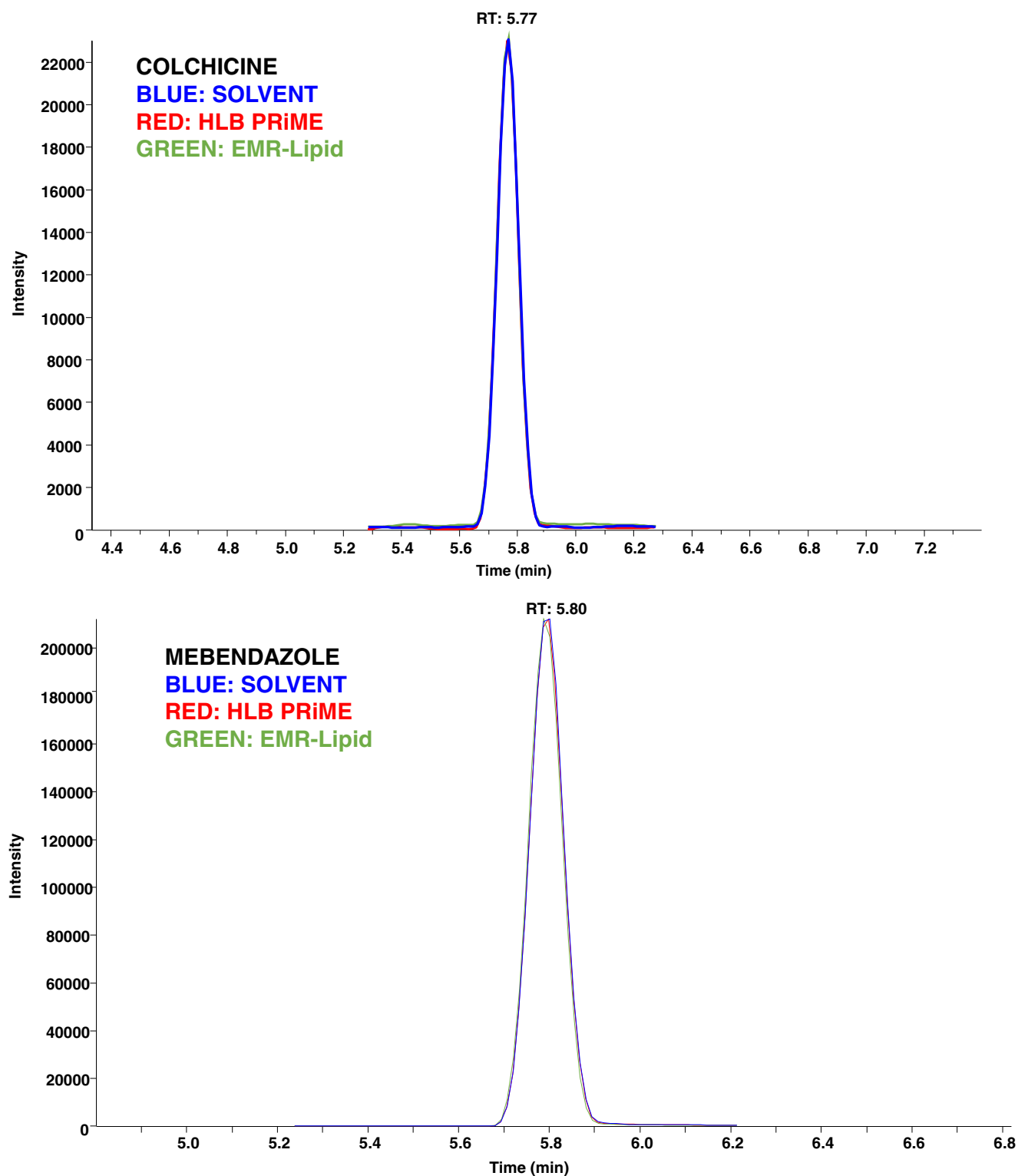


Fig. 4 Extracted ion chromatograms of mebendazole and colchicine corresponding to a standard in solvent at  $1 \mu\text{g L}^{-1}$  and milk matrix spiked at  $50 \mu\text{g kg}^{-1}$  using both sample treatments

$CC\alpha$  and  $CC\beta$  (ESM Table S5) were also calculated for all compounds examined following Commission Decision 2002/657/EC guidelines [28]. For compounds with established MRLs,  $CC\alpha$  was calculated as the MRL plus

1.64 times the standard deviation of the interday precision at the MRL level. For compounds with no set MRL,  $CC\alpha$  was calculated as the concentration at the  $y$ -intercept plus 2.33 times the standard deviation of the reproducibility at the



lowest concentration level.  $CC\beta$  was estimated as the decision limit plus 1.64 times the standard deviation of the reproducibility at the corresponding concentrations.

Repeatability (intraday precision) and intermediate precision (interday precision) were also examined. Repeatability was assessed by means of repetitive application of the entire procedure to five whole milk samples (experimental replicates) spiked at  $50 \mu\text{g kg}^{-1}$  for each veterinary drug. Each sample was injected by triplicate (instrumental replicates) on the same day. Intermediate precision was assessed with a similar procedure, but the samples were analyzed in five different days. Appropriate results—detailed in Table 1 as RSD (%) of peak areas—were obtained in all cases.

**Sample throughput** In light of the results collected, both methodologies provide satisfactory results in terms of matrix effect, sensitivity, recoveries, and precision and being environmentally friendly. Interestingly, the HLB PRiME procedure is faster than EMR-Lipid, as EMR-Lipid dSPE procedure implies a step of sorbent activation, along with centrifugation and a final salting out step. In contrast, HLB PRiME reduces the number of processing steps, which turns out in an average increase of sample throughput. In addition, the tolerance to matrix effect was superior with the pass-through SPE cleanup.

### Analysis of market samples

Twenty-four cow milk samples obtained from local supermarkets were analyzed with the HLB PRiME procedure in order to determine the veterinary drugs chosen in this study. This procedure was chosen because it takes less manipulation steps. It was tried to have different types of milk as whole milk, semi-skim, and skim, with or without lactose and enriched with calcium and omega-3 fatty acids. All the milk samples were kept under recommended conditions in their original packaging before using them. The veterinary drugs concentration was calculated from the corresponding calibration curve and is presented as the mean  $\pm$  standard deviation of four determinations. Traces of danofloxacin were found in two whole cow milk, which are in the range of  $0.7$ – $1.5 \mu\text{g kg}^{-1}$ . However, these concentrations were below the current MRL established [6].

### Conclusions

In this study, two different sample treatment approaches using HLB PRiME SPE and EMR-Lipid have been evaluated in terms of recovery, cleanliness precision, tolerance to matrix effects, and sample throughput. Both approaches have shown to be convenient strategies for the cleanup of veterinary drugs from milk, providing satisfactory recoveries, matrix effects,

and precision. However, HLB PRiME involved fewer manipulation steps, which makes it the more straightforward and suitable option. Consequently, taking into account these facts, it can be concluded that the SPE pass-through cleanup with HLB PRiME is the more convenient sample treatment approach for the determination of the selected compounds.

The analysis of 24 milk samples was carried out to show the applicability of the method, detecting some findings, although at lower levels than MRLs established by the European Union.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

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