Talanta 89 (2012) 201–208

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and validation of a multiclass method for the determination of veterinary drug residues in chicken by ultra high performance liquid chromatography–tandem mass spectrometry

Renata Pereira Lopes[a,b], Rocío Cazorla Reyes[a], Roberto Romero-González[a], Antonia Garrido Frenich[a,*], José Luis Martínez Vidal[a]

[a] Department of Analytical Chemistry, Research Centre for Agricultural and Food Biotechnology (BITAL), Almería University, Agrifood Campus of International Excellence, ceiA3, E-04071 Almería, Spain
[b] Departamento de Química, Universidade Federal de Minas Gerais, Belo Horizonte, MG, 31270-901, Brazil

ARTICLE INFO

Article history:
Received 23 September 2011
Received in revised form
21 November 2011
Accepted 27 November 2011
Available online 9 December 2011

Keywords:
Multiclass analysis
Chicken
Veterinary drug residues
QuEChERS
UHPLC–MS/MS
Validation

ABSTRACT

A multiclass method has been optimized and validated for the simultaneous determination of 20 veterinary drug residues belonging to several classes, as quinolones, sulfonamides, macrolides, anthelmintics, avermectins and diamino derivatives, and benzathine, used as a marker of the presence of penicillin, in muscle chicken. It has been based on QuEChERS methodology (quick, easy, cheap, effective, rugged and safe) and ultra high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry (UHPLC–MS/MS). Several chromatographic conditions were optimized, obtaining a running time <8.5 min. The developed method was validated on the basis of international guidelines. Mean recoveries ranged from 70 to 120%, except for benzathine (65.6% at 20 $\mu g\,kg^{-1}$) and sulfadimidine (69.0% at 100 $\mu g\,kg^{-1}$). Repeatability was lower than 20.0% except for sulfachlorpyridazine (22.1% at 20 $\mu g\,kg^{-1}$) and tylosin (20.5% and 20.6% at 30 and 50 $\mu g\,kg^{-1}$, respectively), whereas reproducibility was lower than 25% except for flumequine (27.4% at 20 $\mu g\,kg^{-1}$) and benzathine (37.8% and 27% at 20 and 50 $\mu g\,kg^{-1}$, respectively). Limits of detection (LODs) and quantification (LOQs) ranged from 3.0 to 6.0 $\mu g\,kg^{-1}$ and 10.0 to 20.0 $\mu g\,kg^{-1}$, respectively, except for tylosin that showed a LOD and LOQ of 9.0 and 30.0 $\mu g\,kg^{-1}$. Decision limit (CC_α) and detection capability (CC_β) were calculated and CC_β ranged from 24.1 $\mu g\,kg^{-1}$ (mebendazole) to 423.6 $\mu g\,kg^{-1}$ (flumequine). Finally, the method was applied to real samples and traces of some compounds were found in eight samples of chicken and benzathine was detected in one sample at 29.9 $\mu g\,kg^{-1}$.

1. Introduction

Veterinary drugs are administered on a large scale in current farm practices and they are mainly used to control diseases, or as growth promoters, of farm animals such as pigs, cows, turkeys or chicken [1–4]. Nowadays, the most common veterinary drugs used include β-lactams, sulfonamides, macrolides and quinolones [5]. These compounds can accumulate in edible tissues, which can be very problematic because their residues can cause allergic reactions in some hypersensitive individuals, and they can delay or destroy the growth of fermenting bacteria [6–9]. Furthermore, different studies indicate that low-level doses of veterinary drugs for long periods could result in bacteria resistance [9–12].

To protect consumer health and to ensure high quality in edible tissues destined for human consumption, European Union (EU) has established maximum residue limits (MRLs) of veterinary drug residues in livestock [13]. These limits require the development of sensitive and specific methods for the determination of veterinary drug residues in food. In order to detect such residues in food and animal tissues, microbiological or bioassay techniques (test kits) are widely used as screening methods [14–16]. These generally do not distinguish between members of a class of veterinary drug, but provide a semi quantitative estimation of 'total' residues present in the sample. However, they are still used because of their simplicity and low cost. Additionally, in case of positive results, more accurate methods are usually required by government regulatory agencies to confirm the identity and amount of veterinary drug [14,15,17].

In the last decades, liquid chromatography (LC) coupled to mass spectrometry (MS) has become an essential technique in food analysis laboratory, but most of the reported methods are applied for a

* Corresponding author. Tel.: +34 950015985; fax: +34 950015483.
E-mail address: agarrido@ual.es (A.G. Frenich).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.11.082

single analyte or analytes belonging to the same class of veterinary drug [18]. However, to improve cost-effectiveness, multiresidue and multiclass methods are necessary to maximize the number of analytes that may be determined by a single procedure, i.e. by a single analysis [19,20]. Recently, selective techniques such as tandem mass spectrometry (MS/MS) and time of flight mass spectrometry (TOF/MS) have been coupled with certain advances in chromatographic technology such as ultra high performance liquid chromatography (UHPLC). These techniques have made possible the development of multiresidue methodologies covering many contaminants at trace levels [18,21–26]. Moreover, UHPLC has been used for the analysis of veterinary drugs in animal products [27–30], bearing in mind that high resolution and sensitivity can be obtained, as well as running time can be reduced.

Despite of the use of selective detection techniques such as MS, sample preparation is still the major bottleneck in any analytical procedure for the determination of chemical residues in food products. Extraction strategies for the determination of multiresidue and multiclass compounds of veterinary drugs in different matrices (as meat, milk, honey and others) have been used, such as solid–liquid extraction (SLE) [31], solid-phase extraction (SPE) [32–36], matrix solid phase dispersion (MSPD) [37], liquid–liquid extraction with fast partition at very low temperature (LLE-FPVLT) [38] and QuEChERS methodology (quick, easy, cheap, effective, rugged and safe) [39]. The QuEChERS multiresidue procedure has some advantages because it simplifies and reduces the time taken for the extraction and clean-up processes. Many papers report the use of this technique for analysis of pesticides in food [40,41]. However, few studies have been reported for the analysis of veterinary drugs in food from animal origin such bovine milk and liver [42], shrimps [43] and chicken breasts [20]. For instance, this one reports the influence of buffers, salts and sorbent during the extraction of sulfonamides, fluoroquinolones, quinolones, nitroimidazoles, ionophores and dinitrocarbanilide. The final procedure consisted of a single extraction for all compounds based on QuEChERS procedure, including an additional clean up step to improve the extraction of nitroimidazoles. Moreover, the chromatographic run was approximately 30 min, which increases the analysis time.

