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Development and validation of a multiclass method for the analysis of antibiotic residues in eggs by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A multiclass method for the analysis of residues, in egg matrices, of 41 antimicrobial agents belonging to seven families (sulfonamides, diaminopyridine derivates, quinolones, tetracyclines, macrolides, penicillins and lincosamides) was developed and validated according to the requirements of European Commission Decision 2002/657. Compounds were extracted with a pressurized liquid extraction (PLE) technique using a 1:1 mixture of acetonitrile and a succinic acid buffer (pH 6.0) at 70 °C. As this resulted in clear extracts, no further clean-up was necessary. Analytes were determined by ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPL–MS/MS) in a chromatographic run of 13 min. Calibration was carried out with spiked blank samples subjected to the entire analytical procedure. Five compounds, two of them isotopically labelled, were used as internal standards. Most analytes were quantified with errors below 10%. Precision in terms of reproducibility standard deviation was between 10% and 20% in most cases. CCα values were in the range 0.5–3.8 μg kg^{-1} for the non-authorized compounds. The proposed method would enable an experienced analyst to process about 25 samples per day.

1. Introduction

Veterinary drugs are used in agricultural practices in order to prevent and treat diseases in food-producing animals. In the past, considerable amounts of drugs were also used as growth promoters. However, this practice has been progressively prohibited in the European Union (EU) and, since January 2006 [1], the use of antimicrobial agents as feed additives has been banned.

Antibiotic residues in food can lead to allergic reactions in some hypersensitive individuals and may compromise the human immune system. In addition, the presence of sub-therapeutic doses of these drugs in foodstuffs for long periods has led to the appearance of bacterial strains that are resistant to drugs used in human medicine. In order to address these problems and to increase food safety the EU has laid down a set of policies and measures, including the establishment of maximum residue limits (MRLs) for some antimicrobials, a network of reference laboratories and a system of alert notifications, among others [2]. A complete list of pharmacologically active substances and their MRLs is available in the Annex to Commission Regulation 37/2010 [3]. Furthermore, because some drugs have been shown to have accumulated in eggs [4], the use of sulfonamides, quinolones, penicillins, some macrolides and certain tetracyclines has been explicitly prohibited in the treatment of animals which produce eggs for human consumption.

At the international level the Codex Alimentarius Commission, one of the main reference organizations on food safety, has established a number of measures designed to control residues of veterinary drugs in food samples [2], including a list of MRLs and a compendium of analytical methods that are deemed suitable to support Codex MRLs [5]. The inclusion of a set of suggested analytical methods distinguishes this approach from the EU strategy, which establishes the requirements for analytical methods [6] but does not recommend specific approaches.

Despite the abovementioned measures, however, fraudulent or improper uses of veterinary drugs cannot be ruled out. Consequently, suitable analytical methods are an essential tool in the context of inspections carried out by health authorities. For reasons of efficiency and economy, the development of multiclass methods, which are able to detect, confirm and quantify as many compounds as possible, has become a significant trend in the analysis of residues and contaminants in food samples. This has been possible thanks to the enormous progress made by mass spectrometry (MS) techniques [7]. Indeed, triple quadrupole mass spectrometry has become the cornerstone technique for the screening and confirmation of food contaminants and residues. Recently, a number of studies have used time of flight (ToF)-MS to screen hundreds of compounds in food matrices within one run [8–11]. However, methods based on ToF-MS are less sensitive than those based

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on triple quadrupole instruments, and they are not considered in Council Decision 2002/657 [6] regarding confirmatory methods.

In terms of the analysis of veterinary drug residues in eggs, most published methods deal with one class of compounds; many of them are based on microbial screening assays [12], liquid chromatography (LC) with fluorescence detection [13–15] or diode array detection [16], although some of them perform determinations by LC–MS [17–22]. Only a few papers to date have described approaches dealing with several classes of veterinary drugs in eggs [10,23,24].

Because of differences in the physicochemical properties of analytes, the extraction process and clean-up is the most challenging step in a multiclass method for the analysis of residues. Due to their high lipid and protein content, eggs constitute a very complex matrix, as some analytes bind to lipoproteins, thereby hindering their extraction, while several organic solvents form emulsions and foams with the matrix [25]. Acetonitrile is considered the best extraction solvent because it precipitates proteins and denatures enzymes, which could degrade drug residues during sample treatment. As regards clean-up, different approaches have been assayed, including solid phase extraction (SPE) [10,23,24], QuEChERS [23] and matrix solid phase dispersion [23]. However, satisfactory recovery has not been obtained for all compounds and a compromise has had to be adopted.

Eggs are a source of cheap and highly nutritious food that is present in all diets. World production of eggs in 2008 reached 65 million tons [26]. In the period January 2000–August 2010 the Rapid Alert System for Food and Feed (RASFF) of the EU [27] registered eight alert notifications and 11 information notifications concerning the presence of veterinary drugs in eggs and egg products, with nitrofurans, quinolones and macrolides being the substances detected.

The objective of the present study was to develop a multiclass method for the analysis of residues of antimicrobial agents in egg matrices, one that could be implemented as part of the routine work and included in the scope of accreditation of the laboratory of the Public Health Agency (Laboratori de l'Agència de Salut Pública) in Barcelona. Analytes were determined by ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPL–MS/MS), using a previously established method [28] for the analysis of the same residues in meat samples. A new extraction procedure based on PLE was developed. The matrix effects on the MS signal were studied.

