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Quechers methodologies as an alternative to solid phase extraction (SPE) for the determination and characterization of residues of cephalosporins in beef muscle using LC–MS/MS

R. Pérez-Burgos [a], E.M. Grzelak [b], G. Gokce [c], J. Saurina [a], J. Barbosa [a], D. Barrón [a,*]

[a] Department of Analytical Chemistry, Faculty of Chemistry, University of Barcelona, Avda. Diagonal, 645, E-08028 Barcelona, Spain
[b] Department of Chromatographic Methods, University of Maria Curie – Skłodowska, M. Skłodowska Sq. 3, 20-031 Lublin, Poland
[c] Department of Elementary Science Education, Faculty of Education, Cumhuriyet University, 58140 Sivas, Turkey

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ABSTRACT

This work was focused on the comparison of two clean-up methods to be used for the simultaneous determination of seven cephalosporins in cow muscle. In particular, the performance of novel dispersive solid phase extraction (d-SPE) procedures based on QuEChERS methodologies was assessed and compared with conventional SPE. The separation and detection of the analytes using both methods was carried out by LC–MS/MS to reach enough sensitivity to be compatible with the detection of the maximum residue limits (MRL) of cephalosporins as regulated by EU directives. The optimization of the clean-up step relied on experimental design in order to find the most suitable conditions with a reduced number of assays. Besides, multi-objective responses were used to reach an overall compromise in the recovery of all analytes simultaneously. The validation of the two methods was done according to the Directive 2002/657/EC. Linearity, decision limit, detection capability, detection and quantification limits (4–50 $\mu g\,kg^{-1}$), precision (RSD less than 15% except for PIR) and recoveries were determined and adequate results with comparable values using QuEChERS and SPE methodologies. LOQ were better for SPE method (0.1–10 $\mu g\,kg^{-1}$) but both methods show LOQ below MRL values. Precision was slightly better for the QuEChERS method, that also presents better recoveries, higher than 85% except for cephalexin.

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1. Introduction

β-Lactams are probably the most widely used class of antibiotics in veterinary medicine for the treatment of bacterial infections of animals used in livestock farming [1,2]. The β-lactam antibiotics comprise two main classes of thermally labile compounds, the penicillins and the cephalosporins. Both penicillins and cephalosporins have a β-lactam ring in common although they differ in the basic structure. Cephalosporins contain the 7-amino cephalosporonic acid nucleus and a six-membered dihydrothiazine ring fused to the β-lactam portion, while penicillins contain a five-membered thiazolidine ring fused to the β-lactam portion [3,4].

The widespread and intense use of antibiotics has led to an increase of the potential risk for human and animal health mainly because of hypersensitivity of some individuals to the parent compounds and metabolites, as well as development of resistance against antibiotics of certain pathogenic bacteria [5,6]. Reliable, efficient and robust methods for monitoring these antibiotic food residues are increasingly demanded to ensure that they are not present at levels that may pose health risks to humans. The European Community has established maximum residue limits (MRL) and requirements concerning the performance of analytical methods and the interpretation of the results. In this sense, Council Directive 37/2010 reports guidelines for residue control and divides pharmacologically active substances into forbidden or banned and authorized substances with established tolerance levels (these values range from 50 $\mu g\,kg^{-1}$ for cephapirin to 1000 $\mu g\,kg^{-1}$ for ceftiofur) as can be seen in Table 1 for beef muscle, while Commission Decision 2002/657/EC [7,8] gives criteria to perform residue analysis. In particular, LC–MS or LC/MS/MS are the preferred techniques in the determination of β-lactams residues. A number of authors have reported multiresidue methods for the determination of β-lactams in animal tissues or milk [6,9–17], but only a few of these methods include some cephalosporins as targets of study [9–11,16,17].

Current methods available for antibiotic determination have shown several steps in common. The extraction and clean-up of the drugs from the complex matrix has been recognized as one of the most difficult steps required for antibiotic analysis [18–20]. Conventionally, liquid–liquid extraction (LLE) and

* Corresponding author.
E-mail address: dolores.barron@ub.edu (D. Barrón).

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Table 1
MRL of cephalosporins (Council Directive 37/2010) in beef tissues.

Antibiotic	MLR ($\mu g\,kg^{-1}$)	Tissues
Cephapirin (PIR)	50	Muscle, fat
	100	Kidney
	60	Milk
Cefquinome (QUI)	50	Muscle, fat
	100	Liver
	200	Kidney
	20	Milk
Cephalexin (LEX)	200	Muscle, fat, liver
	1000	Kidney
	100	Milk
Cephalonium (LON)	20	Milk
Cephazolin (ZOL)	50	Milk
Cefoperazone (PER)	50	Milk
Ceftiofur (TIO)	1000	Muscle
	2000	Fat, liver
	6000	Kidney
	100	Milk

solid-phase extraction (SPE) have been used as sample preparation techniques, being LLE gradually replaced by SPE. Proper performance of SPE requires knowledge of the practice and theory of the technique because common factors, like the adjustment of the flow rate affect the reproducibility of the results [21,22]. Hence, new straightforward approaches involving fewer and simpler steps would be welcome for a more effective clean-up of complex matrices such as meat samples. In this way, QuEChERS (quick, easy, cheap, effective, rugged and safe) has been checked elsewhere for the extraction of veterinary drugs such as quinolones, tetracyclines, macrolides, penicillins or sulphonamides [13,22–26], but to date, only one work focused on the determination of cephalosporines in animals tissues using QuEChERS has been published [17].