In this paper, we present the development, optimization and validation of a rapid multiresidue and multiclass UHPLC–MS/MS method using QuEChERS procedure, capable of quantifying several classes of veterinary drugs such as quinolones, sulfonamides, macrolides, anthelmintics, avermectins and diamino derivatives, in chicken samples, using a single extraction and clean up procedure. Furthermore, benzathine was also included in this study as a marker of the presence of penicillin, bearing in mind that it is usually used to stabilize penicillins.

2. Materials and methods

2.1. Chemicals and reagents

Benzathin penicillin, oxfendazole, tilmicosin, oxolinic acid, tylosin phosphate, fenbendazole, thiabendazole, trimethoprim and sulfadimidine were supplied by Sigma–Aldrich (Madrid, Spain). Albendazole was supplied by LGC Standars (Barcelona, Spain). Emamectin benzoate, mebendazole, levamisole hydrochloride, sulfachlorpyridazine, sulfadimethoxine and sulfaquinoxaline were obtained from Riedel de Haën (Seelze, Germany). Sulfathiazole, josamycin and erythromycin were supplied by Fluka (Steinheim, Germany). Finally, sulfadiazine and flumequine were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Stock standard solutions of individual compounds (with concentrations between 200 and 300 mg L^{-1}) were prepared in methanol, acetonitrile or acetonitrile:water (1:1, v/v). Stock standard solutions were stored at refrigerator ($T < 5\,^{\circ}C$). A multicompound working standard solution of the selected compounds (4 mg L^{-1}) was prepared by appropriate dilution of the stock solution with acetonitrile and it was stored under refrigeration ($T < 5\,^{\circ}C$). All reagents were of analytical grade. HPLC-grade acetonitrile and methanol and sodium citrate dibasic sesquihydrate were supplied by Sigma–Aldrich. Formic acid (assay > 98%) was purchased from Fluka. Anhydrous magnesium sulfate was purchased from Panreac (Barcelona, Spain). Sodium citrate dihydrate were obtained from J.T. Baker (Deventer, Holland). Ethylene diamine tetraacetic acid disodium salt (Na_2EDTA) was purchased from Merck (Darmstadt, Germany). Primary–secondary amine (PSA) bonded silica (particle diameter of 40 μm) was supplied by Scharlab (Barcelona, Spain). Ultrapure water was obtained from a Millipore Milli-Q system (Milford, MA, USA). Purified samples were filtered through Millex-GN nylon filters (0.20 μm, Millipore, Carrightwohill, Ireland).

2.2. Samples and sample preparation

Chicken meat samples were obtained from local supermarkets (Almeria, Spain) and it was confirmed they were free of targeted analyte residues by UHPLC–MS/MS after sample preparation. All tissue samples were finely chopped and homogenized using a kitchen blender, and stored at −30 °C until analysis. Samples were fortified with the targeted compounds during the optimization and validation of the developed procedure. Veterinary drugs were extracted from chicken using an extraction procedure based on QuEChERS methodology. The procedure was as follows: 5.0 g of the sample was weighed in a polypropylene tube followed by addition of 5.0 mL of pure water and 10.0 mL of 1% of acetic acid in a solution of acetonitrile:water (80:20, v/v). Then, the mixture was stirred in a shaker for 15 min. Afterwards, 0.5 g of sodium citrate dibasic sesquihydrate, 1.0 g sodium citrate dihydrate and 4.0 g of anhydrous magnesium sulfate were added and the tubes were shaken for 15 min. After centrifugation at 5000 rpm (4136 × *g*) during 5 min, 1.0 mL of the acetonitrile layer was transferred to an Eppendorf tube containing 150 mg of PSA followed by manual agitation for 30 s and centrifuged again under the same conditions described above. The supernatant was filtered through a Millex-GN nylon filter. Finally, 500 μL of filtrate was diluted with 500 μL of a solution of formic acid 0.1% in acetonitrile:water (50:50, v/v) prior to chromatographic analysis. Five μL of the extract were injected into the UHPLC–MS/MS system.

2.3. Instrumental and chromatographic conditions

Chromatographic analyses were performed using an Acquity UHPLC system (Waters, Milford, MA, USA) and separations were achieved using an Acquity UHPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size) from Waters. The chromatographic separation was carried out with gradient elution using 0.1% (v/v) formic acid in acetonitrile (eluent A) and 0.1% (v/v) formic acid in water (eluent B) at a flow rate of 0.3 mL min^{-1}. The elution started at 10% of eluent A for 0.5 min and then it was linearly increased up to 100% of eluent A in 5 min, held constant for 1.5 min and returned to the initial conditions in 1.5 min. Finally, the total run time, including the conditioning of the column to the initial conditions was 8.5 min. The injection volume was 5 μL and the column temperature was set at 30 °C.

Mass spectrometry analysis was carried out using a Waters Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using electrospray ionization (ESI) in positive ion mode. The data acquisition

Table 1
Retention time windows (RTWs) and MS/MS conditions of the selected compounds.

Analyte	RTW (min)	Voltage cone (V)	Quantification transition[a]	Confirmation transition[a]	Ion ratio (%)
Albendazole	3.21–3.43	32	266.0 > 234.2 (20)	266.0 > 191.1 (35)	37
Benzathine	0.76–0.96	30	241.6 > 91.3 (25)	241.6 > 134.3 (15)	95
Emamectin	4.56–4.66	60	886.6 > 158.2 (30)	886.6 > 82.2 (30)	6
Erythromycin	3.35–3.46	35	717.1 > 158.2 (30)	717.1 > 116.2 (45)	19
Fenbendazole	3.64–3.82	32	300.0 > 268.2 (20)	300.0 > 159.1 (35)	92
Flumequine	3.48–3.72	20	262.3 > 244.3 (20)	262.3 > 202.2 (20)	6
Josamycin	3.60–3.70	55	829.3 > 174.2 (32)	829.3 > 109.1 (40)	84
Levamisole	1.97–2.23	36	205.0 > 123.1 (29)	205.0 > 117.2 (27)	60
Mebendazole	3.11–3.41	37	296.2 > 264.2 (25)	296.2 > 77.1 (46)	80
Oxfendazole	2.87–2.89	35	315.9 > 191.3 (22)	315.9 > 159.2 (35)	38
Oxolinic acid	2.87–3.25	25	262.3 > 244.3 (20)	262.3 > 216.2 (34)	9
Sulfachlorpyridazine	2.71–2.99	32	285.1 > 156.2 (15)	285.1 > 80.2 (50)	8
Sulfadiazine	1.71–2.23	20	251.0 > 156.0 (17)	251.0 > 92.0 (25)	84
Sulfadimethoxine	3.01–3.39	60	311.1 > 156.2 (20)	311.1 > 245.3 (18)	12
Sulfadimidine	2.36–2.60	35	279.1 > 92.1 (30)	279.1 > 124.2 (20)	56
Sulfaquinoxaline	3.06–3.34	32	301.2 > 156.1 (35)	301.2 > 108.1 (30)	7
Sulfathiazole	2.03–3.13	30	256.2 > 156.1 (15)	256.2 > 92.2 (25)	72
Thiabendazole	1.93–2.13	30	201.8 > 175.2 (27)	201.8 > 131.2 (32)	73
Tilmicosin	2.76–2.92	18	870.4 > 174.3 (45)	870.4 > 696.9 (45)	5
Trimethoprim	2.16–2.24	20	291.4 > 261.3 (25)	291.4 > 230.2 (25)	61
Tylosin	3.02–3.22	35	917.4 > 174.3 (18)	917.4 > 101.1 (45)	17