2. Experimental

2.1. Chemicals and reagents

Josamycine (JOS) and oxolinic acid (OXO) were obtained from Fluka (Buchs, Switzerland). Penicillin G potassium salt (PEN G), penicillin V potassium salt (PEN V), sulfadoxine (SDX), sulfamerazine (SMR), sulfapyridine (SPD), ciprofloxacin (CIP), enrofloxacin (ENR), norfloxacin (NOR), danofloxacin (DAN), sarafloxacin hydrochloride (SAR), difloxacin hydrochloride (DIF) and flumequine (FLU) were obtained from Riedel-de Haën (Seelze, Germany). Amoxicillin (AMO), ampicillin sodium salt (AMP), cloxacillin monohydrate sodium salt (CLO), dicloxacillin monohydrate sodium salt (DIC), oxacillin monohydrate sodium salt (OXA), piperacillin sodium salt (PIP), sulfisoxazole (SFX), sulfadiazine (SDZ), sulfamethazine (SMZ), sulfadimethoxine (SDMx), sulfathiazole sodium salt (STZ), sulfamethoxipyridazine (SMPZ), sulfaquinoxaline sodium salt (SQ), sulfachloropyridazine (SCP), sulfamethizole (SMTZ), sulfamethoxazole (SMX), sulfamonomethoxine (SMM), trimethoprim (TMP), spiramycin (SPI), erythromycin (ERY), roxitromycin (ROX), tylosin tartrate (TYL), tilmicosin mixture of isomers (TIL), lincomycin hydrochloride hydrate (LIN), oxytetracycline dihydrate (OTC), tetracycline hydrochloride (TC), chlortetracycline hydrochloride (CTC), doxycycline hyclate (DC) and demeclocycline hydrochloride (DMC) were obtained from Sigma (St. Louis, MO, USA). [$^{13}C_6$]Phenylsulfamethazine (SMZ-^{13}C) was purchased from Cambridge Isotope Labs. (Andover, MA, USA) and [$^{2}D_5$]norfloxacin (NOR-D_5) from Witega (Berlin, Germany).

Acetonitrile and methanol were HPLC gradient grade and purchased from Carlo Erba (Val de Reuil, France). Formic acid and ammonia solution 25% were from Merck (Darmstadt, Germany). Phosphoric acid 85% was from Panreac (Castellar del Vallès, Spain) and acetic acid 99–100% was from J. T. Baker (Deventer, Netherlands). The solid reagents used were all analytical grade; oxalic acid 2-hydrate and ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Panreac. Sodium hydroxide and succinic acid were from Merck, and sodium dihydrogen phosphate monohydrate was from Fluka. Double-deionized water (Milli-Q, Millipore, Molsheim, France) of 18.2 MΩ cm^{-1} resistivity was used. Diatomaceous earth was purchased from Dionex (Sunnyvale, CA, USA). Extran AP 13 (alkaline solid with detergents) containing sodium hydroxide was from Merck.

Stock standard solutions (1000 mg L^{-1}) of all analytes and internal standards were prepared by dissolving the compounds in an appropriate solvent, which was generally methanol (ENR, DAN, SAR, DIF, MAR, TC, OTC, CTC, DC, DMC, JOS, TIL, LIN, PIP, SDZ, SQ and TMP) or acetonitrile (SPD, SMZ, SCP, SDX, SDMx, SMR, SMPZ, SFX, SMX, SMM, SMTZ, STZ, FLU, SPI, ERY, TYL, ROX and SMZ-^{13}C). Penicillins were dissolved in water, OXO and NOR in 0.1 mol L^{-1} sodium hydroxide, CIP in 0.1 mol L^{-1} formic acid, and NOR-D_5 in chloroform. Six intermediate standard solutions containing several analytes (40 mg L^{-1}) grouped according to their LOQ and MRL were prepared by dilution of the stock solutions with water:acetonitrile (75:25, v/v). Another intermediate standard solution was prepared for the five internal standards [SMZ-^{13}C (6 mg L^{-1}), NOR-D_5 (60 mg L^{-1}), ROX (20 mg L^{-1}), PIP (20 mg L^{-1}) and DMC (20 mg L^{-1})] by dilution of the stock solutions with acetonitrile. All solutions were kept at −20 °C in dark glass bottles for one year.

Working solutions containing all analytes with variable concentrations, according to their LOQ and MRL, were prepared by mixing the appropriate amounts of the intermediate standard solutions and diluting with water:acetonitrile (75:25, v/v). These solutions were kept at −20 °C in dark glass bottles for a month. A working solution containing the five internal standards was prepared by dilution of the corresponding intermediate solution with acetonitrile. This solution was kept at −20 °C in dark glass bottles for a month.

pH 3.0 phosphoric acid buffer: 3.4 mL of 85% phosphoric acid were diluted in water and made up to 1 L with more water. 3 mol L^{-1} sodium hydroxide solution was added until reaching a pH of 3.0.

pH 4.0 oxalic acid buffer: 1.26 g oxalic acid 2-hydrate were dissolved in water and made up to 1 L with more water. 3 mol L^{-1} sodium hydroxide solution was added until reaching a pH of 4.0.

pH 5.0 acetic acid buffer: 0.6 mL acetic acid were diluted in water and made up to 1 L with more water. 3 mol L^{-1} sodium hydroxide solution was added until reaching a pH of 5.0.

pH 6.0 succinic acid buffer: 11.8 g succinic acid were dissolved in water and made up to 1 L with more water. Ammonia solution 25% was added until reaching a pH of 6.0.

pH 7.0 phosphate buffer: 1.38 g sodium dihydrogen phosphate monohydrate were dissolved in water and made up to 1 L with more water. 3 mol L^{-1} sodium hydroxide solution was added until reaching a pH of 7.0.

Mobile phase A: 0.13 g oxalic acid 2-hydrate were dissolved in water and 200 μL formic acid were added before making up to 1 L with more water.

Mobile phase B: 1 mL formic acid was diluted with acetonitrile and made up to 1 L with more acetonitrile.

2.2. Instrumentation

LC–ESI-MS/MS measurements were carried out with a Waters Acquity UPLC system (Chicago, IL, USA) coupled to a Quattro Premier triple quadrupole mass spectrometer from Micromass (Waters), using an electrospray source. The column used was C_{18} Acquity UPLC BEH (100 mm × 2.1 mm; 1.7 μm particle diameter) from Waters. Instrument control and data processing were carried out by means of Masslynx 4.1 software.

Pressurized liquid extractions of the analytes from the egg matrix were performed with an ASE 200 system (Dionex, Sunnyvale, CA, USA), equipped with 5 mL stainless steel cells and lined with cellulose filters from Dionex. A turboVap LV evaporation system (Caliper, Hopkinton, MA, USA) was used for the evaporation of the extracts. A vortex shaker from Comecta (Abrera, Spain) and a Hettich refrigerated centrifuge (Tuttligen, Germany) were also used in the treatment of the sample. The pH was measured with a Crison GLP 21 pH meter (Alella, Spain) equipped with a Crison 52-02 Ag/Ag Cl combined glass electrode.

2.3. Samples

Blank egg samples for extraction development and validation studies were obtained from a farm in Catalonia (Spain) on which no antibiotics were used. They were stored at 4 °C before analysis.