The optimization of procedures for the extraction of antibiotics in animal tissues is time-consuming and tedious due to number of variables that may affect the process as well as the number of substances to be extracted. As described in the scientific literature [27,28], the optimization issue can be facilitated by using chemometric approaches to achieve more successful results and information from a reduced series of experiments. Hence, experimental design can be used to find the most relevant factors influencing on the process and study possible interactions among them. On the other hand, the simultaneous optimization of conditions affect a wide variety of analytes with different physicochemical behaviour may be solved from the definition of multi-objective responses [29].

This work aims at studying the extraction of the series of cephalosporins, regulated by EU, using dispersive SPE by QuEChERS. One of the goals is evaluation of the performance of the approach as a sample treatment in the determination of this family of compounds in cow tissue by LC–MS/MS. Besides, results have been compared with another well-established technique such as conventional SPE to ascertain if the QuEChERS procedure could result in a suitable alternative. The extraction of the substances from the matrix has been optimized here using experimental design to obtain the best results in a more efficient way. The two methods have been validated according to the European normative.

2. Experimental

2.1. Reagents and materials

Cephalosporins standards cephazolin (ZOL), cephapirin (PIR), ceftiofur (TIO) and the internal standards piperacilline (PIPE), used in LC–MS studies, and cephalotin (LOT), used in LC–UV experiments, were purchased from Fluka (Buchs, Switzerland). Cephalexin (LEX) and cefoperazone (PER) were supplied by Sigma (St. Louis, MO, USA), cefquinome (QUI) were from AK Scientific (Union City, CA, USA) and cephalonium (LON) was graciously provided by Schering-Plough Animal Health Corporation (Ireland). Fig. 1 shows the chemical structures of all cephalosporins studied.

Solvents to be used for the extraction and preparation of the mobile phase were of HPLC grade. Acetonitrile (MeCN) and methanol (MeOH) were supplied by Merck (Darmstadt, Germany). Formic acid, acetic acid (HAc), sodium hydroxide, magnesium sulphate, sodium chloride and sodium dihydrogenphosphate were also purchased from Merck. Ultrapure water was generated by the Mili-Q system (Millipore, Billerica, MA, USA).

SPE cartridges used in this study were as follows: Bond Elut C18 (3 mL, 200 mg) from Varian (Harbor City, CA, USA), OASIS HLB (3 mL, 200 mg) from Waters (Milford, MA, USA), Strata X (3 mL, 200 mg) from Phenomenex (Torrance, CA, USA) and Isolute ENV+ (3 mL, 200 mg) from Biotage (Uppsala, Sweden).

QuEChERS sorbents were provided as dispersive SPE kits and bulk sorbents. Bulk sorbents of primary–secondary amine (PSA) and C18 were purchased from Agilent Technologies (Santa Clara, CA, USA). Dispersive SPE kits fruits and vegetables with fats and waxes (15 mL, 150 mg PSA, 150 mg C18, 900 mg $MgSO_4$) were from Agilent Technologies (Santa Clara, CA, USA).

2.2. Standards and stock solutions

Individual stock solutions of cephalosporins were prepared at a concentration of 250 mg L^{-1} in water. Standard solutions of internal standards (IS) were prepared in water at a concentration of 80 mg L^{-1} for LOT and 40 mg mL^{-1} for PIPE.

The working solutions used in preliminary experiments were prepared as a mixture of all cephalosporins at a concentration of 80 mg L^{-1} in water. For the validation of the method, working solutions to be spiked to the meat samples of muscle were prepared at concentrations of 20 MRL and 100 MRL for each cephalosporin (i.e., concentrations of 1, 4 and 20 $\mu g\,L^{-1}$ for the 20 MRL working solutions and 5, 10 and 1000 $\mu g\,L^{-1}$ for the 100 MRL working solutions). Cephalosporins without regulated MRL values for beef muscle samples were added to the working solutions at the lowest MRL value regulated for the other cephalosporins. Mili-Q water was used to prepare all the standard solutions.

Solutions were stored in the freezer at −20 °C.

2.3. Instruments

An HP Agilent Technologies 1100 LC system (Santa Clara, CA, USA) equipped with an autosampler and coupled to an API 3000 triple-quadrupole mass spectrometer (PE Sciex) with a turbo ionspray source was used. The system was controlled by Analyst software (v.1.4.2) supplied by Applied Biosystems (Foster City, CA, USA). The analytical column was a Zorbax Eclipse XDB-C8 column (5 μm, 4.6 mm × 150 mm) from Agilent Technologies, using a precolumn Kromasil C8 (5 μm, 4.6 mm × 15 mm) supplied by Akady (Barcelona, Spain). The gradient used for separation is explained in Section 2.4.4 of chromatographic conditions.

Preliminary assays were carried out with an HP Agilent Technologies 1100 LC system equipped with an autosampler and a diode array detector (DAD). The system was controlled by the ChemStation software from Agilent Technologies (Santa Clara, CA, USA).

A Rotanta 460RS centrifuge (Hettich Zentrifuguen) was used for phase separation during the extraction. The SPE procedure was carried out on a Supelco vacuum manifold for 12 cartridges and a Supelco vacuum manifold with disposable liners for 24 cartridges (Bellefonte, PA, USA) connected to a Supelco vacuum tank. Finally, a TurboVap LV evaporator from Caliper LifeSciences (Hopkinton, MA, USA) was used to obtain the dry residue after extraction.

