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Method Optimization and Performance Characteristics for the Determination of Twelve Veterinary Drugs in Food of Animal Origin Using Modified Quechers and LC-MS/MS QTRAP Detection

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Abstract

A sensitive, robust, and selective analytical method for instantaneous determination of (Chloramphenicol, Tetracyclines and sulfonamides) in Food of Animal Origin by cutting- edge LC-MS/MS QTRAP has been established and validated. Sample treatment was based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) with several adjustments depending on the matrix and drugs group, Double extractions using acetified acetonitrile with 1% acetic acid are common practice in sample preparation of Sulfonamides (SAs) and Chloramphenicol (CAP) while extraction for Tetracyclines (TCs) was by (98 % methanol / 2 % hydrochloric acid). Extracts were cleaned-up using PSA for the dispersive solid phase extraction step for interference removal. Various conditions such as MS compatible solutions, LC column, mobile phase and gradient elution were tested in order to save analysis time and for efficient chromatographic separation. Optimization of the various MS/MS experimental parameters (Declustering potential, collision cell exit potential, de-solvation temperature, gas flow and collision energy) was also carried out by full scan and selective reaction monitoring (SRM) with direct injection of single and mixed standard solutions. Matrix interferences were monitored in extracts of honey and chicken muscles for 12 antibiotics using recently developed and validated methods with Ultra-High LC-MS/MS QTRAP. Although a significant dispersion in the results were observed for most of the compounds, ion suppression was the major issue. The optimized method was validated, obtaining suitable results for all validation parameters in the evaluated matrices. Recovery values ranged from 70 % to 120 % meanwhile repeatability and reproducibility were obtained at values lower than 20 %. The limit of quantification (LOQ) was established at 0.20, 10 and 15 µg/kg for CAP, SAs and TCs respectively, which were lower than the maximum limit legally established by the European Union (EU). Reference materials (CRMs) and proficiency tests (PTs) samples were simultaneously performed to improve confidence in the measurement results. A full in-house validation of the method intended for routine analysis to support regulatory enforcement. Finally, the method was applied to 150 Samples from different matrices (Honey, chicken, Liver etc.).

Introduction

Animal breeding and agriculture have been chief human activities, but off late they have evolved into a significant economic activity and they have a relevant impact on food safety.

With an increasing interest developed towards maximizing the quantity of food product and concurrently reducing the cost. It is of utmost importance to cover the needs for food supplies of a growing world population, and to adhere with legal standards regarding contaminants and veterinary drugs used. With the advent of new practices in animal breeding which were designed by controlling various factors such as genetics, nutrition, health, management and the environmental conditions. During the last decades, a large number of veterinary drugs have been used at therapeutic levels in the systems of livestock breeding in order to improve animal health and prevent stress-induced animal death but also as growth promoters for intensive animal production [1]. Antibacterials (including sulfonamides, tetracyclines, beta-lactams, macrolides etc.) are widely used by farmers to be proactive and prevent bacterial infections [2,3]. Furthermore, other families of veterinary drugs, such as anthelmintic and coccidiostats, are used for the treatment of parasitic diseases and coccidiosis (an infectious disease caused by a microscopic protozoan parasite), respectively [4,5]. Their inappropriate use, non-respect of withdrawal periods, and cross-contamination can result in the presence of residues of veterinary drugs mainly antibiotic agents in food of animal origin. Non-altered parent compound may also be included in these residues as well as metabolites and/or conjugates. These may have direct toxic effects on consumers, e.g. allergic reactions in hypersensitive individuals. Furthermore, indirect effects of resistant strains of bacteria may result in development of bacterial resistance in humans [6-8]. Because of this, an increasing concern expressed for the safeguarding of the public health. To tackle this, issue several associations and international systems of legal control are working on the quality assurance and control of the animal products entering the food supply. In order to ensure food safety, several international organizations, such as European Union, have established Maximum Residue Limits (MRLs) of veterinary drugs in foodstuffs from animal origin [9]. Hence it is necessary to develop sensitive analytical methods that comply with the current legislation, allowing for the simultaneous determination of several classes of veterinary drugs in food. This will ensure the safety of the supplied products. Many analytical methods have been described in the literature for the determination of veterinary drugs in food. ELISA an Immuno chemical methods has been developed for the rapid determination of antibiotic residues [10,11]. Because such methods ae only capable of semi quantitative analysis there is the need for other techniques, such as Liquid Chromatography (LC) which have been coupled to different detection systems such as UV or diode array [12], fluorescence [13] or Mass Spectrometry (MS) [14] to increase the number of analytes determined simultaneously. LC coupled to tandem mass spectrometry (MS/MS) [15-16] has become one of the most promising techniques for the analysis of veterinary drugs in food products due to high sensitivity for the determination of banned substances that require low limits of detection as possible. The sensitivity can be increased when Ultra-High-Pressure Liquid Chromatography (UHPLC) is combined with tandem mass spectrometry (MS/MS), allowing a reduction of the chromatographic time as well [17,18]. The most difficult task for the determination of veterinary drugs in food products is the sample treatment, because drugs may bind to the lipoproteins and the extraction solvents form emulsions with the matrix [10]. One of the main objectives during the extraction procedure is the removal of lipids and proteins before the chromatographic analysis. To reduce the matrix effects as much as possible from the extraction step, a wide variety of sample preparations, such as liquid-liquid extraction, Solid Phase Extraction (SPE), Accelerated Solvent Extraction (ASE), matrix solid-phase dispersion, and dilute and shoot approaches, have been reported [19]. Nevertheless, the present method is easy and fast, fulfilling the need of developing analytical methods that allow the quantification of several veterinary drugs with high reproducibility and sensitivity. In the current research, veterinary drugs have been quantified by applying a new analytical method based on ultra-high performance liquid chromatography coupled with QTRAP mass spectrometry (LC-MS/MS QTRAP), which has been developed and validated in different matrices (pork, chicken and honey). Sample treatment was based on QUEChERS with several modifications depending on the matrix and type of drugs post evaluating their respective recoveries and precision.