Spiked samples were prepared by adding the correct amount (range 12.5–200 μL) of a solution containing each of the analytes at the suitable concentrations, and 100 μL of solution containing the five internal standards (0.6 mg L^{-1} SMZ-^{13}C, 6 mg L^{-1} NOR-D_5, 2 mg L^{-1} ROX, 2 mg L^{-1} PIP and 2 mg L^{-1} DMC), to each portion of the weighed samples, which were gently homogenized before analysis.

Incurred eggs were obtained from laying hens fed with a commercial standard diet and bred at an experimental farm of the Institut de Recerca i Tecnologia Agroalimentaria (IRTA) (Prat de Llobregat, Catalonia, Spain). Forty-five laying hens were divided into three groups of 15 hens each. The first group did not receive any veterinary drug (Control). The second group was treated with a daily dose of 75 mg/kg body weight of tylosin tartrate (Oratil, SP. Veterinaria, Tarragona, Spain) in drinking water over a period of five days. The third group received a daily dose of 150 mg/kg body weight of doxycycline hyclate (Doxi 100, SP. Veterinaria, Tarragona, Spain) in drinking water, also over a period of five days. Egg samples from each animal were taken both during the treatment and after the withdrawal period (five days). Samples were immediately refrigerated (4 °C) and stored at −80 °C until analysis.

2.4. Procedures

2.4.1. Extraction

Prior to the extraction procedure, diatomaceous earth was treated with a 0.1 mol L^{-1} solution of EDTA (150 mg EDTA per gram diatomaceous earth) and subsequently dried at 100 °C.

Samples weighing 1 g of the homogenized whole eggs (spiked with analytes and internal standards if required) were mixed in a ceramic mortar with the diatomaceous earth containing 150 mg g^{-1} of EDTA (approximately 1.5 g). The mixture was then placed in 5 mL stainless steel extraction cells containing cellulose filters. The extraction buffer consisted of a 1:1 (v/v) mixture of acetonitrile and 0.01 mol L^{-1} succinic acid buffer pH 6.0 (adjusted with concentrated ammonia). The PLE program started with a cell heating with approximately 10 mL of extraction buffer for 5 min at 70 °C and 1500 psi; this was followed by a static cycle of 3 min with the same 10 mL of extraction solvent at the same temperature and pressure and by the addition of an extra 1.5 mL of extraction buffer. This caused the evacuation of an equivalent volume to the collection vial. A second cycle was carried out at the same conditions. The cell was then purged for 1 min with nitrogen and all the extract was collected into the same vial (approximately 13 mL). Extraction cells were cleaned between each run by sonication for around 15 min in an alkaline solution (Extran AP 13), then 15 min in water and, finally, 15 min in acetone.

After the extraction all extracts were made up to a final volume of 20 mL with a 1:1 (v/v) mixture of acetonitrile and pH 6.0 buffer, placed in centrifuge tubes and centrifuged for 10 min at 3500 rpm and 10 °C. Next, 4 mL aliquots of the clear extracts were evaporated near to dryness at 50 °C with a nitrogen evaporator (turboVap LV) and, subsequently, re-dissolved by vortex mixing in 1 mL of water. Finally, the injection extracts were filtered through 0.45 μm membrane filters (Durapore, Millipore) and 10 μL of each one was injected into the UPLC system.

2.4.2. Liquid chromatography–mass spectrometry

A binary mobile phase with a gradient elution was used for the chromatographic separation of the veterinary drugs in the sample extracts. Mobile phase A was an aqueous solution of 0.02% formic acid and 1 mmol L^{-1} oxalic acid, while mobile phase B was acetonitrile with 0.1% formic acid. The separation was performed at 40 °C and the following gradient program was applied: 0–1 min, 5% B; 1–5 min, linear increase to 25% B; 5–8.5 min, linear increase to 90% B; 8.5–9.5 min, 90% B; 9.5–10 min, decrease to 5% B; and finally, 10–13 min, 5% B. The mobile phase flow rate was 0.3 mL min^{-1} and was directed to the mass spectrometer without splitting. Samples were kept in the autosampler at 15 °C.

The electrospray ionization source was operated in the positive mode under the following working conditions: capillary voltage of +3.5 kV; source block and desolvation temperatures of 120 °C and 400 °C, respectively; desolvation and nebulizer gas (nitrogen) flow rates of 650 L h^{-1} and 50 L h^{-1}, respectively; and argon pressure in the collision cell of 4×10^{-3} mbar.

The selected values of dwell time (d.t.), cone voltage (C.V.) and collision energy (C.E.) optimized for each compound are given in Supplementary Table S1. Full scan spectra were obtained in MS scan mode over a variable range of *m*/*z*, depending on the analyte molecular mass, at a cycle time of 500 ms every 1 s and with an interscan time of 100 ms.

Data for quantification and confirmation were acquired in the multiple reaction monitoring (MRM) mode, splitting TIC acquisition into six windows to achieve greater sensitivity. Two transitions were monitored for identification, although only one was used for quantification (Supplementary Table S1). Identification was based on retention time, while confirmation was performed according to the ion ratio criteria in Decision 2002/657/EC.

Calibration curves for quantification were produced by extracting blank egg fractions of 1 g spiked with analytes at five different concentrations and internal standards. The internal standards used were SMZ-^{13}C (for sulfonamides and trimethoprim), PIP (for penicillins and lincomycin), NOR-D_5 (for quinolones), DMC (for tetracyclines) and ROX (for macrolides).