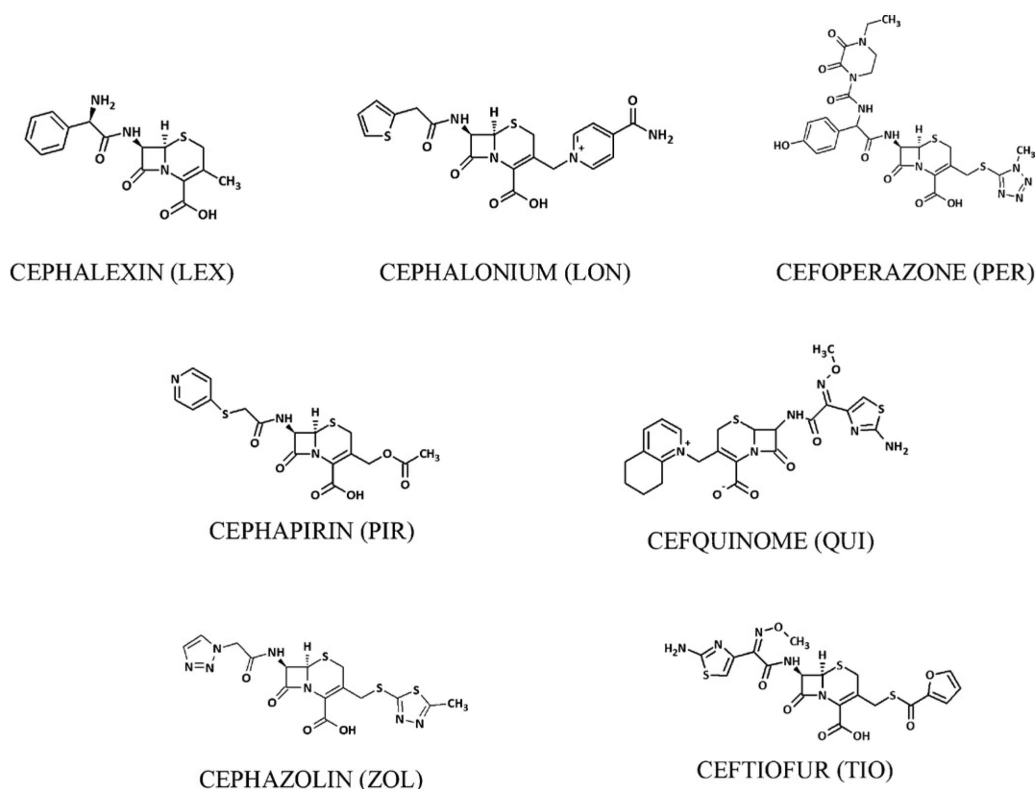

Fig. 1. Chemical structures of the cephalosporins studied.

2.4. Procedure

Two different methods were developed for the determination of cephalosporins in beef muscle samples. In the two cases, methods comprised three stages: sample extraction, clean-up and LC–MS/MS analysis. Although the first and last steps were the same, differences arose in clean-up methodologies based on either conventional SPE or dispersive SPE by QuEChERS (see summary scheme in figure presented as supplementary data).

2.4.1. Sample extraction

A beef muscle sample free of antibiotic was used as matrix. Commercial meat was cut in small pieces and comminuted with an electric grinder to achieve good sample homogeneity and to ensure that a 4 g subsample is representative for the analysis. Appropriate volumes of cephalosporin standards were added to 4.000 g of muscle. PIPE was also added as IS at a level of 1000 $\mu g\,L^{-1}$. After standard additions, samples were kept in dark for 30 min. 15 mL of a mixture of MeCN and water (80:20, v/v) was added to extract the analytes from the samples. After shaking during 2 min on the vortex, samples were centrifuged during 5 min at 3500 rpm, and the liquid phase was taken for further assays.

2.4.2. Clean-up using the QuEChERS method

10 mL of the extract solutions obtained as in Section 2.4.1 were poured into the dispersive SPE kits, which contained 150 mg of PSA sorbent, 150 mg of C18 sorbent and 900 mg of $MgSO_4$. The dispersive SPE kits were shaken vigorously for 5 min. Centrifugation was done for 5 min at 3500 rpm in order to separate the liquid phase containing the analytes in solution from the solid phase with the matrix components retained in the sorbents. A 5 mL aliquot was withdrawn to be dried under nitrogen stream (at 25 °C) with a Turbo Vap evaporator until dryness. Then, samples were reconstituted with 200 μL of water and analysed by LC–MS/MS.

2.4.3. Clean-up using the SPE method

The MeCN in the extract solutions (Section 2.4.1) were dried under a stream of nitrogen. Four millilitres saturated solution of NaCl were added to avoid the foaming during the evaporation. Prior to SPE processing, a phosphate solution (pH 5) was added to the sample up to a final volume of 30 mL.

ENV+ cartridges used in this study were preconditioned with 2 mL MeOH, 2 mL water and 2 mL of 0.05 M sodium dihydrogenphosphate solution at pH 5. The sample solutions were forced to pass through the cartridge. Subsequently, cartridges were washed with 3 mL phosphate solution (pH 5) and 1 mL water. The analytes were eluted with 4 mL MeCN:MeOH:H_2O (45:45:10, v:v:v). The resulting solution was evaporated to dryness under a nitrogen stream, rebuilt with 200 μL of water and analysed by LC–MS/MS.

