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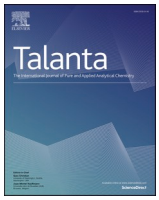

Comparison of conventional and dispersive solid phase extraction clean-up approaches for the simultaneous analysis of tetracyclines and sulfonamides in a variety of fresh vegetables

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ABSTRACT

The extensive use of antibiotics in agriculture has led to the occurrence of residual drugs in different vegetables frequently consumed by humans. This could pose a potential threat to human health, not only because of the possible effects after ingestion but also because the transmission of antibiotic-resistant genes could occur. In this work, two accurate sample preparation procedures were developed and validated for the simultaneous analysis of sulfonamides (SAs) and tetracyclines (TCs) in four of the most widely consumed vegetables (lettuce, onion, tomato, and carrot) in Europe. The evaluated protocols were based on QuECHERS for extraction and subsequent clean-up by SPE (solid phase extraction) or dispersive SPE. Parameters affecting both extraction and clean-up were carefully evaluated and selected for accuracy of results and minimal matrix effect. Overall, apparent recoveries were above 70% for most of the target analytes with both analytical procedures, and adequate precision (RSD<30%) was obtained for all the matrices. The procedural limits of quantification (LOQ_{PRO}) values for SPE clean-up remained below 4.4 μg kg^{-1} for TCs in all vegetables except for chlortetracycline (CTC) in lettuce (11.3 μg kg^{-1}) and 3.0 μg kg^{-1} for SAs, with the exception of sulfadiazine (SDZ) in onion (3.9 μg kg^{-1}) and sulfathiazole (STZ) in carrot (5.0 μg kg^{-1}). Lower LOQ_{PRO} values (0.1–3.7 μg kg^{-1}) were obtained, in general, when dSPE clean-up was employed. Both methods were applied to twenty-five market vegetable samples from ecological and conventional agriculture and only sulfamethazine (SMZ) and sulfapyridine (SPD) were detected in lettuce at 1.2 μg kg^{-1} and 0.5 μg kg^{-1}, respectively.

1. Introduction

Despite the vital importance of antibiotics in improving human welfare, in recent decades there has been great concern about the presence of antibiotics in the environment and the rise of antibiotic-resistant genes, which are closely related to the misuse of these drugs in recent years [1,2]. One of the misuses of antibiotics is related to the massive use in the livestock industry [3–7]. Although the use of antibiotics as growth promoters was banned in the European Union in 2006 [8], there are still many countries where antibiotic uses on animals are presently not regulated.

Once ingested, antibiotics cannot be effectively metabolised by livestock and therefore 30–90% of the administered drug is excreted to the environment through the faeces and urine [5,9–11] as the main antibiotic compound or as active/non-active metabolites [2–4,12–15]. The presence of antibiotic residues in livestock manure has been recurrently reported [2,11,16], especially the presence of tetracyclines (TCs) and sulfonamides (SAs), as they are the first and third most commonly employed antibiotic families, respectively, in veterinary therapies [3,17,18]. Since manure and slurry from treated livestock are frequently used as land fertilisers [5,9,12], antibiotics can enter the food chain through their accumulation in plants [4,19–22], mainly in roots

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and leaves [11,23]. Boxall et al. [19] demonstrated that after the application of veterinary medicines to soils at environmental concentrations, antibiotics were taken up at detectable levels in lettuce and carrot, whereas He et al. [5] detected oxytetracycline in cucumber and cabbage at 30.4 μg kg^{-1} and 126 μg kg^{-1} concentration levels, respectively.

The accumulation of antibiotics in plants directly affects their growth and safety [24] and may even be hazardous to human health through the food chain [4,9,10,25]. Long-term ingestion of antibiotic residues in food could lead to toxicity and other side effects [26], such as anaphylaxis, carcinogenesis, teratogenesis, and mutagenesis. In addition, drug accumulation may also increase the drug tolerance of pathogenic bacteria [6]. Therefore, the occurrence of antibiotic residues in vegetables has come under the spotlight of researchers.