2.4.3. Validation procedure

The method was validated in egg matrix according to European Commission Decision 2002/657 and at four concentration levels. The first level was the limit of quantification (LOQ) for each analyte, estimated as the concentration at which the signal-to-noise ratio was equal to or above 10. For analytes with an established MRL (OTC, TC, CTC, TYL, ERY and LIN) the other three levels corresponded to 0.5, 1 and 1.5 MRL. For the remaining analytes, for which neither MRL nor MRPL have been established, the selected

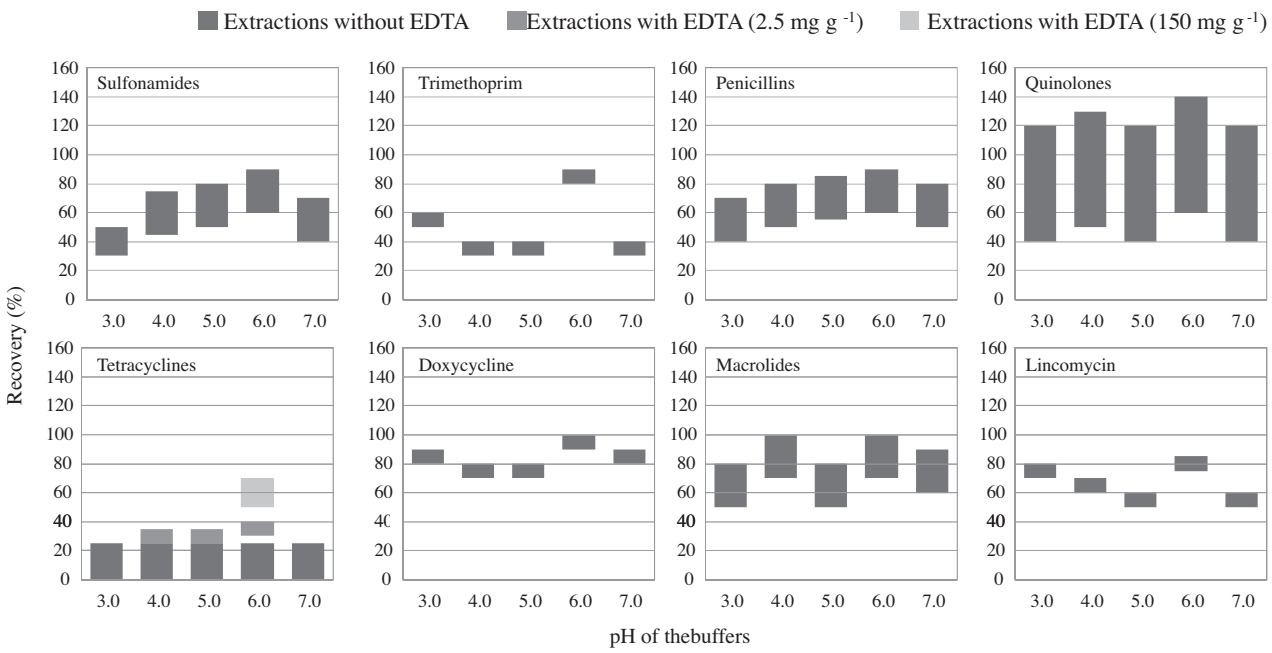

Fig. 1. Influence of pH on analyte recovery. The range shown at each pH value encompasses the recoveries of all the compounds in the group of drugs.

levels were 12.5, 25 and 37.5 μg kg^{-1}. This gave four concentration levels and six repetitions for each one, resulting in 24 extractions for each compound.

Fractions of 1 g of blank samples were fortified at LOQ, using 12.5 μL of a spike solution containing all analytes at different concentrations, depending on the individual LOQ level. To quantify these samples, a calibration curve was prepared by extracting five 1 g fractions of blank egg fortified with variable volumes (12.5–200 μL) of the same spike solution. The procedure was repeated on two additional days.

In a similar way, 1 g fractions of blank sample were fortified at each validation level of 0.5 MRL or 12.5 μg kg^{-1}, MRL or 25 μg kg^{-1}, and 1.5 MRL or 37.5 μg kg^{-1}, using variable volumes (12.5, 25 and 37.5 μL, respectively) of another spike solution that contained all the analytes at the appropriate concentrations, depending on the MRL level and on whether the analytes were banned substances. The calibration curve was developed by fortifying five 1 g fractions of blank egg with volumes from 5 μL to 40 μL of the same spike solution used. This procedure was also repeated on two additional days.

From the data obtained, precision (repeatability and intra-laboratory reproducibility), decision limit (CCα), detection capability (CCβ) and linear range were calculated. The specificity was assessed by analyzing six blank egg samples.

In order to calculate the recovery values of the extraction process the results obtained from three extractions at each validation level were compared with those obtained when spiking the blank egg extract just before the injection, in the re-dissolved step, at the corresponding concentrations.

Finally, the matrix effect was studied by evaluating the ionic suppression and enhancement effects, comparing calibration curves for all analytes prepared with and without egg matrix.

3. Results and discussion

3.1. Extraction procedure

The present study evaluated different factors related to the PLE procedure (pH of the extraction buffer, EDTA addition to the extraction cell and extraction temperature) with the aim of improving the extraction of all 41 studied analytes. The number of cycles and static time of each cycle were optimized in previous studies devoted to the extraction of nine quinolones from whole egg [29]. The best results were obtained when performing PLE with two cycles of 3 min each. The temperature used in the pH and EDTA addition studies was 70 °C, which was the optimal temperature in the previous studies with quinolones.

The pH of the extraction buffer was studied by performing, in triplicate, extractions of fresh whole egg spiked at 100 μg kg^{-1} with all analytes. The extraction buffers consisted of 1:1 (v/v) mixtures of acetonitrile and five different aqueous buffer solutions: pH 3.0 phosphoric acid buffer 0.05 mol L^{-1}, pH 4.0 oxalic acid buffer 0.01 mol L^{-1}, pH 5.0 acetic acid buffer 0.01 mol L^{-1}, pH 6.0 succinic acid buffer 0.01 mol L^{-1} and pH 7.0 phosphate buffer 0.01 mol L^{-1}. The optimal pH for each antibiotic family was monitored from the recovery values (Fig. 1). For sulfonamides and penicillins, best recoveries were at pH 5.0 and 6.0, for trimethoprim and lincomycin they were at pH 6.0, and for macrolides at pH 4.0 and 6.0. For quinolones similar recoveries were found at all pH, while among tetracyclines only doxycycline was extracted. In general, therefore, the buffer extraction solutions at pH 4.0, 5.0 and 6.0 seemed to be the best.

Tetracyclines can form complexes with metal ions present in the sample extraction medium, leading to loss of these analytes during the extraction procedure. To avoid such losses, extractions were performed at pH 4.0, 5.0 and 6.0, adding 100 μL of 0.01 mol L^{-1} EDTA solution to the egg/diatomaceous earth mixture (approximately 2.5 mg EDTA per gram of diatomaceous earth). Oxytetracycline, tetracycline and chlortetracycline were extracted more effectively at pH 6.0, obtaining recoveries between 30% and 40%. In an attempt to improve the recovery of tetracyclines, a higher concentration of EDTA was tested: extractions using buffer at pH 6.0 were performed, mixing spiked whole egg with diatomaceous earth containing 150 mg g^{-1} EDTA, previously prepared. This led to improved recoveries (in the range 50–70%) for oxyitetracycline, tetracycline and chlortetracycline. Thus, better recoveries for most of the 41 analytes were obtained when performing extractions with pH 6.0 buffer and diatomaceous earth containing 150 mg g^{-1} EDTA.