2.4.4. Chromatographic conditions

The mobile phase used in LC–MS/MS consisted of 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in MeCN. The initial eluent composition was water/MeCN (85:15, v/v) at pH 3.2, during the first 2 min of LC analysis. At the minute 4, the proportion of aqueous mobile phase increases to 45% and at the minute 7 increases to 56%. At these points, the mobile phases return to the initial 85:15 and steel constant until the end of the analysis (stop time 10 min). The flow-rate was 1 mL min^{-1} and the injection volume was 20 μL.

As an example, Fig. 2 shows the chromatogram of the separation of analytes studied in this work. It can be seen that a good separation of all components is achieved in less than 8 min.

2.4.5. Mass spectrometry conditions

Tandem mass spectrometry working in multiple reaction monitoring (MRM) in the positive ionization mode was used for detection. Fragmentations in MRM mode were produced by collision-activated dissociation (CAD) of molecular ions separated

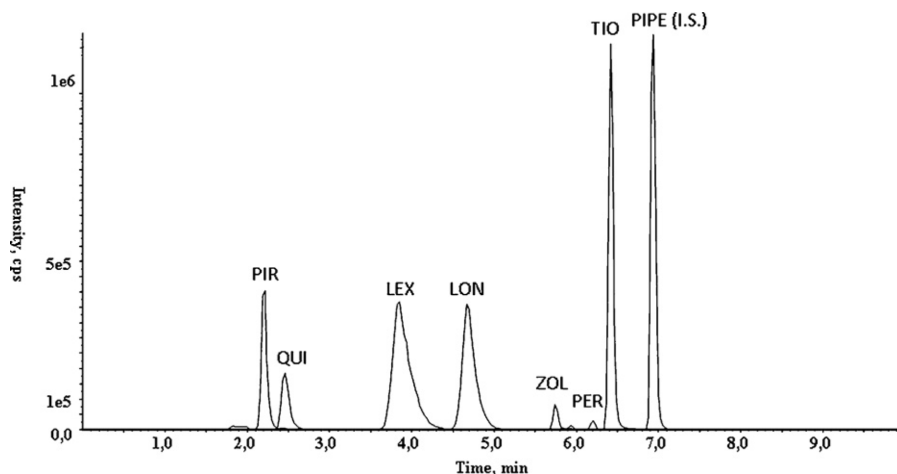

Fig. 2. Separation of cephalosporins studied by LC–MS/MS. This separation is obtained with matrix-matched solution at MRL concentration for all cephalosporins.

in the first quadrupole. Two transitions were followed for each analyte, one was used for confirmation and the other for identification purposes. All the transitions selected and their conditions are shown in Table 2.

2.5. Quality parameters

Validation of the two methods was done according to the EU Commission Decision 2002/657/EC [8] and the FDA guidelines [30]. The quality parameters established were: linearity, recovery, inter-day precision, intra-day precision, limit of detection (LOD), limit of quantification (LOQ), limit of decision (CCα) and limit of capability (CCβ).

The linearity of analytical responses was assessed from the analysis of seven samples spiked at different concentrations and with two independent replicates at each level. The range of concentrations was from the LOQ to three times the maximum residue limit (MRL) value defined by European regulation for muscle samples. Analytes without MRL value were tested at the minimum MRL value of the other regulated cephalosporins, in this case PIR.

Recovery values were calculated for evaluating the efficiency of extraction treatment as well the absence of interferences. Recovery determination was based on the comparison of two calibration curves resulting from the analysis of samples in which the standards were spiked before and after performing extraction procedure. Responses corresponding to samples prepared from the addition of standards after extraction process were assumed to represent the 100% recovery.

The precision of the method was evaluated from intra-day and inter-day variability assays. Intra-day precision was estimated from five independent samples at three different concentrations (MRL value, 1/2 MRL value and 2 MRL value) analysed in the same day. For inter-day precision, samples were analysed in three different days. In the two cases, results were expressed as relative standard deviations (%RSD).

Limit of detection (LOD) and limit of quantification (LOQ) expressed the lowest concentration of analyte that the method can

Table 2
Quantification and confirmation transitions of cephalosporins and the PIPE (IS) with the respective collision energy applied and the proposed fragmentation pathway.

Substance	$[M+H]^+$	Quantification transition	Collision energy (V)	Fragmentation	Confirmation transition	Collision energy (V)	Fragmentation
LEX	348	348 → 140	35	$[F_8-2CO-COOH]^+$	348 → 158	15	$[F_3+H]^+$
LON	459	459 → 152	30	$[F_8-F_1-COOH]^+$	459 → 337	20	$[F_2]^+$
PER	646	646 → 290	35	$[F_8]^+$	646 → 530	20	$[F_2]^+$
PIR	424	424 → 292	20	$[F_2-CO-COOH]^+$	424 → 181	35	$[F_4-CO+H]^+$
QUI	529	529 → 134	20	$[F_1+H]^+$	529 → 396	20	$[F_2+H]^+$
TIO	524	524 → 285	30	$[F_3+2H]^+$	524 → 241	25	$[F_4+H]^+$
ZOL	455	455 → 323	15	$[F_2]^+$	455 → 295	25	$[F_2-CO]^+$
PIPE (IS)	518	518 → 143	25	$[F_{10}+2H]^+$	518 → 359	15	$[F_9+H]^+$

detect and quantify, respectively. These two parameters were estimated from the signal-to-noise (S/N) ratio of samples containing low concentrations of analytes. In particular, S/N = 3 was used for LOD and S/N = 10 for LOQ.