The different physicochemical properties of the various antibiotic families, together with the complexity of vegetable matrices (pigments, fat, cellulose and wax constituents), difficult the development of sensitive and accurate analytical methods [4,5,10,12]. In addition, some antibiotics have shown to bind with silanol groups on glass surfaces [11,27,28] or to undergo epimerisation processes, especially in the case of TCs [29,30], which directly affects the sensitivity of the method. Therefore, the current problem establishes the need to develop accurate analytical methods for the quantification of antibiotics in foodstuff to ensure food safety and public health [16].

The literature gathers a variety of procedures to extract antibiotics from vegetable matrices, including extraction using pressurized liquid extraction (PLE), solid phase micro-extraction (SPME), stir-bar sorptive extraction (SBSE), or ultrasound-assisted extraction (UAE) [4,11,12,31]. However, the use of QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) is gaining attention since its first application by Anastassiades and co-workers in 2003 on watery vegetables for the determination of pesticides [32–34]. The procedure consists of an initial salting-out extraction promoted by the addition of salts such as sodium chloride (NaCl) and anhydrous magnesium sulphate ($MgSO_4$) as dehydrating agent, traditionally in a 4:1 (w/w) ratio [32]. Nonetheless, scientific works have justified the loss of TCs during the sample extraction procedure by the tendency of TCs to form chelate complexes with divalent metal ions like Mg^{2+} [11,28,29], potentially diminishing the extraction efficiency. The use of anhydrous sodium sulphate (Na_2SO_4) has been considered as a possible alternative to avoid the problem; however, most of the already developed methods applied the traditional QuEChERS salt packets including $MgSO_4$, with few exceptions [4]. Hence, to our knowledge, there is not any study that evaluates and compares the effectiveness of both salts in the extraction.

Regardless of the extraction approach used, a clean-up step of the extract is often required to eliminate potential interferences. Solid-phase extraction (SPE) protocols are the most commonly employed ones, using different purification sorbents. The efficiency of several sorbent materials (i.e., hydrophilic-lipophilic balance (HLB), C_{18} bonded silica, and NH_2) was evaluated by Feng et al. and based on their observations HLB cartridges provided the most efficient clean-up of the vegetable samples [12] for the analysis of a wide range of acidic, basic and neutral compounds [35,36].

When QuEChERS approach is used, the subsequent clean-up of the extract is often performed via dispersive SPE (dSPE). Common sorbents for the dSPE step include primary secondary amine (PSA), C_{18}, and graphitized carbon black (GCB) [4,37]. PSA helps to remove various polar interferences [37]; nevertheless, it has been reported to provide problems with carotenoid- and chlorophyll-rich samples [6]. For example, He et al. reported decreased TC recoveries when high amounts (25–50 mg) of PSA were used, due to strong adsorption to the sorbent [5]. C_{18} aids the removal of non-polar substances, such as lipids; and GCB removes sterols and pigments, such as the aforementioned chlorophyll [6,37], and could therefore potentially be used for the purification of antibiotics in vegetables.

Within this context, the present work aimed to develop an accurate analytical method consisting of a QuEChERS-based extraction and ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) detection for the simultaneous analysis of four TCs and five SAs in four vegetables (lettuce, onion, tomato, and carrot) usually consumed raw, which is relevant in the context of antibiotic-resistant gene transmission. The dSPE and SPE clean-up strategies were evaluated and for the first time compared, as well as the different parameters involved in the analyte recovery, including the efficiency of extraction salts or the target compound losses occurring during the evaporation step due to adsorption on glass surfaces and epimerisation. Both optimised protocols were employed for the analysis of vegetable samples from conventional and ecological agriculture.

2. Experimental procedure

2.1. Reagents and materials

The distributor and specific physicochemical properties of the nine antibiotics are gathered in Table S1. In the case of the labelled compounds used as surrogates, ([$^2H_4$]-sulfamethazine ([$^2H_4$]-SMZ (99%)) and [$^{13}C_6$]-sulfamethoxazole ([$^{13}C_6$]-SMX (100%)) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), whereas [$^2H_6$]-tetracycline ([$^2H_6$]-TC (80%)) was acquired from Toronto Research Chemicals (Toronto, Ontario, Canada). Individual solutions for all of them were monthly prepared at 1000 mg kg^{-1} in UHPLC-quality methanol (MeOH, 99.9%, Scharlau, Sentmenat, Catalonia, Spain) or acetonitrile (ACN, 99.9%, Avantor Performance Materials, Gliwice, Silesia, Poland) in the case of [$^2H_4$]-SMZ and [$^2H_6$]-TC. Further combined dilutions were weekly prepared in ACN at 100 mg kg^{-1} and 5 mg kg^{-1} for sample fortification. Most concentrated solutions (1000 mg kg^{-1} and 100 mg kg^{-1}) were stored at −20 °C, while the most diluted ones were kept at 4 °C.