Materials and Methods

The sample preparation procedures provided ensures reliable and highly sensitive results of SAs, CAP and TCs analysis. Honey samples should be stored in dark and dry places at ambient temperature (around 22°C ± 3) and in their original containers but other samples like chicken, meat and fish should be freezed in freezer at -20°C. Trials were done for SAs and CAP by the use of single extraction and double extraction with and without clean-up.

Single Extraction Method for SAs and CAP

Fresh or thawed samples were grinded with a homogenizer. The homogenized samples (2.0 ± 0.04 g) were weighed in 50 ml centrifuge tubes, dissolved with 8 ml De-ionized water (DIW). Samples should be mixed well and sonicated for 15 min at ultrasound bath and then diluted with 10 ml (0.1 % acetic acid in Acetonitrile), vortex for 1min. and then sonicated with ultrasound bath for 15 min. QUECHERS kits were added and tubes shaken slowly, centrifuged for 10 mins with 10000 r/min. Extractant was transferred to dispersive Solid Phase Extraction (SPD) tubes with

PSA for clean-up and interference removal. 4 ml extractant was transferred to glass tubes for evaporation at 40ºC. The dry residues were dissolved in 800 µl (10 % methanol, 0.1 % formic acid) and then filtered through 0.20 µm disposable syringe filter, transferred to HPLC vials 1 ml and samples were run with calibration standards on 6500 Q trap LC/MS-MS AB-SCIEX. Dilution factor for this method = 1.

Double Extraction Method for SAs and CAP

Fresh or thawed samples were grinded with a homogenizer. The homogenized samples (2.0 ± 0.04 g) were weighed in 50 ml centrifuge tubes, dissolved with 8 ml De-ionized water (DIW). Samples should be mixed well and sonicated for 15 min at ultrasound bath and then diluted with 10 ml (0.1 % acetic acid in Acetonitrile), vortex for 1min. and then sonicated with ultrasound bath for 15 min. QUECHERS kits were added and tubes shaken slowly, centrifuged for 10 mins with 10000 r/min. Another centrifuge tubes (2nd tube) prepared to transfer the supernatant from 1st tube to 2nd tube. Double extraction by transferring another 10 ml (0.1 % acetic acid in Acetonitrile) to the 1st tube, vortexed and centrifuged for 10min at 10000 r/min. Supernatant were transferred from 1st tube to 2nd tube. Extracts were cleaned-up using PSA for the dispersive solid phase extraction step for interference removal by transferring only 7 ml from extractant from 2nd tube to SPD tube then vortexed and centrifuged for 10 mins with 10000 r/min. 4 ml extractant were transferred to glass tubes for evaporation at 40ºC. The dry residues were dissolved in 800 µl (10 % methanol, 0.1 % formic acid) and then filtered through 0.20 µm disposable syringe filter, transferred to HPLC vials 1 ml and samples were run with calibration standards on 6500 Q trap LC/MS-MS AB-SCIEX.

Extraction Method for TCs

Fresh or thawed samples were grinded with a homogenizer. The homogenized samples (2.0 ± 0.04 g) were weighed in 50 ml centrifuge tubes then samples were diluted with 10 ml (2 % HCl in methanol), vortexed for 1min and then sonicated with ultrasound bath for 15 min. Samples were shaken slowly and then centrifuged for 10 mins with 10000 r/min. Supernatant was transferred to SPD tube to purify and clean up the samples from matrices after that SPD tubes were vortexed and centrifuged for 10 mins with 10000 r/min. Samples were filtered through 0.20 µm disposable syringe filter and transferred to HPLC vials 1 ml . Samples were run with calibration standards by 6500 Q trap LC/MS-MS AB-SCIEX.

Chemicals and Reagents

Analytical standards of Sulfacetamide (SMD), Sulfadiazine (SDZ), Sulfamerazine (SMZ), Sulfamethazine (SMT), Sulfamethoxazole (SMX), Sulfapyridine (SPD), Sulfathiazole (STZ), Chloramphenicol (CAP), Tetracycline (TC), Chlortetracycline (CTC), Oxytetracycline (OTC) And Doxycycline (DC) were obtained from Sigma-Aldrich, stored at room temperature as indicated in certificate.