Limit of decision (CCα) and limit of capability (CCβ) are two parameters defined by the EU regulation. CCα values were determined by analysing 20 blank samples fortified with cephalosporins at MRL level. CCβ was calculated as the decision limit CCα plus 1.64 times the corresponding standard deviation (β = 5%), supposing that the standard deviation at the MRL was similar to that obtained at the CCα level.

3. Results and discussion

3.1. Sample treatment

The sample treatment consisted of two steps: (i) extraction of the cephalosporins from the food matrix and (ii) the clean-up of the extracted solutions in order to minimize the impact of interferences and matrix effects on the analysis. In this part, both SPE and dispersive-SPE (d-SPE) by QuEChERS approaches were evaluated to find the most suitable conditions in terms of higher recoveries and lower interferences.

The optimization of the sample treatment relied on experimental design and multicriteria decision making. In these studies, muscle samples were spiked at a level of $4\,mg\,kg^{-1}$ of each cephalosporin. Then, spiked samples were subjected to the treatment procedure under study and extracts were further analysed by LC–MS/MS. Peak areas of each target compound were used as analytical data to calculate recoveries for encountering the best experimental conditions.

3.1.1. Optimization of the extraction procedure

The extraction solvent was composed of a mixture of acetonitrile (MeCN) and water. MeCN was chosen as the organic solvent because its good miscibility with water, and its ability to recover the analytes without extracting high quantities of lipophilic material [21]. As it can be found in the literature, MeCN is one of the most popular solvents for the extraction of β-lactams [11,16,17,31]. Water was selected as the other extraction solvent to facilitate the extraction of the most polar components. Complementarily, the use of an acid modifier (e.g., acetic or formic acid) may be also recommendable for improving the performance of the extraction step [32].

Some significant variables to be considered in the optimization of the extraction step comprised: (i) MeCN/water ratio in the extraction solution (i.e., percentage of MeCN), (ii) total volume of extracting solvent (V), and (iii) addition of a low quantity of HAc as a modifier.

The optimization of these three variables was preliminarily tackled according to a 3-factor at 2-level cube design with two replicate experiments in the cube centre (i.e., 10 experiments were carried out). High and low levels of each variable were defined as follows: MeCN, 80 and 100%; extracting solvent volume, 6 and 12 mL; HAc modifier, 0 and 0.1%. The centre point was 90% MeCN, 9 mL extracting solvent and $5\,mL\,L^{-1}$ acetic acid. The principal objective of this optimization was the maximization of the overall extraction taking into account all the analytes simultaneously. Hence, the mean extraction percentage accounted from the seven cephalosporins was calculated as an overall response.

Extracts from the experimental design were further treated by dispersive SPE (see below) and the resulting cleaned extracts were analysed chromatographically. Then, the mean recovery from all cephalosporins was used as the analytical information for the estimation of effects and interactions. As shown in Fig. 3, all the factors considered here (i.e., MeCN, extracting volume, and HAc addition) were significant. The negative sign of MeCN and HAc main effects indicated that the increase in the magnitude of these factors led to a decrease in the efficiency of the extraction. As a result, a high percentage of MeCN was found to be unfavourable to carry out the analyte extraction. In a similar way, the addition of HAc to the solvent was not recommendable. In contrast, the sign of extracting volume was positive so a high volume of solvent was favourable for increasing the recovery of cephalosporins. A similar behaviour was obtained for the two types of clean-up.

The interaction between MeCN and extracting volume was relevant as well. This finding indicated that the optimization of these two variables should be carried out simultaneously as the effect of each one depended on the level of the other. This was here assessed from a 2-factor at 3-level grid design considering the following levels: MeCN, 60, 80 and 100%; volume (V) 6, 12, 18 mL. The mean recoveries from all the analytes were used to obtain the overall response function as depicted in Fig. 4. The best extraction conditions corresponded to 15 mL of MeCN/water mixture (80:20, v:v) and the use of HAc as a modifier was discarded.

3.1.2. Optimization of the clean-up of extracts

In this step, both conventional SPE and dispersive SPE (QuEChERS) procedures were compared to ascertain the most convenient strategy to carry out the sample clean-up. Muscle samples, spiked at $4\,mg\,kg^{-1}$ each cephalosporin, were subjected to the extraction procedure as developed in the previous section and clean extracts were analysed chromatographically. The optimization criterion relied on minimizing overall analyte losses. For this purpose, mean recovery values at each experimental point were calculated as an estimation of the performance of the extraction.

3.2. QuEChERS method

In the novel QuEChERS methodology, clean-up is accomplished by a modification of traditional SPE through the so-called dispersive-SPE mode (d-SPE). The sorbent, as a dispersive medium, is used to retain those non-desirable components co-extracted from the matrix while target compounds remain in solution. After separation of the dispersive phase by centrifugation, the resulting extracts are ready to be injected into the chromatographic system.

The most common sorbents to be used in d-SPE for the treatment of muscle samples are C_{18} and PSA (primary and secondary amine). It has been described elsewhere that for other kind of matrices such as fruits and vegetables, Graphitized Carbon Black is also widely used to remove pigments. Magnesium sulphate is another typical component of d-SPE systems that may serve to improve the separation of the organic and aqueous phases as well as to remove water from the organic layer [21].