[a] Collision energy (eV) is given in parentheses.

was performed using MassLynx 4.1 software with QuanLynx program (Waters). The ionization source parameters were: capillary voltage 3.0 kV, extractor voltage 2 V, source temperature 120 °C, desolvation temperature 350 °C, cone gas flow 80 L h^{-1} and desolvation gas flow 600 L h^{-1} (both gases were nitrogen). Collision-induced dissociation was performed using argon as the collision gas at the pressure of 4×10^{-3} mbar in the collision cell. The specific MS/MS parameters for each compound are shown in Table 1.

2.4. Validation procedure

Performance characteristics of the optimized method were established by a validation procedure according to the criteria laid down by the European Commission Decision [44]. Analytical characteristics evaluated were sensitivity, linearity, trueness through recovery studies, intra and interday precision, uncertainty, limits of detection (LODs) and quantification (LOQs), decision limit (CC_α) and detection capability (CC_β) and selectivity. Linearity was evaluated using matrix-matched calibration (MMC), spiking extracted blanks at six concentration levels between 10 and 250 μg kg^{-1}. This range included the lower MRLs established for the analytes studied. LODs and LOQs were estimated by fortifying blank chicken samples with veterinary drugs (1, 2, 5, 10, 20 and 30) μg kg^{-1} and applying the extraction procedure prior to chromatographic determination. LODs and LOQs were determined as the amount for which signal-to-noise ratio (*S/N*) was higher than 3 and 10, respectively. CC_α and CC_β parameters were calculated based on a linear regression model analyzing spiked blank samples at six concentration levels, according to BS ISO 11843-2 [45]. Recovery and repeatability (intraday precision) was performed spiking blanks at three concentration levels (20, 50 and 100) μg kg^{-1}, using five replicates for each concentration level in one day, except for tylosin, which lower level was 30 μg kg^{-1}. To evaluate interday precision (reproducibility), the same concentration levels were studied, spiking blanks during five consecutive days. Finally, uncertainty was also evaluated using the data obtained from the validation of the method [46]. Thus, expanded uncertainty (*U*) was obtained by multiplying the relative combined uncertainty by a coverage factor of 2, which is related to a confidence level of 95%.

3. Results and discussion

3.1. Optimization of the analytical method

UHPLC coupled to MS/MS is the most suitable technique for the simultaneous determination of multiclass veterinary drugs, allowing the reliable analysis of this type of compounds at low levels in complex matrices.

First, for MS/MS detection, ESI in positive ion mode was used, and two transitions per compound were monitored. The MS/MS parameters for each compound are shown in Table 1.

Then, the chromatographic conditions were studied in order to provide overall optimum peak shape and resolution. Thus, the mobile phase composition was investigated to maximize the method sensitivity and resolution. Several experiments were performed to evaluate different mobile phases consisting of methanol or acetonitrile as organic phase and water, with different concentrations of formic acid (0.01 and 0.1%, v/v). Acetonitrile provided overall better sensitivity than methanol. Moreover the highest concentration of formic acid (0.1%, v/v) in acetonitrile provided the best sensitivity for UHPLC–MS/MS analysis of the selected compounds. Furthermore, the gradient was optimized in order to provide a good separation of the selected compounds in less than 9 min. Other parameters such as column temperature, flow rate and injection volume were tested in order to get a fast and reliable separation, obtaining the best results with the conditions described in Section 2. Using these conditions, the analytes were distributed in nine overlapping acquisition functions, containing a maximum of seven compounds (14 transitions) per function. Good peak shape and suitable *S/N* were obtained when 0.025 s was used as dwell time, except for benzathine, flumequine, fenbendazole and josamycin, which were monitored using a dwell time of 0.05 s.

To prevent carry-over effect during UHPLC–MS/MS analysis, different compositions of aqueous solutions of methanol or acetonitrile were tested for the weak and strong solvent used during the washing procedure of the sample needle. It was observed that the composition of 800 μL acetonitrile:water 10:90 (v/v) and 600 μL acetonitrile:water 90:10 (v/v), as weak and strong solvent respectively, provided the best results.

The critical step during the development of a multiresidue antibiotic method is the extraction and clean-up procedure. It must be stressed that QuEChERS was developed for the extraction of pesticides from matrices with high water content (approx. 90%).