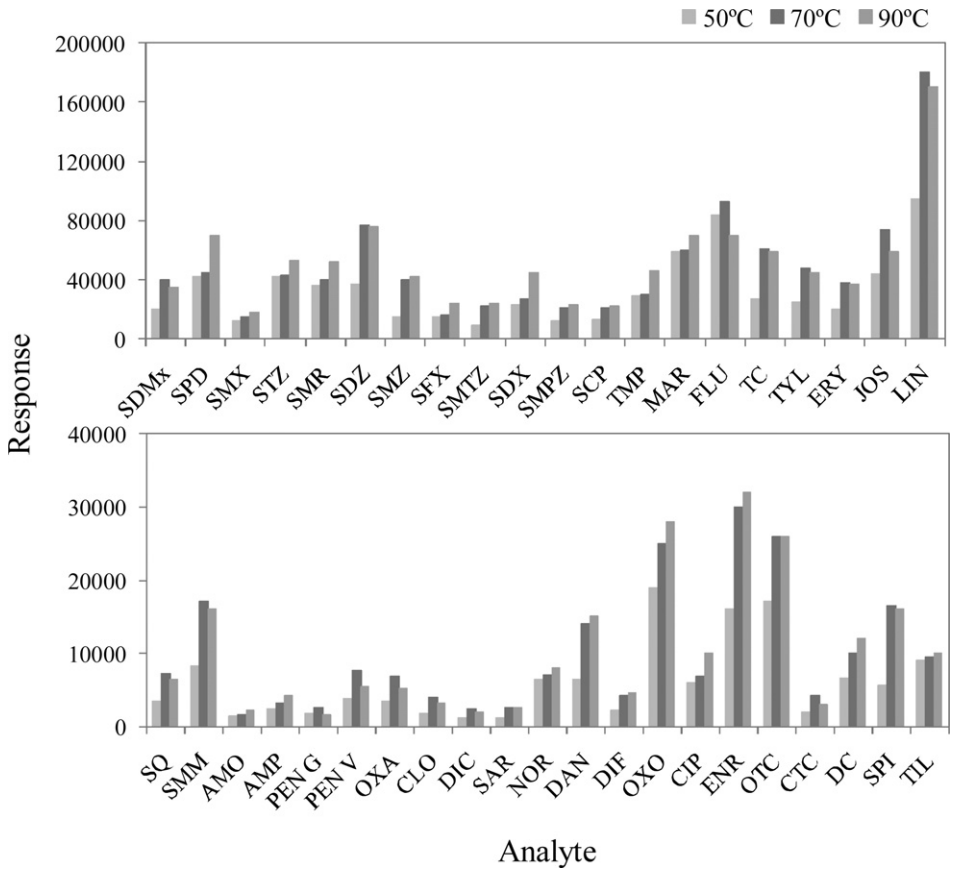

Fig. 2. Influence of temperature on the extraction of analytes.

In general, the use of high temperatures in PLE gives better results because analytes are easily extracted. However, very high temperatures can lead to degradation of analytes or to an increased extraction of matrix interferences. One previous study has reported that when temperatures of about 100 °C are applied, a larger fraction of egg soluble organic matter is extracted, thereby obtaining extremely dirty extracts [30]. To study the effect of temperature, extractions were performed in triplicate at 50 °C, 70 °C and 90 °C. Better responses were obtained at 70 °C than at 50 °C, while extractions at 90 °C did not improve responses significantly (see Fig. 2). Therefore, 70 °C was the optimized temperature in this method.

Contamination of cells with the analytes was observed after the first extractions performed in the study. Two different cell cleaning procedures were assayed. The first involved sonication of cells for 15 min in a 0.05 mol L^{-1} phosphoric acid solution and sonication in water for 15 min, while the second consisted of sonication for around 30 min in methanol. However, neither procedure was able to fully remove the contamination. The concentration ranges of the obtained contaminations were: 0.1–1.2 μg L^{-1} in extract solution for sulfonamides and trimethoprim; 0.1–3 μg L^{-1} for tetracyclines; 0.5–10 μg L^{-1} for quinolones; and below 0.8 μg L^{-1} for macrolides. Finally, a cleaning procedure consisting of sonication for around 15 min in an alkaline solution of Extran AP 13, followed by sonication in water for 15 min and a final sonication in acetone for 15 min gave successful results, avoiding any carry-over contamination.

3.2. Liquid chromatography–mass spectrometry method

The chromatographic analysis was based on a previously reported UPLC–MS/MS methodology [28], one which was implemented as a routine method in the "*Laboratori de l'Agència de la Salut Pública de Barcelona*" for the control of antibiotic residues in animal tissues. Two new antibiotics were included in the present study: lincomycin and tilmicosin.

For lincomycin and tilmicosin, mass spectrometry parameters were optimized by infusion of standard solutions of these analytes in acetonitrile. Mass spectrometry parameters for sulfamethizole were newly optimized by infusion of a standard solution, because egg matrix interferences made its determination more difficult.

Mass spectrometry parameters and retention times for the 41 studied analytes and the five internal standards used are shown in Supplementary Table S1.

3.3. Validation results

The method was validated according to European Commission Decision 2002/657. This Decision establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results obtained by laboratories. Guidelines have been published [31] in order to clarify ambiguous aspects of Commission Decision 2002/657. The fact that some analytes are authorized (with corresponding MRL values) while others are prohibited makes it more difficult to design the validation procedure for multi-residue methods.

The validation parameters measured to evaluate the method were specificity, linear range, trueness, precision (repeatability and intra-laboratory reproducibility), CCα, CCβ and recoveries.

Specificity was assessed by analyzing six blank egg samples and checking the absence of background peaks (above a signal-to-noise ratio of 3) at the retention times of the target compounds. The chromatogram of a blank egg sample is shown in Supplementary Fig. S1.