The optimization of the QuEChERS clean-up was focused on the study of the composition of the sorbent employed. Handmaiden d-SPE cartridges with different compositions of C_{18} and PSA were prepared according to a grid design of 2 factors at 5 levels as follows: C_{18} amounts were 0, 50, 150, 250 and 400 mg; PSA amounts were 0, 50, 150, 250 and 400 mg. Extracts were then treated by d-SPE using the set of 5^2 cartridges corresponding to the experimental design. Recoveries of each cephalosporin were determined chromatographically in the solutions resulting after this cleanup.

Fig. 5 summarizes the results obtained in the optimization of the sorbent composition of the d-SPE systems. The representation of the average signal from all the cephalosporins against the different amounts of C_{18} and PSA sorbents shows that best experimental conditions were reached with amounts of PSA between 150 and 250 mg per cartridge. Regarding C_{18} sorbent, it did not effect significantly to the adsorption of the target compounds. As a result, a mixture of 150 mg of C_{18} and 150 mg of PSA was chosen as the

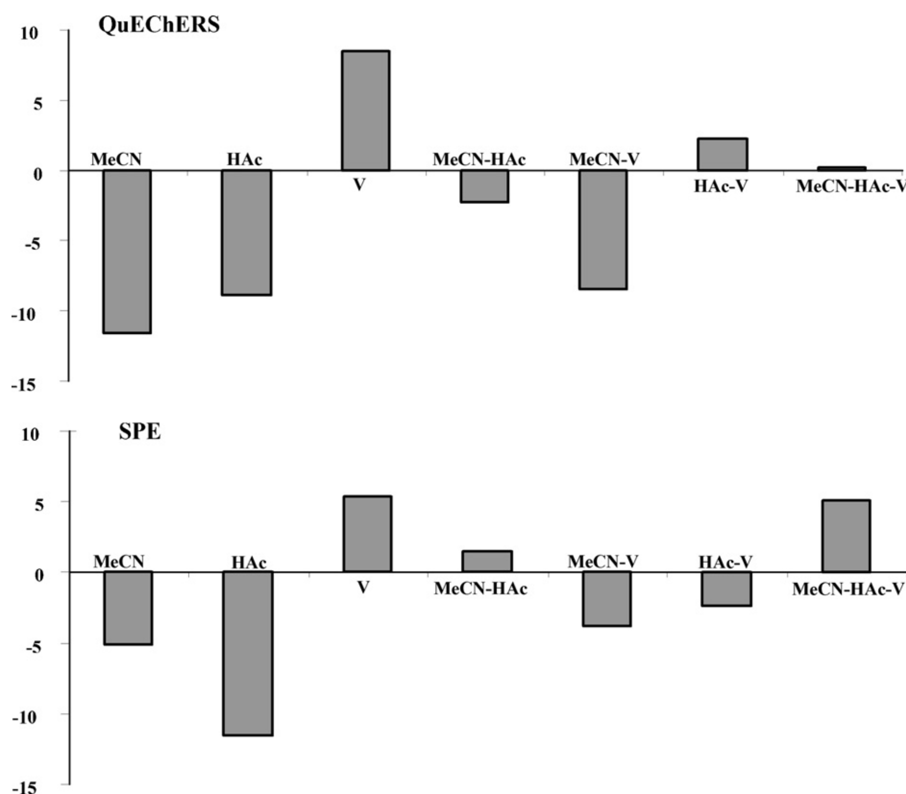

Fig. 3. Estimation effects and interaction in SPE and QuEChERS method. MeCN: acetonitrile; HAc: acetic acid; V volume; MeCN–HAc: interaction between factors acetonitrile and acetic acid; MeCN–V: interaction between factors acetonitrile and volume; HAc–V: interaction between factors acetic acid and volume; MeCN–HAc–V: interaction between factors acetonitrile, acetic acid and volume.

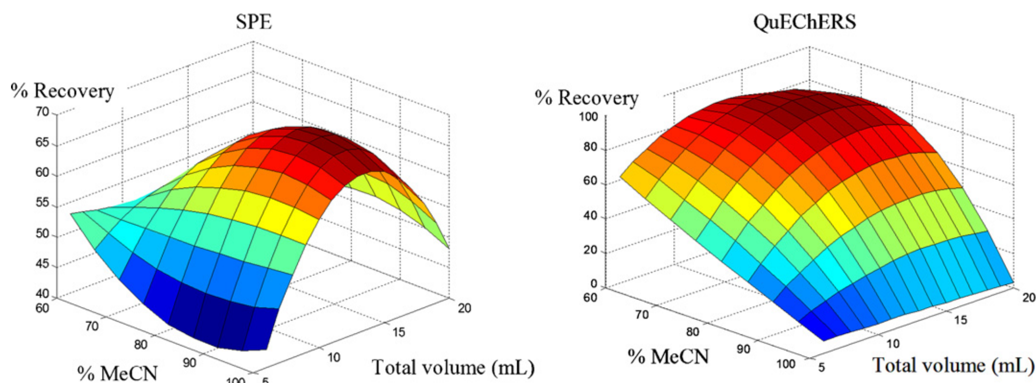

Fig. 4. Overall response function for the recovery of cephalosporins by SPE and QuEChERS.