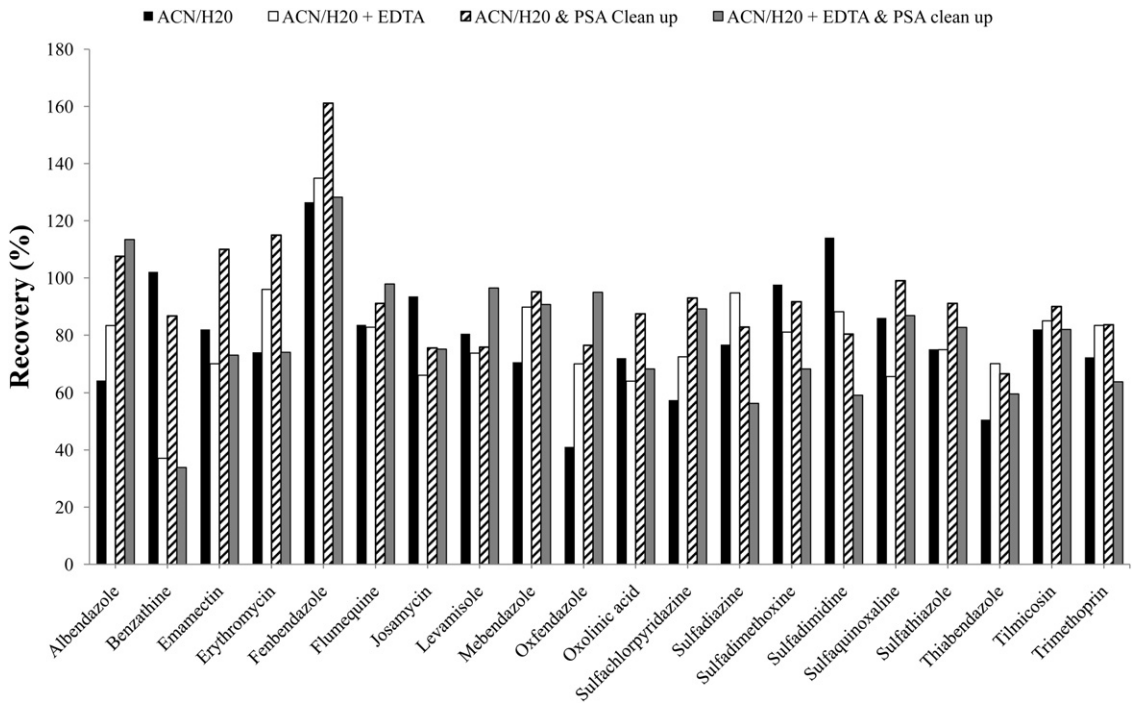

Fig. 1. Effect of different extraction solutions, with or without clean up steps, in the QuEChERs procedure for chicken samples.

Bearing in mind that muscle contains 70% of water, 5 mL of water was added to the sample in order to favor the extraction of the compounds, as it was indicated in other applications where several types of compounds were extracted from matrices with low water content applying QuEChERS procedure [47,48].

First, the extractant solvent was evaluated. Several solvents acidified with acetic acid (1%, v/v) such as acetonitrile, methanol and a mixture of acetonitrile–methanol (1:1, v/v) were evaluated. Better recoveries were obtained for most of the compounds when acetonitrile was used, whereas lower recoveries (<70%) were obtained if a mixture of acetonitrile:methanol or methanol was used, except for flumequine and oxolinic acid, which shown better recoveries if a mixture of acetonitrile:methanol was used. Furthermore, it was observed that if a mixture of acetonitrile:water (80:20, v/v) was used, there was a slight improvement in the recoveries of the analytes, specially for flumequine, josamycin and oxfendazole, and it was used for further experiments.

Considering that ethylene diamine tetraacetic acid (EDTA) can improve the extraction of some veterinary drugs [28], the addition of 10 mL of Na_2EDTA solution (0.125 M) to the extraction solution was evaluated. It can be observed (Fig. 1) that the addition of EDTA only improves the recovery of benzimidazoles (albendazole, mebendazole, oxfendazole and thiabendazole), two sulfonamides (sulfachlorpyridazine and sulfadimidine), two macrolides (erythromycin and tilmicosin) and trimethoprim, whereas the extraction of benzathine was significantly reduced.

A clean-up procedure was evaluated, because interferent compounds can be co-extracted during the extraction, reducing the lifetime of the chromatographic column as well as interfering veterinary drug detection. Therefore, dispersive solid phase extraction (d-SPE) with PSA was evaluated, and 1.0 mL of the acetonitrile layer was transferred to an Eppendorf tube containing 150 mg of PSA. This clean-up step was evaluated with/without the use of EDTA during the extraction process, obtaining the results shown in Fig. 1. It can be observed that the addition of PSA significantly improves the results, except for sulfadiazine and sulfadimidine, if EDTA was added during the extraction process. If EDTA was not added better recoveries were obtained, except for benzathine, josamycin, levamisole, sulfadimethoxine and sulfadimidine. For these compounds recoveries decrease, but they remain higher than 70%. In general, the addition of the clean-up step provided better results if EDTA was not added during the extraction process except for albendazole, flumequine, levamisole and oxfendazole. Therefore, EDTA was not added in the clean-up procedure that was used for further experiments.

Finally, Fig. 2 shows the extracted ion chromatograms (XIC) from representative compounds for each class of veterinary drug (quantifier transition was only shown), spiking a blank sample at 100 $\mu g\,kg^{-1}$.

3.2. Method validation

A validation procedure was carried out to evaluate several performance characteristics of the method, such as linearity, trueness, repeatability (intraday precision), reproducibility (interday precision), LODs, LOQs, CC_α, CC_β and uncertainty.

To evaluate matrix effect, the slopes obtained in the calibration with MMC were compared with those obtained with solvent standards, injecting several concentrations from 10 to 250 $\mu g\,kg^{-1}$, except for benzathine, flumequine, oxolinic acid, sulfadimidine, thiabendazole, tilmicosin and trimethoprim (range 20–250 $\mu g\,kg^{-1}$) and tylosin (range 30–250 $\mu g\,kg^{-1}$). Then, matrix/solvent slope ratios for each compound were obtained (Fig. 3) considering a signal enhancement or suppression effect as acceptable if the slope ratio ranged from 0.8 to 1.2. Slope ratios higher values than 1.2 or lower than 0.8 indicate a strong matrix effect. It can be observed that a significant matrix effect was noticed for benzathine, emamectin, flumequine, josamycin, sulfadiazine and sulfaquinoxaline, whereas tolerable matrix effect was observed for the rest of compounds.

Then, linearity was evaluated by MMC at the same ranges described above. Calibration curves were obtained by least-squares linear regression analysis of the peak area versus concentration. The calibration curves showed good linearity with determination coefficients (R^2) higher than 0.990 in all the cases. Furthermore, deviations of the individual points from the calibration curve were lower than 20%.