NaCl (100%) and disodium ethylenediaminetetracetate dihydrate (Na_2EDTA, 100%) salts were obtained from PanReac AppliChem (Castellar del Vallés, Catalonia, Spain), anhydrous citric acid H_3Cit (99.5%) and anhydrous Na_2HPO_4 (98%) from Scharlau, anhydrous Na_2SO_4 (99%) from Merck (Darmstadt, Hesse, Germany) and anhydrous $MgSO_4$ (99.5%) from Alfa Aesar (Kandel, Rhineland-Palatinate, Germany). Extractants included UHPLC-grade MeOH and ACN, ultra-pure water (Milli-Q water purification system, model 185, <0.05 μS/cm, Millipore, Bedford, Massachusetts, USA) and a citrate buffer consisting of an aqueous solution of anhydrous NaH_2Cit (99%) and $Na_2HCit{\cdot}1.5H_2O$ (99%) (Honeywell Fluka, Charlotte, North Carolina, USA). Concerning the clean-up, PSA, Bondesil-C_{18} (40 μm, Agilent Technologies, Santa Clara, CA, EEUU) and GCB (37–125 μm, Superclean ENVI-Carb, Merck) sorbents and Oasis HLB cartridges (200 and 500 mg, 6 cc, 30 μm) purchased from Waters (Milford, Massachusetts, USA) were employed. Oxalic acid (100%, Merck) and formic acid (HCOOH, 98%, PanReac AppliChem) were used in the final extract reconstitution. UHPLC quality water (Optima LC-MS, Fisher Scientific, Waltham, Massachusetts, USA) was used for mobile phase preparation.

In total, twelve fresh lettuces, two onions, two tomatoes and nine carrots from ecological and non-ecological agriculture were acquired in local markets and department stores. Along sample preparation, the Multi Reax shaker by Heidolph (Schwabach, Bavaria, Germany) and a 5840R centrifuge by Eppendorf (San Sebastián de Los Reyes, Madrid, Spain) were used.

2.2. Cleaning procedure

New glass test tubes were heated at 350 °C for an hour in an HD-230 muffle furnace by Hobersal (Caldes de Monbui, Catalonia, Spain). Once room temperature was reached, they were washed with an aqueous solution of 5% (w/w) Na_2EDTA in an ultrasound bath (J.P.SELECTA, Abrera, Catalonia, Spain) to avoid the possible interaction of the target analytes with the silanol groups on the glass surface [11,27,28], then,

rinsed with Milli-Q water to remove Na_2EDTA residue, and further ultrasonicated with Milli-Q water. Finally, test tubes were dried at 100 °C. Ceramic homogenisers were ultrasonicated with Milli-Q water and dichloromethane subsequently. Eventually, they were heated at 350 °C for an hour before use.

2.3. Sample pre-treatment and extraction

The different conditions tested during the optimisation of the extraction step are summarized in Table 1. Under optimal conditions, 10 g of fresh, crushed and homogenised vegetable (lettuce, onion, tomato or carrot) samples were weighed in a 50 mL polypropylene centrifuge tube. Samples were fortified with 200 μL of a 5 mg kg^{-1} stock solution containing the surrogate compounds. Afterwards, samples were vortexed (2000 cycles·min^{-1}, 10 min) and kept in the darkness for 30 min at room temperature. ACN (10 mL), a ceramic homogeniser and the salts (4 g anhydrous Na_2SO_4, 1 g NaCl, 0.5 g anhydrous H_3Cit and 0.049 g anhydrous Na_2HPO_4) were added. The mixture was then shaken manually and degasified by opening the centrifuge tube, until no gas was released. All samples were vortexed (2000 cycles·min^{-1}, 8 min) and centrifuged (4000 rpm, 5 min) at 10–15 °C.