Water used throughout the method is Milli pore, Milli-Q, Gulf scientific corporation. Methanol solvent with (Purity ≥ 99.9 %), acetonitrile solvent with (Purity ≥ 99.9 %), acetic acid, (Purity = 99 %) and formic acid, (purity 98 %), LC/MS grade were obtained from sigma Aldrich.

10 % methanol, 0.1 % formic acid; prepared by transferring 100 ml methanol, 1 ml formic acid to a 1 L volumetric flask, complete volume with water and mix well.

50 % methanol; prepared by transferring 50 ml methanol to a 100 ml volumetric flask, complete volume with milli pore water and mix well. Extraction solvent for SAs and CAP was 1 % acetic acid / Acetonitrile; prepared by transferring 10 ml acetic acid to a 1 L volumetric flask, bring to volume with acetonitrile and mix well.

Extraction solvent for TCs (2% Hydrochloric Acid / Methanol) was prepared by transferring 20 ml HCl to a 1 L volumetric flask, bring to volume with Methanol and mix well.

Mobile phase was composed of the following components, component A- 0.1 % formic acid in water; prepared by transferring 1ml formic acid to a 1 L volumetric flask and bring to volume with milli pore water and mix and component B- 0.1 % formic acid in methanol which was prepared by transferring 1 ml formic acid to a 1 L volumetric flask and bring to volume with methanol and mix, mobile phase degassed with ultrasonic bath to remove bubbles.

Stock solutions for SAs, CAP and TCs (1000 mg / L) were prepared individually by weighing 0.01 g to a 10 ml volumetric flask, dissolved in about 5 ml of (50 % methanol), bring to a volume and mix well. Flasks should be sonicated for 30 minutes to be well dissolved and stored at -20°C.

Three different mixed working solutions for SAs, CAP and TCs (100 mg / L) were prepared by diluting 1 ml of each stock solutions to a 10 ml volumetric flask with (50 % methanol), mixed well and stored at -20°C.

Three different mixed working solutions for SAs, CAP and TCs (10 mg / L) were prepared by diluting 1 ml of mixed working solutions for SAs, CAP and TCs (100 mg / L) to a 10 ml volumetric flask with (50 % methanol), mixed well and stored at -20°C.

Three different intermediate mixture of SAs, CAP and TCs standard solution (1 mg / L) were prepared by diluting 1 ml of 10 mg / L mixed working solution to a 10 ml volumetric flask with (50 % methanol), mixed well and stored at -20°C.

Multi-level calibration curve used for quantitation of SAs and TCs were prepared by serial dilution of six levels which are (1 µg / L, 5 µg / L, 10 µg / L, 25 µg / L, 50 µg / L and 100 µg / L) and was (0.1 µg/L, 0.5 µg/L, 1 µg/L, 5 µg/L, 10 µg/L and 20 µg/L) for CAP.

Apparatus and Software

Chromatographic analysis was performed using high performance liquid chromatography Agilent 1260 infinity series equipped with analytical HPLC column C18, Phenomenex (synergi 2.5 µ fusion-RP 100A, 50 × 2.00 mm 2.5 micron). Separation, identification and quantification of compounds were done by 6500 Q trap liquid chromatography - mass spectrometry applied bio systems (Q trap LC-MS/MS AB-SCIEX), equipped with Ion Source, Turbo V Ion drive and data station with ANALYST software and MULTIQUANT for quantitation.

Analytical balance, Mettler Toledo, 0.0001 g sensitivity up to 210 g. Refrigerator with temperature up to -20°C. Ultra sound bath (Sonicator), fisher brand, Germany. Turbo Vap, Biotage (controlled by Temperature from 10-100°C and N2 gas evaporator). N2 gas cylinder with 99.9995 % purity. Vortex mixer, model VM-1000, dig system lab. Instruments INC. Centrifuge, Beckman coulter, Allerga 64 R, (50 ml tube carriers and adaptors for 15 ml tubes with speed up to 10000 r/min. Centrifuge tubes, polypropylene, disposable, 15 ml and 50 ml. Syringe filters, PVDF, 0.20 µm, Agilent. Pipettes, transfer 5 ml and10 ml disposable polyethylene. Beakers (50 ml, 250 ml), Certified grade A. Volumetric flasks (5 ml, 10 ml, 1 L), Certified grade A. QUECHERS extract tubes, EN method, Agilent, USA. QUECHERS dispersive solid phase dispersion EMR-Lipids (dSPE) 15 ml, Lipids, Agilent, USA. Glass tubes bottom type-round 12 × 100 mm. Micropipettes 20 µl, one from 20-200 µl and other one from 100-1000 µl, Brand, Germany. Pipette tips for 100-1250 µl pipettes and for 20-200 µl pipettes.