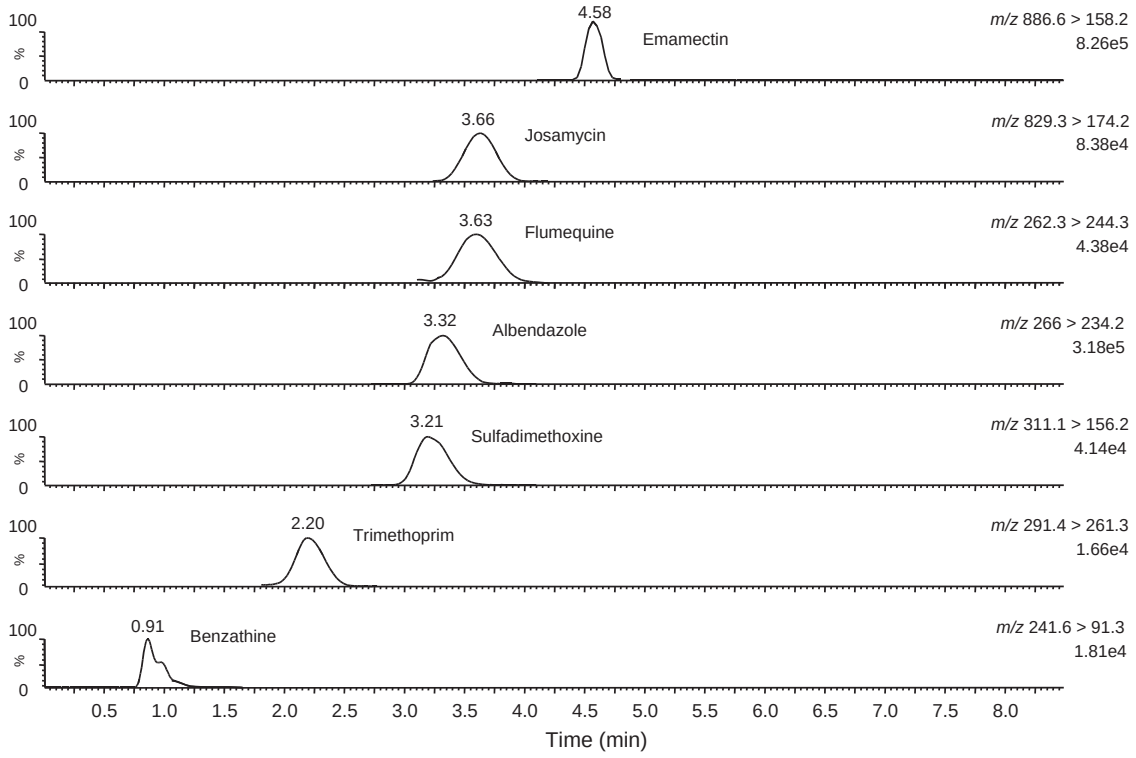

Fig. 2. UHPLC–MS/MS chromatograms from different compounds belonging to several classes of veterinary drugs spiked at 100 μg kg^{-1} in a blank chicken meat sample.

Trueness was estimated through recovery studies, applying the extraction procedure described previously. Table 2 shows the obtained results and it can be seen that satisfactory results were found, with recoveries between 70 and 120%, for all the assayed compounds at the three concentration levels, except for benzathine, which showed a recovery of 65.6% at 20 μg kg^{-1} and sulfadimidine with a recovery value of 69.0% at 100 μg kg^{-1}, concluding that recovery was acceptable for all compounds studied at the three levels assayed.

The precision of the method was studied by performing repeatability (intraday precision) and reproducibility (interday precision) experiments and the results obtained are shown in Table 2. For

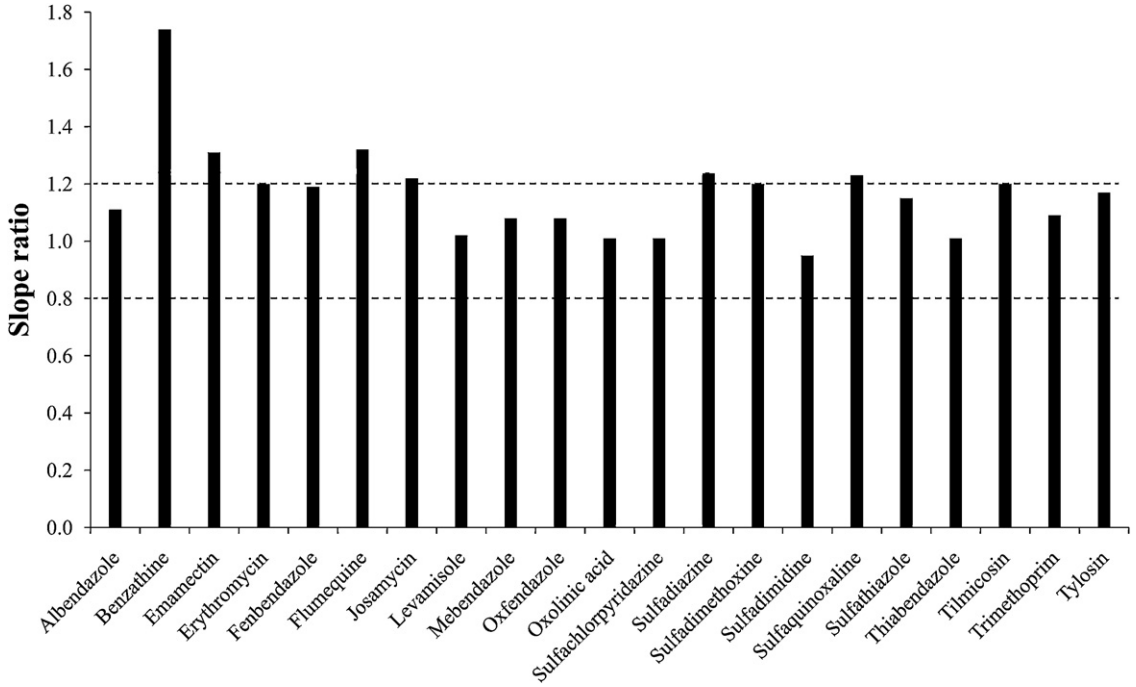

Fig. 3. Slope ratios between matrix-matched and solvent calibration. The compliance interval covering the range of slope ratios between 0.8 and 1.2, for tolerable matrix effect, has been plotted.

Table 2
Validation parameters of the optimized UHPLC–MS/MS method.