2.4. Clean-up

2.4.1. SPE

For SPE clean-up optimisation, different amounts and sorbents of the SPE cartridges (100 mg PSA, 100 mg C_{18} with or without 50 mg GCB and 200 mg or 500 mg Oasis HLB sorbents), sample loading volume and elution volume were studied. Under optimal conditions, a representative aliquot of 1 mL of the supernatant was isolated, diluted with 20 mL of citrate buffer (0.05 mol L^{-1}, pH 4) and loaded to the previously conditioned (using 10 mL of ACN, 10 mL of Milli-Q water and 10 mL of citrate buffer) 500-mg Oasis HLB SPE cartridges. The cartridges were washed with 5 mL of water and dried under vacuum. The elution was carried out using 9 mL of ACN and the extracts were evaporated to 1 mL in a nitrogen-flow TurboVap LV evaporator device (Caliper Life Sciences, Hopkinton, Massachusetts, USA). Aliquots of 125 μL were reconstituted in 250 μL of a 1:1 (v/v) ACN:oxalic acid (aq., 0.01 mol L^{-1}, pH 2) and filtered through 0.22 μm polypropylene filters (Clarify-PP, Phenomenex, Torrance, California, USA) before UHPLC- MS/MS analysis.

Table 1
Combinations of salts and extractants evaluated for the QuEChERS extraction.

Condition No.	Salts	Extractant	Reference(s)
1	4 g anhydrous $MgSO_4$ 1 g NaCl 0.5 g anhydrous H_3Cit 0.049 g anhydrous Na_2HPO_4	ACN	[10,38]
2	4 g anhydrous Na_2SO_4 1 g NaCl 0.5 g anhydrous H_3Cit 0.049 g anhydrous Na_2HPO_4	ACN	[4,10,38] (adapted)
3	4 g anhydrous $MgSO_4$ 1 g NaCl 0.5 g anhydrous H_3Cit 0.049 g anhydrous Na_2HPO_4	MeOH:citrate buffer (0.05 mol L^{-1}, pH 4) (1:1, v/v)	[10,38] (adapted)
4	4 g anhydrous $MgSO_4$ 1 g NaCl 0.5 g anhydrous H_3Cit 0.049 g anhydrous Na_2HPO_4	ACN:citrate buffer (0.05 mol L^{-1}, pH 4) (1:1, v/v)	[10,38] (adapted)

2.4.2. dSPE

In the case of the dSPE clean-up strategy, two sorbent combinations were evaluated, PSA (10 mg) and C_{18} (25 mg), with or without GCB (2.5 mg) addition, together with 150 mg anhydrous Na_2SO_4 in all the cases. Under optimal conditions, an aliquot of 1 mL of the extractant was transferred to a 50 mL centrifuge tube containing 10 mg PSA, 25 mg C_{18} and 150 mg de Na_2SO_4, and the mixture was then vortexed (2000 cycles·min^{-1}, 1 min) and centrifuged (4000 cycles·min^{-1}, 5 min) at 10–15 °C. Aliquots of 500 μL were reconstituted in 1 mL of a 1:1 (v/v) ACN:oxalic acid (aq., 0.01 mol L^{-1}, pH 2) according to the optimisation and filtered before their analysis, as previously explained.

2.5. UHPLC-MS/MS analysis

Chromatographic separation was performed in an Agilent 1290 Infinity II UHPLC device (Agilent Technologies), equipped with a degassing system, a binary pump, and an automatic sampler. A Kinetex C_{18} polar 100 Å (2.1 × 5 mm, 2.6 μm) pre-column and a Kinetex C_{18} polar 100 Å (2.1 × 50 mm, 2.6 μm) column, both by Phenomenex (Alcobendas, Spain), were used. Mobile phase components consisted of UHPLC quality water (A) and UHPLC quality methanol (B), both containing 0.1% (v/v) in HCOOH. Column flow was set at 0.3 mL min^{-1}, the temperature at 35 °C and the optimal injection volume was fixed at 3 μL (see section 3.1). Agilent 6430 Triple Quad tandem mass-spectrometer by Agilent Technologies was used as the detector. Quantification was carried out in dynamic multiple reaction monitoring (DMRM) acquisition mode using nitrogen (99.999%, Air Liquide, Paris, Île-de-France, France) as nebulizer and drying and collision gas (99.999%, Messer, Bad Soden am Taunus, Hessen, Germany). The electrospray ionisation source worked in the positive mode (ESI+) for all the analytes, at a capillary voltage of 3000 V, with a drying flow rate of 8 L min^{-1}, setting the temperature of the gas at 300 °C. The nebulizer worked at a pressure of 50 psi. The parameters related to the mass spectrometry (fragmentor voltage, collision energy and collision cell accelerator) were fully optimised using a standard containing all the target compounds at a concentration level of 2.5 μg mL^{-1} through the specific Agilent MassHunter Optimizer software (10.0 version). Both target analytes and surrogates were considered. Optimum values are summarized in Table 2. Data was acquired and treated using Agilent MassHunter Workstation software (Quantitative Analysis for QQQ, 10.0 version) by Agilent Technologies.