Results and Discussion

LC-MS/MS QTRAP optimization

The applied methodology consisted of liquid chromatography coupled to a QTRAP mass spectrometry. For the MS characterization of the studied analytes, an aliquot of each compound (10 µg/L) was analyzed by UHPLC. In previous studies [20,21], a comparison between the ESI and APCI sources was performed in several complex matrices. One of them concluded that APCI showed better results [21], meanwhile the other one indicated that ESI provides better sensitivity [20]. Therefore, in this study the analytes were monitored by ESI in both ionization modes, positive and negative. All the compounds were monitored in positive Electrospray Ionization (ESI+), meanwhile CAP could also be monitored in negative Electrospray Ionization (ESI-). Nevertheless, the identification of the studied compounds was carried out in ESI+ because better sensitivity and reduce mass error were achieved for most of the analytes, meanwhile to achieve maximum sensitivity for all compounds, MS/MS parameters (Declustering potential, collision cell exit potential, de-solvation temperature, and gas flow and collision energy) were optimized by direct infusion of standard solutions into the MS.

The precursor ion and their product ions were selected for each analyte. (Table 1) presents the m/z ion transition monitored for SAs, CAP and TCs and the associated parameters. The use of an acidic mobile phase adjusted with 0.1% formic acid promoted positive ionization, which improved the detection of most compounds since only chloramphenicol is negatively ionized. Ion source parameters set to be fit for our purpose and these parameters as in the (Table 2). For the optimization of the LC method, the initial chromatographic conditions were adopted from a previous study. Then, in order to improve the peak form and sensitivity, different parameters were evaluated: gradient mode (different percentage of mobile phase), mobile phases 0.1% (v/v) formic acid and ammonium format (5 mM) in methanol, 0.1% (v/v) formic acid and ammonium format (5 mM) in acetonitrile as well as methanol or acetonitrile as the organic phase), but no significant differences were observed, and the conditions described in Section 4 were used for further experiments. Moreover, two chromatographic columns Zorbax Eclipse Plus C18 column (100 mm × 2.1 mm, 1.8 "m particle size) and HPLC column C18, Phenomenex (synergi 2.5 µ fusion-RP 100A, 50 × 2.00 mm 2.5 micron) were tested, and the, Phenomenex provided better peak shape than Zorbax and it was used for further experiments, gradient program described in (Table 3).

Parameter	Q1 mass (Da)	Q3 mass (Da)	Time (m.sec)	Decluttering potential (DP)	Collision energy (CE)	exit potential (CXP)
Sulfacetamide (1)	215.10	156.10	200	75	15	15
Sulfacetamide (2)	215.10	108	200	75	25	15
Sulfadiazine (1)	251.10	156	200	95	20	15
Sulfadiazine (2)	251.10	108	200	95	30	15
Sulfamerazine (1)	265.10	108	200	75	35	12
Sulfamerazine (2)	265.10	92	200	75	35	12
Sulfamethazine (1)	279.20	92.10	200	90	35	12
Sulfamethazine (2)	279.20	108	200	90	30	12
Sulfamethoxazole (1)	254.10	156.10	200	70	20	12
Sulfamethoxazole (2)	254.10	108	200	70	30	12
Sulfapyridine (1)	250.10	156.10	200	75	25	12
Sulfapyridine (2)	250.10	108	200	75	35	12
Sulfathiazole (1)	256.10	156.10	200	75	15	12
Sulfathiazole (2)	256.10	108	200	75	25	12
Chloramphenicol (1)	321.10	152.0	200	-81	-35	-10
Chloramphenicol (2)	321.10	121.0	200	-81	-35	-10
Tetracycline (1)	445.20	410	200	80	25	12
Tetracycline (2)	445.20	154	200	80	35	12
Chlortetracycline (1)	479.30	444	200	80	30	12
Chlortetracycline (2)	479.30	303	200	80	55	12
Oxytetracycline (1)	461.20	426.10	200	70	30	12
Oxytetracycline (2)	461.20	201	200	70	50	12
Doxycycline (1)	445.20	428.30	200	85	25	12
Doxycycline (2)	445.20	149.10	200	85	55	12

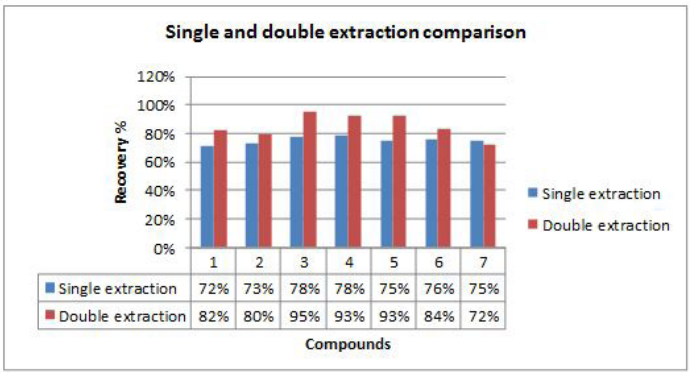
Table 1: MRM acquisition conditions for each antibiotic.