LOQs were determined as explained in Section 2.4.3. Different LOQ values were obtained for the analytes: 2 μg kg^{-1} (TMP and sulfonamides, except SQ and SMM), 4 μg kg^{-1} (SQ, SMM, penicillins, quinolones, macrolides and tetracyclines, except CTC) and 8 μg kg^{-1} (CTC).

As described in Section 2.4.3, calibration curves for each compound were produced using five 1 g fractions of blank egg samples spiked in different concentration ranges. The linear regression analysis was carried out by plotting the peak area ratio of the analyte to internal standard versus the analyte concentrations. Correlation coefficients were $r \geq 0.99$ for all the analytes in all the concentrations ranges. Residuals were below 20% at all points of the calibration curves for all analytes, showing good linearity.

Accuracy of the method was evaluated by estimating errors and precisions for all analytes. The error was calculated at each validation level as the relative difference between the obtained mean and the nominal concentration (%). Results were below 10% in most cases, increasing in only a few cases up to 16%. Values of repeatability (RSD_r) and intra-laboratory reproducibility (RSD_R) were calculated by applying a one-way (day) ANOVA for a 95% confidence level in order to obtain inter-session variation (s_L), intra-session variation (s_r) and intra-laboratory total standard deviation (s_R). According to Decision 657/2002, the calculated RSD_R must not exceed the level calculated by the Horwitz equation. However, for concentrations lower than 100 μg kg^{-1}, application of the Horwitz equation gives unacceptably high values, and hence these RSD_R should be as low as possible. In this validation the RSD_R obtained for authorized substances at concentrations equal to or above 100 μg kg^{-1} did not exceed the corresponding Horwitz levels. When concentrations were lower than 100 μg kg^{-1}, obtained RSD_R values were below 27%. All these results corresponding at the MRL or 25 μg kg^{-1} level are summarized in Table 1.

The CCα, defined as the concentration above which it can be concluded that a sample is non compliant with an error probability α, was calculated using two different approaches, i.e. for banned substances and for MRL substances. For compounds with established MRLs, the CCα was calculated as the MRL plus 1.64 times the corresponding standard deviations of the intra-laboratory repro-

Table 1
Accuracy and precision of the method.

Compound	MRL (μg kg^{-1})	MRL μg kg^{-1} or 25 μg kg^{-1}		
		Error (%)[a]	RSD_r (%)/ RSD_R (%)[b]	% recovery
Sulfonamides				
SDMx	–	5	10/15	67
SPD	–	10	9/11	75
SMX	–	7	13/15	78
SQ	–	12	12/20	74
STZ	–	12	8/9	65
SMR	–	8	10/15	66
SDZ	–	5	8/9	73
SMZ	–	7	10/15	68
SFX	–	14	12/13	58
SMTZ	–	13	11/22	66
SDX	–	7	9/13	65
SMM	–	4	10/10	63
SMPZ	–	8	11/19	71
SCP	–	17	13/15	62
Diaminopyridine derivates				
TMP	–	4	8/13	105
Penicillins				
AMO	–	2	8/22	47
AMP	–	16	11/15	57
PEN G	–	7	10/23	71
PEN V	–	13	17/23	65
OXA	–	9	10/23	71
CLO	–	9	14/15	74
DIC	–	1	11/20	85
Quinolones				
SAR	–	3	6/16	127
NOR	–	0.0	9/15	170
MAR	–	0.3	7/8	190
DAN	–	9	12/17	320
DIF	–	5	8/10	144
FLU	–	14	12/26	91
OXO	–	9	12/17	89
CIP	–	5	12/12	122
ENR	–	8	11/23	280
Tetracyclines				
OTC	200	12	6/15	57
TC	200	11	8/15	71
CTC	200	8	12/14	49
DC	–	1	9/16	85
Macrolides				
TYL	200	1	5/7	92
SPI	–	5	10/14	145
ERY	150	4	12/15	79
JOS	–	6	9/10	96
TIL	–	1	11/18	130
Lincosamides				
LIN	50	15	9/17	73

[a] Error (%): difference between the obtained mean and the nominal concentration.
[b] RSD_r (%): repeatability and RSD_R (%): intra-laboratory reproducibility.

Table 2
CCα and CCβ values.

Compound	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
Sulfonamides		
SDMx	1.0	1.5
SPD	2.0	2.5
SMX	0.5	1.0
SQ	1.0	1.8
STZ	1.8	2.3
SMR	1.7	2.2
SDZ	1.0	1.5
SMZ	1.4	1.9
SFX	1.4	1.9
SMTZ	1.4	1.9
SDX	1.2	1.5
SMM	2.0	2.8
SMPZ	0.5	0.9
SCP	2.1	2.6
Diaminopyridine derivates		
TMP	0.8	1.3
Penicillins		
AMO	0.8	1.5
AMP	2.5	3.1
PEN G	2.1	3.0
PEN V	1.3	2.1
OXA	2.3	2.9
CLO	0.6	1.4
DIC	1.0	1.8
Quinolones		
SAR	1.6	2.4
NOR	2.9	3.5
MAR	0.3	0.9
DAN	1.2	1.9
DIF	1.3	2.2
FLU	3.8	4.5
OXO	3.8	4.6
CIP	3.1	3.8
ENR	3.1	3.7
Tetracyclines		
OTC	223.8	247.6
TC	229.9	259.8
CTC	232.3	264.6
DC	0.6	1.1
Macrolides		
TYL	216.8	233.6
SPI	0.6	1.4
ERY	177.5	204.9
JOS	0.8	1.3
TIL	1.6	2.2
Lincosamides		
LIN	57.6	65.2

Table 3
LC–MS multiclass methods for the analysis of residues of veterinary drugs in eggs.