2.6. Method validation

Both methods (QuEChERS-based extraction followed by SPE and dSPE clean-ups) were validated in four vegetable samples (lettuce, onion, tomato, and carrot) at three concentration levels (5, 25 and 50 μg kg^{-1}) of the target antibiotics by means of UHPLC-MS/MS analysis. The developed methods were validated according to the criteria described in the Regulation (EU) 2021/808 of March 22, 2021 [39] and all the QA/QC parameters were determined as described in the literature [40–43]. Linearity was assessed using a twelve-point external calibration curve (0.25–100 μg kg^{-1}). Trueness was determined by two different approaches: (i) isotopically labelled compounds used as surrogates in order to correct the absolute recovery of the target compounds, and (ii) matrix-matched calibration approach (using a six-point calibration curve in the range of 1–75 μg kg^{-1} prepared in each of the four vegetable matrices that were submitted to the whole procedure). Precision was determined in terms of repeatability by the analysis of spiked-sample replicates and intermediate repeatability [40,43,44].

For each compound, the instrumental limit of quantification (LOQ_{INS}) was calculated as the lowest external calibration point with a relative standard deviation (RSD %) and a systematic error in relation to

Table 2
Fragmentor voltages (V), *m/z* transitions (Da), collision energies (eV) and RT (min) for the target analytes and surrogates.

Compound	Fragmentor voltage (V)	*m/z* transitions (Da)	Collision energy (eV)	RT (min)
CTC	135	479.1 → 462.1	17	6.9
		479.1 → 444.1	21	
DXC	135	445.2 → 428.1	17	7.5
		445.2 → 154.1	33	
OTC	120	461.2 → 426.1	17	5.3
		461.2 → 443.1	9	
TC	135	445.2 → 410.1	17	5.2
		445.2 → 427.1	9	
SDZ	86	251.1 → 156.0	13	2.9
		251.1 → 92.1	29	
SMZ	86	279.2 → 186.0	13	5.0
		279.2 → 124.0	25	
SMX	86	254.1 → 156.0	13	5.9
		254.1 → 108.1	25	
SPD	86	250.1 → 156.0	13	3.3
		250.1 → 108.1	25	
STZ	70	256.1 → 156.0	9	3.2
		256.1 → 108.1	21	
[$^2H_4$]-SMZ	102	283.1 → 185.9	24	5.0
		283.1 → 124.0	33	
[$^{13}C_6$]-SMX	105	260.1 → 162.0	13	6.0
		260.1 → 114.1	25	
[$^2H_6$]-TC	120	451.2 → 433.2	9	4.9
		451.2 → 416.2	17	

the theoretical value below 30%. For that aim, the points between 0.25 and 25 μg kg^{-1} concentration levels were measured in triplicate. Procedural limits of quantification (LOQ_{PRO}) were calculated considering the average signal of the procedural blanks plus ten times their standard deviation as signal, and converted to concentration values in sample using an external calibration and the absolute recovery for each analyte in each matrix.