Source parameters	Setting for SAs, CAP and TCs
Curtain gas (CUR)	35
Collision gas (CAD)	High
Ion spray voltage (IS)	450
Temperature	450°C
Ion source gas 1 (GS1)	40
Ion source gas 2 (GS2)	60

Table 2: Q trap 6500 ion source parameters. 4.2 Evaluation of the Extraction Procedure

Tissue samples are complex matrices, so the optimization of the extraction method is an important issue for the correct identification of antibiotics.

In the present study, QuEChERS method, single and double extraction steps were evaluated in the chicken as a represented matrix. In order to compare these methods a 3 replicates of blank samples of chicken were spiked at 50 µg/kg and analyzed using the two procedures described in Section 3 (avoiding the clean-up step). The results in (Figure 1) showed the average recoveries of replicates with single and double extraction. The recoveries obtained when a single extraction method was applied ranged from 72 to 78 %. On the other hand, when the QuEChERS method was applied with double extraction, recovery values ranged from 72 to 95 %. Thus, QuEChERS with double extraction approach provided better results than QuEChERS with single extraction approach in most of the target compounds and it was selected for further experiments. Due to the complexity of the matrix, a clean-up step was necessary to improve the sensitivity of the proposed method and minimize matrix effect. All of these aspects must be taken into account when selecting the appropriate SPE cartridge, especially as it can be difficult to find one with multi-class selectivity. The use of dispersive SPE containing PSA can strongly interact with acid compounds and remove various co-extractive interferences such as polar organic acids, sugars and fatty acids however it may also interact with target compounds and cause the loss of analytes. Addition of 1% acetic acid on acetonitrile impedes the performance of PSA in the clean-up step preventing the loss of analytes. 1% acetic acid in acetonitrile was an effective extraction solvent.



	1	2	3	4	5	6	7
Single extraction	72%	73%	78%	78%	75%	76%	75%
Double extraction	82%	80%	95%	93%	93%	84%	72%

Figure 1: Comparison between single and double extraction.

Matrix Effect and Selectivity Study

The purpose of matrix effect study is to understand the behavior of analytes in various matrices and to optimize a calibration curve that matches the behavior of the analytes in the respective matrix. In order to quantify the three selected matrices, the evaluation of matrix effect were studied for three different matrices (Honey, Chicken and Pork) for antibiotics and compared with solvent calibration curve. To calculate the matrix effect, the slopes of solvent calibration and the matrix matched calibration were compared (matrix/solvent) using response (area) at the same concentrations from (0.1 to 20 µg/L) for CAP and (1 to 100 µg/L) for SAs and TCs. The calibration for each matrix was based on the matrix itself. A tolerable signal variation between the ratios of both slopes is adequate when the matrix effect ranged from (0.80 to 1.20). The results obtained for CAP ranged from (1.13 to 1.22) for Honey matrix, (0.19 to 0.71) for Chicken matrix and (0.47-0.53) for Pork matrix. Therefore, the results show a significant matrix effect (suppression) for chicken and pork matrices but for honey matrix, this could be considered as negligible. It is a significant matter due to the possibility of performing the quantification of honey samples using solvent calibration instead of matrix-matched calibration. In this case, either pork or chicken can be used as the representative matrix for improving sample throughput in this case for samples other than honey as shown in the (figure 2).

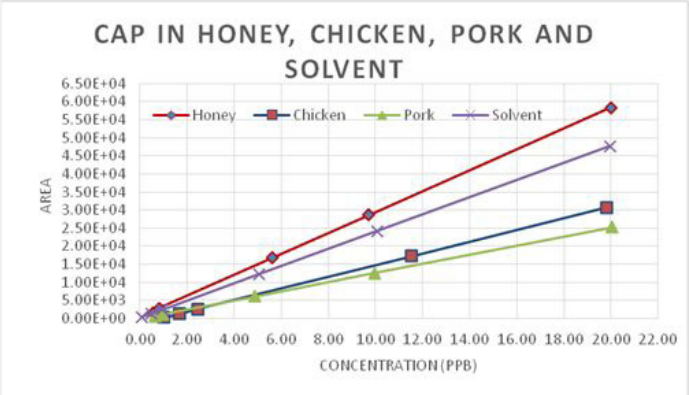
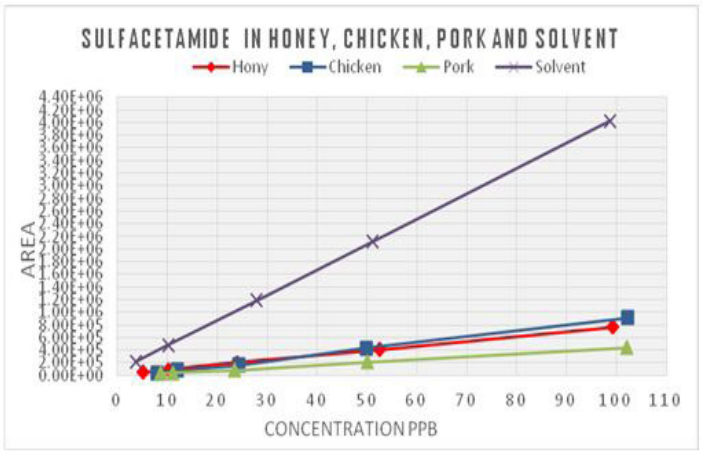
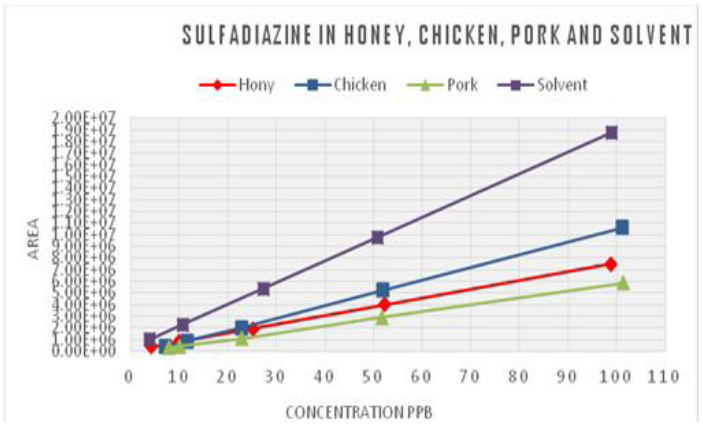