Analyte	Recovery (%)			Interday precision (RSD %)[a]			U (%)[b]
	20 ($\mu g\,kg^{-1}$)[c]	50 ($\mu g\,kg^{-1}$)[c]	100 ($\mu g\,kg^{-1}$)[c]	20 ($\mu g\,kg^{-1}$)	50 ($\mu g\,kg^{-1}$)	100 ($\mu g\,kg^{-1}$)	
Albendazole	91.0 (8.1)	92.5 (3.1)	105.6 (1.6)	12.3	2.7	8.3	9.9
Benzathine	65.6 (8.2)	72.8 (14.8)	81.9 (9.1)	37.8	27.0	20.4	23.9
Emamectin	99.7 (4.7)	96.6 (4.1)	112.5 (1.2)	14.5	10.3	10.2	18.4
Erythromycin	111.9 (9.3)	105.8 (7.8)	117.0 (4.1)	16.0	16.3	4.3	23.1
Fenbendazole	94.7 (5.4)	93.7 (5.5)	110.3 (4.4)	10.5	6.6	7.0	15.6
Flumequine	90.4 (19.2)	89.2 (8.8)	89.0 (13.0)	27.4	21.4	18.7	19.0
Josamycin	95.6 (10.5)	102.0 (4.6)	111.9 (4.6)	4.5	7.2	10.8	14.8
Levamisole	92.4 (17.7)	97.8 (18.6)	111.5 (13.4)	14.9	8.8	21.6	25.2
Mebendazole	97.5 (2.8)	94.0 (7.2)	112.6 (5.4)	19.3	8.9	9.3	10.5
Oxfendazole	96.7 (11.9)	90.2 (6.8)	108.4 (4.3)	9.0	6.6	18.2	10.5
Oxolinic acid	75.7 (19.3)	73.8 (16.6)	118.3 (9.8)	12.2	9.2	27.2	12.0
Sulfachlorpyridazine	78.0 (22.1)	73.3 (11.8)	117.9 (3.0)	10.2	12.7	12.2	10.2
Sulfadiazine	86.5 (21.2)	88.0 (14.7)	107.3 (11.0)	11.9	11.4	14.3	9.4
Sulfadimethoxine	110.2 (7.1)	107.4 (8.9)	106.9 (7.2)	21.0	12.0	8.4	20.1
Sulfadimidine	76.0 (13.0)	82.2 (15.3)	69.0 (12.4)	13.2	5.1	12.9	6.2
Sulfaquinoxaline	96.2 (17.2)	104.9 (14.0)	96.3 (11.5)	13.2	10.3	4.5	15.0
Sulfathiazole	91.0 (18.2)	88.4 (11.3)	109.4 (7.6)	19.8	13.4	23.6	14.7
Thiabendazole	91.1 (7.5)	93.7 (9.9)	114.2 (2.7)	20.5	8.1	13.6	18.3
Tilmicosin	75.5 (11.9)	81.4 (18.1)	90.1 (19.4)	16.5	22.0	16.1	41.0
Trimethoprim	89.6 (16.2)	85.8 (10.5)	88.8 (10.7)	12.8	13.3	16.7	13.9
Tylosin[d]	83.2 (20.5)	91.6 (20.6)	75.4 (14.5)	24.1	19.5	12.7	27.2

[a] Number of replicates = 5.
[b] Expanded uncertainty ($k = 2$) estimated at 50 $\mu g\,kg^{-1}$.
[c] Intraday precision is given in brackets as relative standard deviation ($n = 5$).
[d] The lower level concentration for this compound was 30 $\mu g\,kg^{-1}$.

repeatability, it can be observed that relative standard deviations (RSDs) were always lower than 20% for all the levels assayed, except for sulfachlorpyridazine (RSD = 22.1% at 20 $\mu g\,kg^{-1}$) and tylosine (RSD = 20.5% and 20.6% at 30 and 50 $\mu g\,kg^{-1}$ respectively). For reproducibility, RSD values were lower than 28%, except for benzathine at 20 $\mu g\,kg^{-1}$ (37.8%), indicating the stability of the developed method.

The estimation of expanded uncertainty (U) was calculated by using the data derived from the validation of the method [46]. This includes sample preparation, standards dilution, and chromatographic and MS detection variability, measured as RSD. Table 2 shows the obtained results at 50 $\mu g\,kg^{-1}$, and it can be observed that U was below 27.2% for the assayed compounds, except for tilmicosin (41.0%).

LODs and LOQs were calculated analyzing blank samples spiked at (1, 2, 5, 10, 20 and 30 $\mu g\,kg^{-1}$), and they were determined as the lowest concentration of the analyte for which S/N were 3 and 10 respectively. The results obtained are shown in Table 3. It can be seen that LODs and LOQs were always below 6.0 $\mu g\,kg^{-1}$ and 20.0 $\mu g\,kg^{-1}$ respectively, except for tylosin, which showed a LOD and LOQ value of 9.0 $\mu g\,kg^{-1}$ and 30.0 $\mu g\,kg^{-1}$ respectively. However, these results were below of the MRL of tylosin in meat, which is 100 $\mu g\,kg^{-1}$.

CC_α and CC_β allow the estimation of critical concentrations above which the method can distinguish and quantify a substance taking into account the variability of the method and the statistical risk to take a wrong decision. These parameters were calculated according to BS ISO 11843-2 [45] (Table 3), which allows the determination of both parameters for both non-permitted and permitted compounds. For compounds without MRLs established (albendazole, benzathine, emamectin, fenbendazole, josamycin, mebendazole, oxfendazole and thiabendazole) CC_α and CC_β were calculated from the LOQ established for each analyte. For these compounds, the highest results were obtained for benzathine (33.1 and 46.2 $\mu g\,kg^{-1}$ for CC_α and CC_β, respectively). For compounds with a set MRL, the values ranged from 19.4 (levamisole) to 411.8 $\mu g\,kg^{-1}$ (flumequine, which has a MRL value of 400 $\mu g\,kg^{-1}$) for CC_α and from 28.7 (levamisole) to 423.6 (flumequine) $\mu g\,kg^{-1}$ for CC_β.

The selectivity was evaluated by analyzing control blank chicken samples. The absence of any signal at the same retention time as the analytes indicated that there were no matrix interferences that may give a false positive signal.

Finally, identification of the compounds was carried out by searching in the appropriate retention time windows (RTWs), defined as the retention time ± three standard deviations calculated from the retention time of the compounds, obtained when 10 blank chicken meat samples were spiked at 50 $\mu g/kg$ (Table 1). Furthermore according to Annex I of Directive 96/23/EC [49], substances that exert pharmacological activity are classified in group B (veterinary drugs and contaminants) and a minimum of three points is required for their identification. In accordance with European Commission Decision 657/2002/EC [44], four identification points were obtained (one precursor ion and two product ions). Moreover, the relative intensities of the ions detected were compared with those obtained using fortified blank chicken meat samples. Confirmation was considered reliable if the relative intensities of the product ions was within the criteria laid down in the European Commission Decision 657/2002/EC [44]. Table 1 shows the obtained ion ratios. Thus, the identification and confirmation of a target compound must meet the tolerances for the retention time and the ion ratio of the quantification and confirmation transitions.