Furthermore, matrix effect (ME %) affecting the detection was experimentally determined for both validated methods in each vegetable matrix as indicated in **Equation (1)**; where B is the chromatographic peak area of the analyte in a reference standard and A is the area of the analyte in a sample spiked just before the chromatographic analysis at the same concentration as the standard solution.

$$Matrix\ effect\ (ME\ \%) = (A\ /\ B - 1)\ x\ 100 \quad (1)$$

3. Results and discussion

3.1. UHPLC-MS/MS injection solvent

The optimisation of sample injection conditions was performed to obtain the best peak resolution while minimizing the isomerization of TCs, particularly CTC. The injection volume was initially set at 5 μL. On that basis, pure organic solvents (MeOH and ACN) and aqueous mixtures of them were tested at different proportions (ACN:H_2O 1:9, 2:8, 1:1 and 8:2 (v/v); MeOH:H_2O 2:8 (v/v)), as well as the inclusion of HCOOH and oxalic acid as additives (ACN:HCOOH (aq., 2%, 2:8 (v/v)), ACN:HCOOH (aq., 4%, 2:8 (v/v)) and ACN: oxalic acid (aq., 0.01 mol L^{-1}, pH 2) 2:8 and 1:1 (v/v)).

Overall, high water percentages resulted in the reduction of the chromatographic peak broadening (SDZ: 2.60–2.90 min in ACN:water 2:8 *vs* 2.45–2.95 min in ACN:water 1:1. See Fig. S1), improving chromatographic resolution, but promoting epimerisation of CTC (see Fig. S2). Between both organic solvents tested (ACN and MeOH), ACN seemed to be particularly helpful in the control of the epimerisation process given for CTC (see Fig. S2). According to Liang et al. [45], MeOH plays an important role in the degradation of TCs through the addition and substitution of the functional groups on TCs, and were able to identify more than fourteen degradation products in a TCs MeOH solution. This observation was also supported in the work of Gajda et al. [29], since they use ACN for the preparation of standard solutions.

Regarding the proportion of organic solvent content, the higher the organic solvent content, the broader the peaks for SAs, losing resolution and sensitivity, especially over 50% of organic solvent. Similarly, since peak partitioning or co-elution problems occurred in the presence of MeOH, ACN seemed to be the most suitable solvent for the analysis of SDZ (Fig. S1).

The presence of both, HCOOH and oxalic acid, also helped to diminish epimerisation processes. Nevertheless, the sensitivity of the target analytes decreased substantially when HCOOH was added, especially in HCOOH (aq., 2%) condition (Fig. 1). Hence, ACN:oxalic acid (aq.) at 1:1 (v/v) proportion was established as the optimal injection solvent, as a consensus between sensitivity and peak broadening of SAs which was further controlled by reducing the injection volume to 3 μL.

3.2. Extraction

Lettuce was selected as the vegetable-matrix to optimise the different steps of the whole analytical protocol, and optimum conditions were afterwards applied to the rest of matrices. However, the amount and type of salts used were evaluated for all the studied vegetables (lettuce, onion, tomato, and carrot). For this purpose, antibiotics-free fresh-vegetable samples were fortified (three replicates of each vegetable) with the target antibiotics to obtain a concentration of 50 μg kg^{-1} in the final extract.

Diverse scientific studies [4,5,10] have reported the combination of several QuEChERS salts to perform a salting-out extraction, fixing a 4:1 (w/w) proportion between anhydrous $MgSO_4$ and NaCl. Additional salts included anhydrous Na_2SO_4 and/or trisodium citrate (Na_3Cit). According to the collected information, different solvent mixtures (ACN, MeOH and citrate buffer) as extractants and the use of Na_2SO_4 or $MgSO_4$ as dehydrating agents were evaluated. The conditions tested are gathered in Table 1.

Within the different tested conditions, the ones including citrate buffer (No. 3 and 4) were discarded due to the impossibility to separate the supernatant from the pellet in the centrifugation step, caused by the high aqueous content of the extractants mixture [46]. Among the remaining conditions, the presence of Na_2SO_4 rendered the significantly highest absolute recoveries for both antibiotic families in lettuce (F_{exp} = 16.0>F_{crit} = 6.0 for TCs and F_{exp} = 29.5>F_{crit} = 5.3 for SAs). Similarly, Na_2SO_4 only provided significantly higher recoveries (F_{exp} = 17.6>F_{crit} = 5.3) for the extraction of SAs in onion (see Fig. S3 in the supplementary). In the case of carrot and tomato, no statistical differences were noticed between the analytes' recoveries obtained under conditions No. 1 and 2 (F_{exp} = 2.2–3.5<F_{crit} = 6.0 for TCs and F_{exp} = 3.2–4.2<F_{crit} = 5.3 for SAs). Therefore, condition No. 2 was chosen as optimal and used in further experiments, which included Na_2SO_4 as dehydrating agent.