Figure 2: Slopes of solvent calibration comparing with the matrix-matched calibration curve (matrix/solvent) for Chloramphenicol.

In the same way the following results for SAs were obtained, matrix effect ranged from (0.17 to 0.68) for Honey matrix, (0.17 to 0.91) for Chicken matrix and (0.07-0.47) for Pork matrix. From the above data, the conclusion is the matrix effect behavior is similar for honey and chicken. There is a significant matrix effect (High Suppression) especially in lower concentration. In the pork matrix, the behavior of matrix effect is slightly different (High Suppression) in the entire quantitation range of the Lower Calibration Level (LCL) to the Higher Calibration Level (HCL) as in the (figures 3-5) as example.





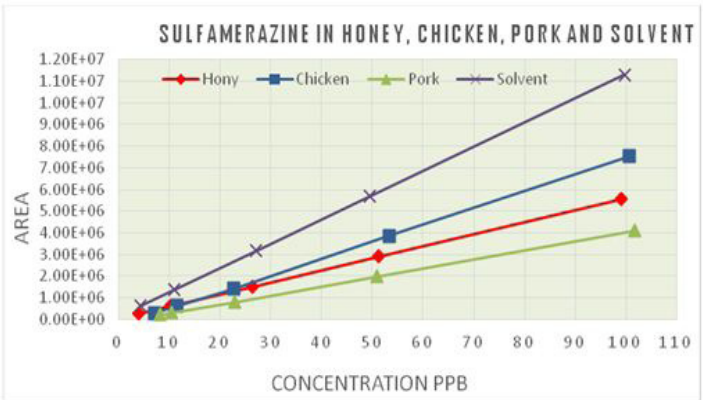
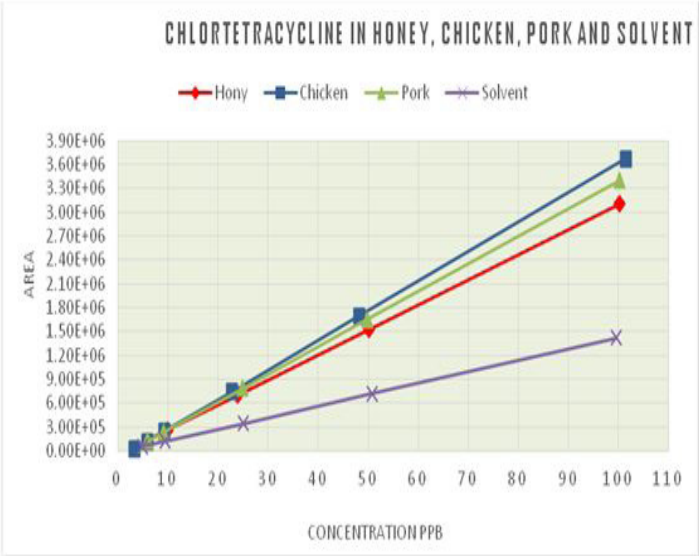


Figure 3-5: Slopes of solvent calibration comparing with the entire quantitation range of the Lower Calibration Level (LCL) to the Higher Calibration Level (HCL).

Therefore, we can conclude that for SAs during quantitation it is required one specific matching calibration curve for each individual matrix.

Similarly, for the tetracycline compounds tetracycline, chlortetracycline and oxy-tetracycline have same behavior. They have high enhancement in all three matrices ranged from (1.23 to 2.59). However, for doxycycline there is a slight suppression ranged from (0.66 to 0.77) in the honey matrix for the whole quantitation range of LCL to HCL. For chicken and pork, it shows a significant enhancement from (1.34 to 3.13) in both matrices as shown in (figures 6-9).