3.3. Sample analysis

The developed method was applied to the determination of veterinary drug residues in eleven chicken samples obtained from local supermarkets in Almeria (Spain). In order to ensure the quality of the results when the proposed method was applied, an internal quality control was carried out in every batch of samples. This quality control consisted of a matrix-matched calibration, a reagent blank and a spiked blank sample at 30 $\mu g\,kg^{-1}$. Furthermore, the retention time and the relative intensities of the detected ions in real samples were compared to those of corresponding calibration standards in the same batch to confirm the identity of the detected analytes using the criteria established by Decision Commission 657/2002/EC [44]. The obtained results are indicated in Table 4. Traces of veterinary drugs (<LOQ) were observed in 8

Table 3
MRL, LOD, LOQ, CC_α and CC_β obtained for the studied compounds.

Analyte	MRL ($\mu g\,kg^{-1}$)[a]	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)	CC_α ($\mu g\,kg^{-1}$)	CC_β ($\mu g\,kg^{-1}$)
Albendazole	–[b]	3.2	10.0	24.5	38.4
Benzathine	–	6.4	20.0	23.1	36.2
Emamectin	–	3.2	10.0	18.5	26.3
Erythromycin	200.0	3.2	10.0	207.8	215.6
Fenbendazole	–	3.2	10.0	17.8	25.0
Flumequine	400.0	6.4	20.0	411.8	423.6
Josamycin	–	3.2	10.0	19.3	28.7
Levamisole	10.0	3.2	10.0	19.4	28.7
Mebendazole	–	3.2	10.0	17.0	24.1
Oxfendazole	–	3.2	10.0	18.6	26.5
Oxolinic acid	100.0	6.4	20.0	108.9	117.8
Sulfachlorpyridazine	100.0	3.2	10.0	107.4	114.7
Sulfadiazine	100.0	3.2	10.0	116.9	133.8
Sulfadimethoxine	100.0	3.2	10.0	106.8	113.6
Sulfadimidine	100.0	6.4	20.0	111.1	122.2
Sulfaquinoxaline	100.0	3.2	10.0	120.1	140.3
Sulfathiazole	100.0	6.4	20.0	106.0	112.1
Thiabendazole	–	6.4	20.0	22.4	33.6
Tilmicosin	75.0	6.4	20.0	111.5	148.1
Trimethoprim	50.0	6.4	20.0	60.0	70.1
Tylosin	100.0	16.0	30.0	108.2	116.5

[a] For analytes that do not have a MRL established, the limit of quantification (LOQ) was used to estimate CC_α and CC_β.
[b] MRL not established for this compound in the matrix evaluated.

Table 4
Concentration of veterinary drugs ($\mu g\,kg^{-1}$) found in real samples.

Analyte	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}
Benzathine	–	–	–	–	–	–	–	–	–	29.9	–
Levamisole	–	<LOQ	–	<LOQ	<LOQ	–	<LOQ	–	–	–	–
Mebendazole	<LOQ	–	<LOQ	<LOQ	<LOQ	–	–	–	<LOQ	–	<LOQ
Sulfadiazine	–	–	–	<LOQ	–	–	–	–	–	–	–
Thiabendazole	–	–	–	–	–	–	–	–	–	<LOQ	–
Trimethoprim	–	–	–	–	–	–	<LOQ	–	–	–	–
Tylosin	–	–	–	<LOQ	–	–	–	–	–	–	–

samples (levamisole, mebendazole, sulfadiazine, tylosin and thiabendazole), and only benzathine was detected above the LOQ in one sample at 29.9 $\mu g\,kg^{-1}$ (Fig. 4). It must be stated that despite no MRL has been established for this compound for chicken tissues, this is usually used to stabilize penicillin. Therefore, the detection of this compound can indicate the use of this type of veterinary drug in the analyzed sample.

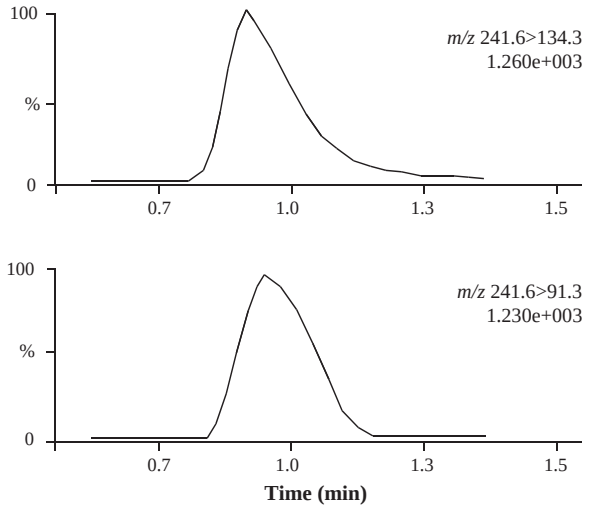

Fig. 4. UHPLC–MS/MS chromatogram for a positive sample of benzathine at 29.9 $\mu g\,kg^{-1}$.

4. Conclusions

In this work, a simple, cheap, fast, reproducible and sensitive multiclass method was developed and validated for the quantification of a large range of veterinary drugs (21 analytes from seven different classes) in chicken samples. The method employs the QuEChERS extraction method (including the clean up step with PSA) and UHPLC–MS/MS. The compounds examined in this study possess a wide range of physicochemical properties indicating the potential of the QuEChERS procedure for the extraction of veterinary residues in chicken. The method was validated according to international guidelines and good validation data were obtained for linearity, recovery, precision, LODs, LOQs, CC_α, CC_β and uncertainty. 15 samples can be extracted in less than 1 h using the proposed method, and the extracts can be analyzed in less than 2.5 h. Bearing in mind that many compounds are determined from a single extraction, the proposed method could be applied in routine analysis. It must be indicated that penicillins were not target compounds in this study. However, benzathine can be used as indicator for these compounds, bearing in mind that it is used to stabilize them.

Acknowledgments

The authors gratefully acknowledge Spanish Ministry of Science and Innovation (MICINN)-FEDER (Project Ref. AGL2010-21370) and the National Council for Scientific and Technological Development (CNPq)-Brazil for financial support. RRG is also grateful for personal funding through the Ramón y Cajal Program (SMSI-ESF).