Analytes/compounds	Extraction	Cleanup	Analytical technique	Calibration	Limits ($ng\,g^{-1}$)[a]	Recoveries[a]	Remarks	Reference
29 compounds:	Aqueous sodium succinate buffer	SPE Oasis HLB 1 evap.	LC ion trap MS	A standard in solvent, a matrix matched standard and a spiked sample were compared to asses recoveries and matrix effects	*Limits of performance*:		Screening purposes	[24]
Sulfonamides					10–20	45–80%		
Quinolones					10–20	70–80%		
Tetracyclines					25–50	45–55%		
β-Lactams					50	25–50%		
76 compounds:	ACN + water	SPE StrataX 1 evap.	LC ToF MS	Spiked blank samples	*CCβ*[b]:		Screening purposes	[10]
Benzimidazoles					5–263	86–114%		
Macrolides					118–566	69–261%		
Penicillines					133–679	70–143%		
Quinolones					5–180	88–119%		
Sulfonamides					15–198	83–118%		
Trimethoprim					60–62	95–109%		
Tetracyclines					251–538	63–120%		
Nitroimidazolen					13–26	84–128%		
Coccidiostat					196–415	76–138%		
Ionophores					50–526	16–190%		
Amphenicols					73–169	89–121%		
25 compounds:	ACN + aqueous citric acid buffer + EDTA	SPE Oasis HLB 1 evap.	LC triple quadrupole MS	Standards in solvent and spiked blank samples	*CCα*:		Only this method was validated	[23]
Anthelmintic					2–13	71–92%		
Tetracyclines					210–220	71–74%		
Quinolones					3–6	84–91%		
Sulfonamides					6–114	73–88%		
Macrolides					12–218	75–97%		
	Modified QuEChERS		LC triple quadrupole MS	Spiked blank samples		62–96%		
						0–15%		
						2–49%		
						51–76%		
	Matrix solid phase dispersion (C18 and Florisil)		LC triple quadrupole MS	Spiked blank samples		2–83%		
						0–7%		
						0–2%		
						0–90%		
						3–72%		
	Aqueous citric acid buffer + EDTA	SPE Oasis HLB 1 evap.	LC triple quadrupole MS	Spiked blank samples		0–131%		
						49–79%		
						36–76%		
41 compounds:	PLE with ACN + succinic acid buffer + EDTA		LC triple quadrupole MS	Standards in solvent and spiked blank samples	CCα:			The present paper
Sulfonamides					0.5–2	41–101%		
Trimethoprim					0.8	62%		
Penicillines					0.6–2.5	48–106%		
Quinolones					0.3–3.8	98–228%		
Tetracyclines					0.6–232	56–100%		
Macrolides					0.6–217	67–274%		
Lincomycin					58	68%		

[a] Limits and recoveries are given in the same order as the families of compounds.
[b] Average values for the matrices tested (meat, fish and egg).

ducibility study at the MRL level. For banned compounds, the calibration curve procedure was followed and the CCα was established as the concentration at the *y*-intercept plus 2.33 its standard deviation in the lowest level using data from the intra-laboratory reproducibility study.

CCβ is defined, for compounds with an established MRL, as the concentration at which the method can detect compliant concentration limits with an error probability β. For banned substances, CCβ is the minimum concentration level at which the method can detect contaminated samples with an error probability β. In both cases, it was calculated as the CCα plus 1.64 times the corresponding standard deviation when analyzing at least 18 egg samples spiked at the CCα level. These deviations were considered to be the same as that obtained at the MRL level, or at the lowest level (LOQ) for banned compounds. CCα and CCβ values are summarized in Table 2.

Recovery indirectly measures the analyte losses during sample extraction. This is also affected by the matrix and might vary as a function of the concentration [32]. Recoveries were calculated, at each validation level, as the response ratio between the analyte amount added before extraction and that added before the injection (%). Table 1 shows the recovery values obtained at the MRL or 25 $\mu g\,kg^{-1}$ level, which were satisfactory (above 50%) for most of the analytes. High recoveries, above 120%, were observed for quinolones, except OXO and FLU, and for SPI and TIL, indicating signal enhancement due to the presence of matrix components. This effect was also observed in a previously published work [9]. This type of problem is usually addressed by using matrix-matched standards, i.e. standards prepared by adding known amounts of analytes to the extracts of blank samples just before injection. In the present work, however, the signals obtained with samples fortified at the beginning of the sample treatment led to results that were significantly different from those obtained with extracts fortified at the end of the process (just before injection). An explanation for this phenomenon can be found in the study performed by Kaufmann et al. about liquid chromatography/electrospray/triple quadrupole fragmentation [33]. They explain that substances like difloxacin may exhibit different protonated ion species depending on the concentration and the process they have been subjected to. The differences in protonation can give origin to different fragmentation patterns and, consequently, different signals. Moreover, this phenomenon was not observed when concentrations of these compounds were near to or above 100 $\mu g\,kg^{-1}$, as here the recoveries were below or equal to 100%.

Finally, matrix effects were evaluated by building calibration curves ($n = 5$) for each analyte with standards in solvent and matrix-matched standards. Thus, calibration curves, prepared with and without matrix, were compared graphically. Supplementary Fig. S2 shows these graphs for some compounds that are representative of each family. Since calibration curves in matrix were identical to those in solvent for SPD, LIN, ERY, JOS, DC and AMP, no matrix effects were observed for these compounds. For almost all sulfonamides, penicillins, tetracyclines, FLU and OXO, suppression matrix effects were observed, since calibration curves in matrix had lower slopes than did calibration curves in solvent. Enhancement matrix effects were observed for almost all quinolones, TMP, TYL, SPI and TIL, as calibration curves in matrix were above the calibration curves in solvent. In the quantification of samples, problems derived from suppression or enhancement effects were avoided by using calibration curves that were built by extracting fortified blank egg samples.

3.4. Application to incurred egg samples

To evaluate the applicability of the validated method, six egg samples obtained from laying hens that were bred at the IRTA experimental farm were analyzed in triplicate. The objective of these analyses was also to quantify the residues present in eggs collected during the treatment of hens with TYL and DC and after the withdrawal period. Two of the six samples corresponded to control samples, which came from hens that did not receive any veterinary drugs and which were free of any analyte. A further two corresponded to samples taken during the treatment with TYL and DC and which were contaminated with 138 $\mu g\,kg^{-1}$ of TYL and 3800 $\mu g\,kg^{-1}$ of DC, respectively. The final two samples corresponded to eggs taken after the withdrawal period in the treatment with TYL and DC and which contained 16 $\mu g\,kg^{-1}$ of TYL and 147 $\mu g\,kg^{-1}$ of DC, respectively. The residues obtained for TYL during treatment and after the withdrawal period were below the corresponding MRL. In the case of DC, a banned substance, residues obtained were far above the CCα of the method.