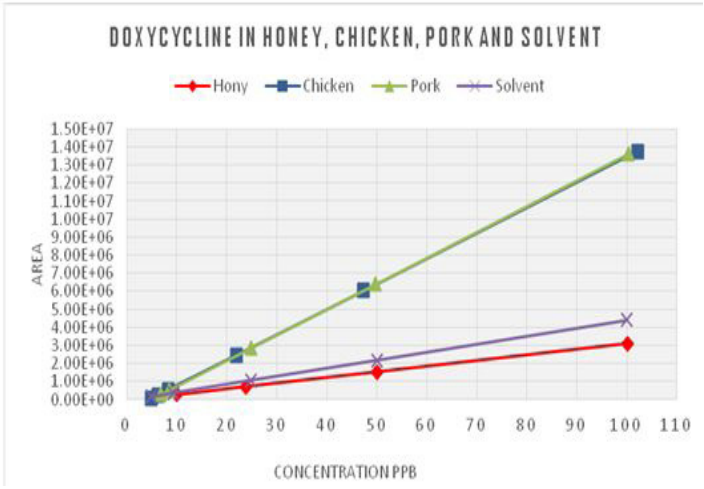


Figure 6-9: Slopes of solvent calibration comparing with the honey matrix for the whole quantitation range of LCL to HCL. For chicken and pork.

From the data obtained we can conclude that honey requires one specific matching calibration curve while chicken and pork have similar behavior therefore during quantitation we can use one representative matrix for both chicken and pork for matching calibration curve.

Method Validation

Once the optimization procedure was carried out, a validation protocol was performed in the selected matrices. SANTE guidelines [23] and Eurachem guide [24] have been used for the validation procedure. Some parameters were evaluated to ensure the suitable identification and quantification of the analytes such as linearity, specificity, recovery, repeatability, reproducibility, CCα, CCβ and LOQs. The evaluation of the linearity was achieved in the range from (10 to 200 µg /kg) for most of the target compounds in the three matrices, except for CAP as it was from (0.2 to 20 µg/kg). The determination coefficients (R2) were higher than 0.99 for all the compounds in the three selected matrices. Deviation of the residuals was always lower than 20%. Specificity was established by analyzing 6 control blank samples for each of the three matrices. The absence of any signal at the same retention time as the target compounds suggested that there were no matrix interferences that may give a false positive signal.

In order to quantify the three selected matrices with just one calibration curve in routine analysis, the recoveries obtained from the different matrices when the calibration curve of each matrix was used, were compared. Adequate recoveries between (70-20 %) at five different concentration levels (0.5 to 20 µg/L) for CAP and (5 to 100 µg/L) for SAs and TCs, shown in (Table 4). As it can be observed, the number of compounds with adequate recovery values, when the chicken and pork calibration curves were applied, were similar, especially at HCL. However, this does not happen when the spiked samples were quantified with the honey calibration curve. This may be due to the classification of the chicken and the pork as white meat while the honey is classified as sugar based matrix. Thus, for routine analysis only one calibration curve would be necessary to analyze specified veterinary drugs in white meat. However, more research is needed to confirm this conclusion and to evaluate the variation between samples from the same type of matrices. Bearing in mind both matrix effect and recoveries comparison using different calibration curves; also in order to minimize the variability between samples, matrix-matched calibration was used for the three matrices evaluated.

Compound	Recovery (%)				
	Level (1)	Level (2)	Level (3)	Level (4)	Level (5)
Honey					
Chloramphenicol	78	93	106	99	100
Sulfacetamide	70	90	97	112	97
Sulfadiazine	99	108	87	107	99
Sulfamerazine	113	107	88	103	100
Sulfamethazine	114	108	89	99	101
Sulfamethoxazole	109	105	86	101	101
Sulfapyridine	113	113	90	101	100
Sulfathiazole	110	107	88	106	99
Tetracycline	85	107	113	91	101
Chlortetracycline	106	94	99	93	88
Oxytetracycline	90	110	114	88	102

Doxycycline	79	91	118	102	99
Chicken					
Chloramphenicol	82	120	72	89	104
Sulfacetamide	93	112	70	117	97
Sulfadiazine	118	112	71	93	103
Sulfamerazine	118	118	74	92	104
Sulfamethazine	117	113	71	91	104
Sulfamethoxazole	120	117	72	93	103
Sulfapyridine	110	114	72	92	103
Sulfathiazole	109	98	70	99	102
Tetracycline	74	105	109	104	99
Chlortetracycline	82	88	105	103	99
Oxytetracycline	79	83	120	109	97
Doxycycline	91	90	100	103	99
Pork					
Chloramphenicol	70	109	115	95	100
Sulfacetamide	73	110	114	99	98
Sulfadiazine	84	118	110	91	100
Sulfamerazine	78	114	114	93	100
Sulfamethazine	75	109	116	94	100
Sulfamethoxazole	79	112	116	90	101
Sulfapyridine	79	114	112	86	100
Sulfathiazole	78	115	117	89	100
Tetracycline	74	100	101	110	97
Chlortetracycline	101	105	90	104	100
Oxytetracycline	80	98	87	114	97
Doxycycline	95	106	101	98	100

Table 4: Recovery recorded for 3 different matrices (Chicken, honey and pork) after fortification with 5 calibration levels: for SAs and TCs (5. 10, 25, 50 and 100 µg/kg) and for CAP (0.5, 1, 5, 10 and 20 µg/kg).Cs (5. 10, 25, 50 and 100 µg/kg) and for CAP (0.5, 1, 5, 10 and 20 µg/kg).