As far as we are concerned, this work is the first to offer a comparison between the extraction recoveries obtained using both salts (Na_2SO_4 or $MgSO_4$). Most of the already developed QuEChERS-based methods included $MgSO_4$ salt in the extraction [5,10]; nonetheless, diverse published works attributed the low extraction recoveries obtained for TCs to the binding of these compounds to Mg^{2+} ion [11,29]. Based on the reported problems given with $MgSO_4$, Chuang et al. [4] used Na_2SO_4 for the extraction of one TC and two SAs, among other pharmaceuticals, in celery and lettuce samples. The results of our work suggested that the extraction efficiency regarding the addition of the different salts is matrix-dependent. In the case of lettuce, lower extraction recoveries were obtained for TCs and SAs in the presence of $MgSO_4$ as well as for SAs in onion. The problem was avoided when Na_2SO_4 was employed, getting a significant improvement in the extraction efficiency. However, no significant differences were noticed for target analytes' recoveries using Na_2SO_4 or $MgSO_4$ in the case of the rest of the vegetable matrices.

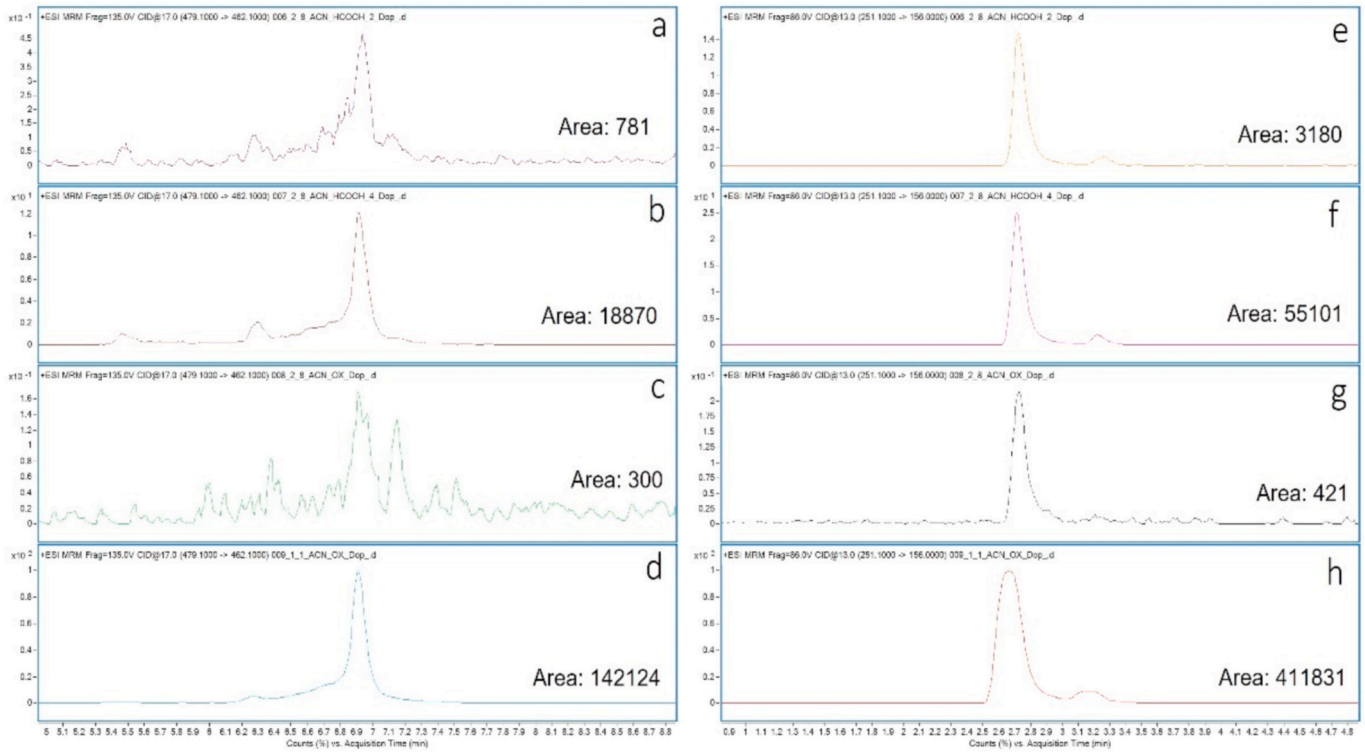

Fig. 1. Chromatograms obtained for CTC (a–d) and SDZ (as representative of the SAs) (e–h) in different injection solvents: ACN:HCOOH (aq, 2%, 2:8 (v/v)), ACN:HCOOH (aq, 4%, 2:8 (v/v)) and ACN:oxalic acid (aq, 0.01 mol L^{-1}, pH 2) 2:8 and 1:1 (v/v)). Injection volume: 3 μL.

3.3. Clean-up

For optimisation of the clean-up, two strategies were evaluated: dSPE [5,10] and conventional SPE [12].

3.3.1. dSPE clean-up protocol

The efficiency of the dSPE clean-up was evaluated by testing different purifying sorbents: PSA (10 mg) and C_{18} (25 mg), with or without GCB (2.5 mg), together with 150 mg anhydrous Na_2SO_4 in all the cases. For that purpose, lettuce samples were processed; three replicates were spiked at the beginning of the procedure, another three just before the analysis and another three were left as procedural blanks. Absolute recoveries were calculated with the early spiked samples, and ME % affecting the detection was calculated employing the replicates spiked after the clean-up (as explained in section 2.5). In all the cases, procedural blank signal correction was applied.