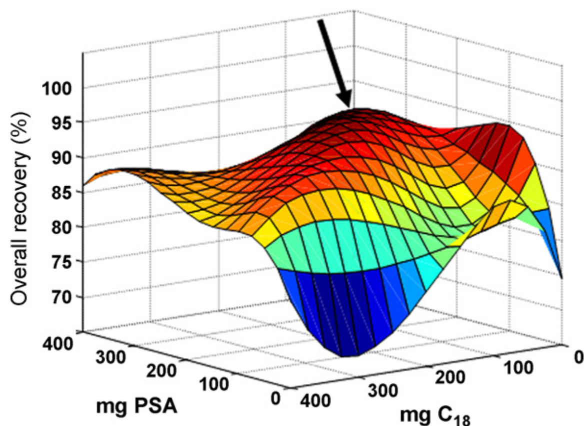

Fig. 5. Optimization of the sorbent composition of the QuEChERS method.

sorbent mixture used in the d-SPE clean-up of our final QuEChERS method.

3.3. SPE method

The optimization of the SPE procedure was focused on (i) the choice of the type of sorbent, (ii) the composition of the buffer solution for cartridge conditioning and washing steps and (iii) the composition of the elution solvent.

First, the performance of the following four commercial cartridges was compared in this study: Isolute ENV+ (hydroxylated polystyrene–divinylbenzene copolymer sorbent), Oasis HLB (hydrophilic–lipophilic-balanced reversed-phase sorbent), Phenomenex Strata-X (reversed phase functionalized polymeric sorbent) and Bond Elut C18 (hydrophobic, bonded silica sorbent). These SPE cartridges were selected as they are some of the most

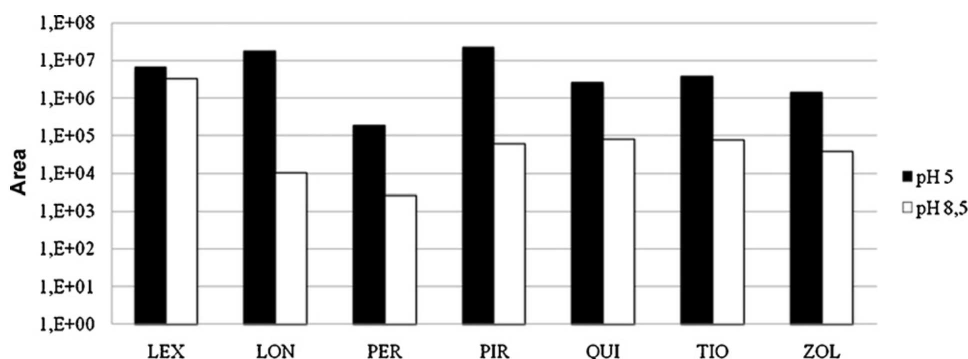

Fig. 6. Influence of the pH on the recovery of cephalosporins by SPE.

Table 3
Validation parameters of the SPE method.

SPE method	LEX	LON	PER	PIR	QUI	TIO	ZOL
Slope	0.1198	1.5847	0.1336	0.9147	0.4723	0.9078	0.2263
Y-intercept	0.0009	−0.006	−0.0004	−0.0005	0.0025	0.032	0.0009
R	0.991	0.994	0.994	0.995	0.993	0.996	0.993
LOD ($\mu g\,kg^{-1}$)	5	<0.5	1,25	4	0.05	<1	0.05
LOQ ($\mu g\,kg^{-1}$)	10	0.5	4	5	0.1	1	0.2
%Recovery (RSD)	62(2)	87(4)	87(4)	18(1)	86(5)	62(3)	68(2)
Inter-day (%RSD)[a]	(6–13)	(8–9)	(6–10)	(18–21)	(8–12)	(4–10)	(5–11)
Intra-day (%RSD)[a]	(11–14)	(8–12)	(8–15)	(17–25)	(10–11)	(10–11)	(10–14)
CCα ($\mu g\,kg^{-1}$)	230	59	59	64	63	1148	60
CCβ ($\mu g\,kg^{-1}$)	260	69	69	78	77	1297	69

[a] The results are shown as a range of the maximum and minimum values obtained in the three levels of concentration analysed.

commonly available on the market and, also, because they have been employed successfully with other types of antibiotics. Besides, the type of sorbents covers a high variety of polarities so they may be useful for dealing with a wide range of analytes.

Similar results were obtained with all four sorbents. Hence, any of these four cartridges could be chosen to carry out the determination of cephalosporins in muscle samples. Anyway, we finally chose Isolute ENV+ SPE cartridges because its performance demonstrated for the analysis of other antibiotics, such as penicillins and quinolones, that might occasionally occur with cephalosporins [15,33–35].

The influence of pH of the solution to be used for cartridge conditioning and washing was assayed with two hydrogenphosphate solutions (pH values adjusted to 5.0 and 8.5). As can be observed in Fig. 6, conditioning and cleaning at pH 5 gave higher signal responses for all cephalosporins. At this pH, the molecules that have a carboxylic group and a basic N in their structure are neutral (as a zwitterion form), so they are expected to interact more efficiently with the polymeric sorbent. Regarding the elution conditions, three different mixtures prepared from MeCN, MeOH and water were compared: MeCN/MeOH (50:50, v:v), MeCN/MeOH/H_2O (45:45:10, v:v:v) and MeCN/MeOH/H_2O (30:40:30, v:v:v). In general, the analyte recoveries were similar in all the cases, but slightly better are obtained if the solvent contains some water. However, considering our interest in a further solvent removal step, the mixture MeCN/MeOH/H_2O (45:45:10, v:v:v) was finally chosen due to its faster and easier solvent evaporation.

So, the selected SPE conditions were as follows: the cartridge was Isolute ENV+, the cartridge conditioning solution was dihydrogenphosphate (pH = 5) and the eluting solution was a mixture of MeCN/MeOH/H_2O (45:45:10, v:v:v).