References

[1] G. Bretschneider, J.C. Elizalde, F.A. Pérez, Livest. Sci. 114 (2008) 135–149.
[2] L. Kelly, D.L. Smith, E.L. Snary, J.A. Johnson, A.D. Harris, M. Wooldridge, J.G. Morris, Int. J. Antimicrob. Agents 24 (2004) 205–212.
[3] G. Ternak, Med. Hypotheses 64 (2005) 14–16.
[4] H.C. Wegener, Curr. Opin. Microbiol. 6 (2003) 439–445.
[5] A.A.M. Stolker, U.A.T. Brinkman, J. Chromatogr. A 1067 (2005) 15–53.
[6] J.L. Albright, S.L. Tuckey, G.T. Woods, J. Dairy Sci. 44 (1961) 779–807.
[7] U. Herz, P. Lacy, H. Renz, K. Erb, Curr. Opin. Immunol. 12 (2000) 632–640.
[8] N. Kemper, Ecol. Indic. 8 (2008) 1–13.
[9] J.L. Martinez, Environ. Pollut. 157 (2009) 2893–2902.
[10] R.H. Gustafson, J. Dairy Sci. 74 (1991) 1428–1432.
[11] H. Sørum, T.M. L'Abée-Lund, Int. J. Food Microbiol. 78 (2002) 43–56.
[12] J. Wallmann, Int. J. Food Microbiol. 296 (2006) 81–86.
[13] EC, Council Regulation (EC) 37/2010/EC of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, Off. J. Eur. Union L15 (2010) 1–72.
[14] C. Cháfer-Pericás, Á. Maquieira, R. Puchades, Trends Anal. Chem. 29 (2010) 1038–1049.
[15] M.G. Pikkemaat, S.O. Dijk, J. Schouten, M. Rapallini, L. Kortenhoeven, H.J. Egmond, Food Control 20 (2009) 771–777.
[16] M.G. Pikkemaat, M.L.B.A. Rapallini, S.O. Dijk, J.W.A. Elferink, Anal. Chim. Acta 637 (2009) 298–304.
[17] M.G. Pikkemaat, S.O. Dijk, J. Schouten, M. Rapallini, H.J. Egmond, Food Control 19 (2008) 781–789.
[18] G. Balizs, A. Hewitt, Anal. Chim. Acta 492 (2003) 105–131.
[19] H.D. Alwis, D.N. Heller, J. Chromatogr. A 1217 (2010) 3076–3084.
[20] G. Stubbings, T. Bigwood, Anal. Chim. Acta 637 (2009) 68–78.
[21] C. Blasco, A.D. Corcia, Y. Picó, Food Chem. 116 (2009) 1005–1012.
[22] T. Goto, Y. Ito, S. Yamada, H. Matsumoto, H. Oka, J. Chromatogr. A 1100 (2005) 193–199.
[23] K. Granelli, C. Elgerud, Å. Lundström, A. Ohlsson, P. Sjöberg, Anal. Chim. Acta 637 (2009) 87–91.
[24] M. Hadjigeorgiou, C. Papachrysostomou, Z. Theodorou, P. Kanari, S. Constantinou, Anal. Chim. Acta 637 (2009) 220–224.
[25] B. Shao, D. Dong, Y. Wu, J. Hu, J. Meng, X. Tu, S. Xu, Anal. Chim. Acta 546 (2005) 174–181.
[26] G. Vyncht, A. Jànosi, G. Bordin, B. Toussaint, G. Maghuin-Rogister, E.D. Pauw, A.R. Rodriguez, J. Chromatogr. A 952 (2002) 121–129.
[27] Y. Deceuninck, E. Bichon, F. Monteau, J.P. Antignac, B.L. Bizec, Anal. Chim. Acta 700 (2011) 137–143.
[28] A. Garrido Frenich, M.M. Aguilera-Luiz, J.L. Martínez Vidal, R. Romero-González, Anal. Chim. Acta 661 (2010) 150–160.
[29] J. Vanden Bussche, L. Vanhaecke, Y. Deceuninck, K. Verheyden, K. Wille, K. Bekaert, B. Le Bizec, H.F. De Brabander, J. Chromatogr. A 1217 (2010) 4285–4293.
[30] M. Whelan, K. Kinsella, A. Furey, M. Moloney, H. Cantwell, S.J. Lehotay, M. Danaher, J. Chromatogr. A 1217 (2010) 4612–4622.
[31] R. Romero-González, J.C. López-Martínez, E. Gómez-Milán, A. Garrido Frenich, J.L. Martínez Vidal, J. Chromatogr. B 857 (2007) 142–148.
[32] P. Mottier, V. Parisod, E. Gremaud, P.A. Guy, R.H. Stadler, J. Chromatogr. A 994 (2003) 75–84.
[33] W. Ben, Z. Qiang, C. Adams, H. Zhang, L. Chen, J. Chromatogr. A 1202 (2008) 173–180.
[34] E. Rodriguez, M.C.M. Bondi, M.D. Marazuela, J. Chromatogr. A 1209 (2008) 136–144.
[35] T.S. Thompson, D.K. Noot, J. Calvert, S.F. Pernal, J. Chromatogr. A 1020 (2003) 241–250.
[36] W.X. Zhu, J.Z. Yang, W. Wei, Y.F. Liu, S.S. Zhang, J. Chromatogr. A 1207 (2008) 29–37.
[37] Y. Yang, B. Shao, J. Zhang, Y. Wu, J. Ying, J. Chromatogr. B 870 (2008) 241–246.
[38] R.P. Lopes, D.V. Augusti, L.F. Souza, F.A. Santos, J.A. Lima, E.A. Vargas, R. Augusti, Anal. Methods 3 (2010) 606–613.
[39] E. Cieslik, A.S. Rociek, J.M.M. Ruiz, M.S. Zadora, Food Chem. 125 (2010) 773–778.
[40] U. Koesukwiwat, S.J. Lehotay, S. Miao, N. Leepipatpiboon, J. Chromatogr. A 1217 (2010) 6692–6703.
[41] A. Wilkowska, M. Biziuk, Food Chem. 125 (2010) 803–812.
[42] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, Anal. Chim. Acta 637 (2009) 196–207.
[43] M. Villar-Pulido, B. Gilbert-López, J.F. García-Reyes, N.R. Martos, A. Molina-Díaz, Talanta 85 (2011) 1419–1427.
[44] EC. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Offic J Eur Comm L221 (2002) 8–36.
[45] BS ISO/11843-2, Capability of detection—part 2: methodology in the linear calibration case, 2000.
[46] EURACHEM/CITAC, Guide Quantifying Uncertainty in Analytical Measurement, 2000.
[47] J.A. Padilla Sánchez, P. Plaza Bolaños, R. Romero González, A. Garrido Frenich, J.L. Martínez Vidal, J. Chromatorgr. A 1217 (2010) 5724–5731.
[48] K. Mastovska, K.J. Dorweiller, S.J. Lehotay, J.S. Wegscheid, K.A. Szpylka, J. Agric. Food Chem. 58 (2010) 5959–5972.
[49] EC. Commission Decision 96/23/EC of 29 April 1196 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC Off. J. Eur. Union L125 (1996) 1–10.