4. Conclusions

As stated earlier, only a few papers to date have proposed multiclass methods for the analysis of residues of veterinary drugs in eggs. Table 3 summarizes the main features of these methods for comparative purposes. Of these, only the method proposed recently by Garrido Frenich et al. [23] fulfils the requirements of EU Decision 657/2002 [6] concerning confirmatory methods. In this regard, the method reported in the present paper complements that of Garrido Frenich et al. in two respects: first, it explores another extraction technique, i.e. PLE, and second, it increases the number of compounds included in the validation scheme, 41 instead of 25 (17 are studied in both papers).

Trueness of the method proposed here is slightly better, since errors are lower than 10% in most cases, while in the study by Garrido Frenich et al. [23] trueness, expressed as recovery, ranges from 60% to 118%. The values of intra-laboratory reproducibility obtained at 37.5 $\mu g\,kg^{-1}$ or at 1.5 MRL levels are comparable with those reported in this previous report [23], which were obtained at 50 $\mu g\,kg^{-1}$, whereas the repeatability values obtained in the present study are slightly lower. In the case of banned compounds, the CCα values obtained here are about one order of magnitude lower than those reported by other authors. As regards the matrix effect observed in the present study, it was absent only in the case of six analytes, and was very pronounced for TIL, SPI, ENR, DAN, MAR, NOR and SAR.

In summary, the present paper reports a method for the analysis of 41 antimicrobial agents in eggs, one that fulfils the requirements of EU Decision 657/2002 concerning accuracy, selectivity, sensitivity, detection and confirmation capabilities. Moreover, the method takes advantage of the automation of PLE and avoids a further clean-up step, thereby allowing the analysis of about 25 samples per day.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.01.021.

References

[1] Council Regulation 1831/2003/EC, Off. J. Eur. Commun., L 268 (2003) 29.
[2] R. Companyó, M. Granados, J. Guiteras, M. Prat, Anal. Bioanal. Chem. 395 (2009) 877.
[3] Commission Regulation 37/2010, Off. J. Eur. Commun., L 15 (2010) 1.
[4] C.A. Kan, M. Petz, J. Agric. Food Chem. 48 (2000) 6397.
[5] Codex Veterinary Drugs Residues in Food online database, http://www.codexalimentarius.net/vetdrugs/data/index.html?lang=en (accessed 29.09.2010).
[6] Council Decision 2002/657 from Directive 96/23, Off. J. Eur. Commun., L 221 (2002) 8.
[7] A.K. Malik, C. Blasco, Y. Pico, J. Chromatogr. A 1217 (2010) 4018.
[8] A. Kaufmann, P. Butcher, K. Maden, M. Widmer, J. Chromatogr. A 1194 (2008) 66.
[9] D. Ortelli, E. Cognard, P. Jan, P. Edder, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877 (2009) 2363.
[10] R.J. Peters, Y.J. Bolck, P. Rutgers, A.A. Stolker, M.W. Nielen, J. Chromatogr. A 1216 (2009) 8206.
[11] A.A. Stolker, P. Rutgers, E. Oosterink, J.J. Lasaroms, R.J. Peters, J.A. van Rhijn, M.W. Nielen, Anal. Bioanal. Chem. 391 (2008) 2309.
[12] M.G. Pikkemaat, P.P. Mulder, J.W. Elferink, A. de Cocq, M.W. Nielen, H.J. van Egmond, Food Addit. Contam. 24 (2007) 842.
[13] J.F. Huang, B. Lin, Q.W. Yu, Y.Q. Feng, Anal. Bioanal. Chem. 384 (2006) 1228.
[14] R.J. Maxwell, E. Cohen, D.J. Donoghue, J. Agric. Food Chem. 47 (1999) 1563.
[15] J.H. Shim, M.H. Lee, M.R. Kim, C.J. Lee, I.S. Kim, Biosci. Biotechnol. Biochem. 67 (2003) 1342.
[16] K.I. Nikolaidou, V.F. Samanidou, I.N. Papadoyannis, J. Liq. Chromatogr. Rel. Technol. 31 (2008) 2141.
[17] S. Bogialli, G. D'Ascenzo, A. Di Corcia, A. Lagana, G. Tramontana, J. Chromatogr. A 1216 (2009) 794.
[18] L. Chen, J. Liu, Q. Zeng, H. Wang, A. Yu, H. Zhang, L. Ding, J. Chromatogr. A 1216 (2009) 3710.
[19] M. Dubois, D. Fluchard, E. Sior, P. Delahaut, J. Chromatogr. B Biomed. Sci. Appl. 753 (2001) 189.
[20] A.F. Forti, G. Scortichini, Anal. Chim. Acta 637 (2009) 214.
[21] G. Hamscher, S. Limsuwan, N. Tansakul, M. Kietzmann, J. Agric. Food Chem. 54 (2006) 9017.
[22] H. Yan, F. Qiao, K.H. Row, Anal. Chem. 79 (2007) 8242.
[23] A. Garrido Frenich, M.d.M. Aguilera-Luiz, J.L. Martínez Vidal, R. Romero-González, Anal. Chim. Acta 661 (2010) 150.
[24] D.N. Heller, C.B. Nochetto, N.G. Rummel, M.H. Thomas, J. Agric. Food Chem. 54 (2006) 5267.
[25] M.M. Aerts, A.C. Hogenboom, U.A. Brinkman, J. Chromatogr. B Biomed. Appl. 667 (1995) 1.
[26] European Egg Processors Association, http://www.eepa.info (accessed 29.09.2010).
[27] Rapid Alert System for Food and Feed, http://ec.europa.eu/food/food/rapidalert/index_en.htm (accessed 29.09.2010).
[28] J. Chico, A. Rubies, F. Centrich, R. Companyo, M.D. Prat, M. Granados, J. Chromatogr. A 1213 (2008) 189.
[29] V. Jiménez, R. Companyó, J. Guiteras, submitted for publication.
[30] S. Herranz, M.C. Moreno-Bondi, M.D. Marazuela, J. Chromatogr. A 1140 (2007) 63.
[31] Guidelines for the implementation of Decision 2002/657/EC, http://ec.europa.eu/food/food/chemicalsafety/residues/cons_2004-2726rev2004_en.pdf (accessed 2029/2009/2010).
[32] A. Kaufmann, Anal. Chim. Acta 637 (2009) 144.
[33] A. Kaufmann, P. Butcher, K. Maden, M. Widmer, K. Giles, D. Uria, Rapid Commun. Mass Spectrom. 23 (2009) 985.