3.4. Structural elucidation

A triple quadrupole mass spectrometer working in the multi residue mode (MRM) was employed to quantify and identify all the cephalosporins. Cephalosporins were detected as protonated molecular ions $[M+H]^+$ using the positive electrospray ionization mode [36]. For all analytes, $[M+H]^+$ was chosen as the precursor ion of transitions to be considered to quantify and confirm all the cephalosporins. In particular, the most intense transition was used for quantification and the second most intense was devoted to identification. Table 2 shows these quantification and identification transitions with the proposed fragments as well as schemes of the general cephalosporin structures and fragmentations. For PIPE (IS), MS/MS transitions m/z 518 → 143 and 518 → 359 were monitored.

3.5. Validation according 657/2002/EU

The two developed methods were validated according the European Commission Decision 657/2002/EU [8]. Experimental details for assessing such validation are given in Section 2.5. Results are described and compared in the following paragraphs.

3.5.1. Linearity

The calibration curve has been prepared between LOQ and 3MRL levels. For LON, ZOL and PER, with no MRL in beef muscle, the same range than for PIR was prepared. Every level of the calibration curves was prepared twice. The IS was added at a concentration of 1 $mg\,kg^{-1}$. The samples were analysed according to the proposed procedures and the calibration curves were constructed using analyte/IS peak area ratio versus analyte/IS concentration ratio. Tables 3 and 4 show the calibration curves for all cephalosporins studies using both methods and the corresponding correlation coefficients *R*. In general, the linearity was excellent, with *R* values higher than 0.990.

3.5.2. Limit of detection and limit of quantification

LODs and LODs of both methods were determined by analysing matrix-matched samples spiked at very low concentration levels of cephalosporins. Although the SPE method provided slightly better LODs and LOQs (see Tables 3 and 4), results obtained for the

Table 4
Validation parameters of the QuEChERS method.

QuEChERS method	LEX	LON	PER	PIR	QUI	TIO	ZOL
Slope	0.096	1.518	0.126	2.731	0.308	0.566	0.515
Y-intercept	−0.017	−0.0101	−0.0003	−0.02889	−0.0009	−0.0519	−0.0012
R	0.994	0.991	0.993	0.980	0.991	0.996	0.991
LOD ($\mu g\,kg^{-1}$)	20	1.25	1.25	0.5	2.5	5	2.5
LOQ ($\mu g\,kg^{-1}$)	50	4	4	1.5	4	25	5
%Recovery (RSD)	65(2)	105(1)	89(0.4)	89(1)	100(0.5)	99(10)	104(1)
Inter-day (%RSD)[a]	(4–6)	(4–5)	(3–5)	(10–20)	(4–7)	(2–4)	(3–9)
Intra-day(%RSD)[a]	(7–10)	(7–9)	(6–12)	(18–20)	(11–13)	(6–9)	(10–11)
CCα ($\mu g\,kg^{-1}$)	229	59	61	62	62	1143	61
CCβ ($\mu g\,kg^{-1}$)	259	69	71	74	74	1287	73

[a] The results are shown as a range of the maximum and minimum values obtained in the three levels of concentration analysed.

QuEChERS approach were entirely satisfactory as they were always below the MRL values defined in the European legislation.

3.5.3. Accuracy

The accuracy of both methods was estimated from recovery assays as indicated in Section 2.5. Recoveries for most of the cephalosporins were higher than 80% as it can be seen in Tables 3 and 4. In general, the QuEChERS method seemed to provide slightly better results than the SPE counterpart, and recovery values were only lower than 80% for LEX. The PIR recovery of the SPE method was rather poor, probably due to a degradation underwent by this cephalosporin when was in contact for a long time with meat samples as some studies have described [37].

3.5.4. Inter-day and intra-day precision

Inter-day and intra-day precision express the reproducibility and repeatability of an analytical method respectively. Results shown in Tables 3 and 4 are expressed as the relative standard deviation (RSD). This study proved the great precision of the two developed methods. Reproducibility values were not higher than 15% at the MRL value, except for PIR. The comparison of results concluded that QueChERS method presented a slightly higher precision than the SPE method for all the cephalosporins studied.

3.5.5. CCα and CCβ

Twenty fortified samples at the MRL level were prepared and analysed with the two developed methods to determine the decision limit (CCα) and the detection capability (CCβ). As it can be seen in Tables 3 and 4, all CCα and CCβ values obtained with QuEChERS and SPE methods were very close to the MRL value. This finding suggested the suitability of both methods to determine cephalosporins in muscle samples according to the European Decision Commission 657/2002.

4. Conclusions

Two methods were successfully developed to detect and quantify the presence of residues of cephalosporins in beef muscle samples. One of the two methods relied on the well-known SPE clean-up technique. The other consisted of a novel QuEChERS methodology based on dispersive-SPE. Both methods were found to be suitable for the determination of all the cephalosporins in beef muscle samples according the EU requirements.

Comparing the figures of merit, it was concluded that the precision and accuracy for the QuEChERS method were slightly higher than for the SPE method. Conversely, LODs and LOQs of the SPE method were better than those of the QuEChERS method. Anyway, both methods showed LOD and LOQ below the MRL values.

Other characteristics of the two methods, such as solvent waste and speed, were also considered. The QuEChERS method showed a lower solvent consumption and it was faster and more straightforward than SPE. The QuEChERS method also presents improved analytical parameters with respect to SPE and lower amounts of samples were required for the analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.05.002.

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