Recoveries ranged from 93 to 103 % with Relative standard deviation (RSD) values ≤ to 10 % for repeatability and < 20 % for reproducibility when honey matrix was tested, recovery values obtained for the chicken matrix ranged from 93 to 101 %, RSD < 20 % for repeatability and ≤ 20 % for reproducibility. In case of pork matrix, recovery values ranged from 95 to 101 %, RSD < 20 % for repeatability and < 20 % for reproducibility as shown in (Table 3). The use of the internal standard is not necessary due to the suitable obtained results, simplifying the proposed method. For most of the compounds, the LOQ value was established at 10 µg/kg, except for CAP at 0.20 µg/kg because European Union decision 2003/181/EC [22] stated that CAP has been prohibited in food of animal origin due to health concern, a legislative minimum required performance level (MRPL) of 0.30 µg/kg has been issued for CAP which means all methods used in the analysis of this compound should be able to, at least, achieve this level. CCα values was ≤ 120 µg/kg for SAs and TCs in case of all matrices but for CAP was 0.25 µg/kg. The CCβ values obtained in studied matrices was ≤ 145 µg/kg for SAs and TCs but for CAP was 0.30 µg/kg which is the MRPL permitted.

Matrix	Compounds	Mean recovery (%)	Repeatability RSD (%)	Reproducibility RSD (%)
Honey	Chloramphenicol	95	7	11
	Sulfacetamide	93	7	16
	Sulfadiazine	100	10	8
	Sulfamerazine	102	6	9
	Sulfamethazine	102	7	9
	Sulfamethoxazole	100	6	9
	Sulfapyridine	103	9	9
	Sulfathiazole	102	7	9
	Tetracycline	99	7	12
	Chlortetracycline	96	10	7
	Oxytetracycline	101	6	12
	Doxycycline	98	7	15
Chicken	Chloramphenicol	93	13	20
	Sulfacetamide	98	10	19
	Sulfadiazine	99	12	19
	Sulfamerazine	101	17	18
	Sulfamethazine	99	18	19
	Sulfamethoxazole	101	12	19
	Sulfapyridine	98	19	17
	Sulfathiazole	96	13	16
	Tetracycline	98	10	14
	Chlortetracycline	95	12	10
	Oxytetracycline	98	17	18
	Doxycycline	97	18	6
Pork	Chloramphenicol	98	12	18
	Sulfacetamide	99	10	16
	Sulfadiazine	101	11	14
	Sulfamerazine	100	16	15
	Sulfamethazine	99	15	16
	Sulfamethoxazole	100	12	15
	Sulfapyridine	98	18	16
	Sulfathiazole	100	14	17
	Tetracycline	96	11	14
	Chlortetracycline	100	12	6
	Oxytetracycline	95	15	14
	Doxycycline	100	19	4

Table 5: Recoveries & RSD for repeatability and reproducibility. 4.5

Sample Analysis

The developed methods were applied to the analysis of 150 Samples of different matrices (Honey, chicken, Liver etc.) collected from different markets. Among the 150 Samples, 43 honey, 90 chicken, 7 duck and 10 Liver samples were analyzed. In order to eliminate false positive and ensure that the system is under Control, an internal quality control was applied during the analysis. Hence, a matrix-matched calibration, two blank samples spiked at 10 and 50 µg/kg, blank control samples and a reagent blank (aliquot of solvents without samples) were evaluated each day of analysis. No antibiotics detected for 120 samples, antibiotics were identified in twenty samples, 6 honey samples are rejected 3 as SAs and 3 as CAP, the concentrations of the rejected compounds were (3.07, 0.85, 1.33µg/kg) for CAP, 123.91, 119.43 and 127.54 µg/kg for total SAs in honey matrix, that was more than the rejected limit (MRL).

Conclusion

The proposed methodology was optimized and validated in three different samples (chicken, pork and honey matrices), noting that QuEChERS with double extraction procedure provides suitable recoveries, applying a clean-up step. Suitable validation parameters were obtained achieving LOQs ranging at 10 µg/kg for most of the compounds except for CAP was 0.20 µg/kg. To prove the applicability of the present method, 150 real samples were analyzed. Among all the samples, no antibiotic detected for 120 samples and four antibiotics were identified in thirty different samples, four honey samples are rejected 3 as sulfonamides and one as chloramphenicol. It is important to highlight the need of quantifying the antibiotic in samples of animals origin in order to know if there are any of these compounds and, therefore, to develop sensitive analytical methods for their determination in food samples of animal origin. Finally, due to the versatility and simplicity of the proposed procedure, this methodology can be implemented in analytical laboratories for routine analysis of the target compounds.

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