The absolute recoveries determined when employing both dSPE sorbents mixtures (115–170% with GCB and 113–137% without GCB) were statistically compared, concluding that there were no significant differences in the analytes' recoveries ($F_{exp} = 1.3 < F_{crit} = 4.5$) despite GCB addition. Nevertheless, the presence of GCB caused a higher matrix effect at the detection for SMZ and DXC, for which a positive matrix effect of 37% and 83%, respectively, were calculated (see Fig. 2). The result for DXC concurred with the strong signal enhancement observed by He et al. for this compound when 2.5 mg of GCB was used [5]. Regarding the condition excluding GCB, the matrix effect was under 30% for all the analytes except for CTC, for which an ion suppression of 32% was determined. Therefore, 10 mg of PSA, 25 mg of C_{18} and 150 mg of anhydrous Na_2SO_4 without the GCB addition, were selected as the optimal sorbents to perform the dSPE clean-up.

3.3.2. SPE clean-up protocol

For the SPE clean-up protocol, cartridges consisted of 100 mg PSA, 100 mg C_{18} with or without 50 mg GCB and Oasis HLB (6 cc, 200 and 500 mg) cartridges were tested. For this purpose, lettuce samples were extracted, three replicates were spiked before the clean-up, another three just before the analysis and another three were left as procedural blanks. Absolute recoveries and ME % affecting the detection were calculated as explained in section 3.3.1.

Opposite to dSPE, with the use of SPE cartridges signal suppression rather than enhancement was observed for almost all the antibiotics (Fig. 2). Both PSA + C_{18} and PSA + C_{18}+GCB cartridges showed a significantly lower matrix effect in comparison to the Oasis HLB cartridges especially in the case of SAs (p-value <0.05). Nonetheless, they were discarded since even if performing two consecutive elution steps, the recoveries obtained for TCs were negligible (data not shown). Regarding Oasis HLB cartridges, the signal suppression was mainly observed for SAs, reflected in the recoveries obtained, in the 26–46% and 23–43% range for 200-mg and 500-mg cartridges, respectively. Both clean-up procedures using Oasis HLB sorbents were statistically compared and no significant differences were detected (p-value >0.05), neither for TCs nor for SAs. Hence, as final extracts from the clean-up with 500 mg were clearer, regarding pigment content, compared to 200 mg cartridges, 500 mg Oasis HLB cartridges were chosen as optimal for the clean-up step.

Furthermore, the loading sample volume to the SPE cartridge was studied. For this purpose, 1 mL and 2 mL loading volumes were evaluated, with and without concentration to 300 μL under a nitrogen flow. Previous to SPE loading, concentrated and non-concentrated samples were diluted with the necessary pH 4 aqueous buffer volume to obtain sample solutions with less than 5% (v/v) of organic solvent [47], that is to say, extracts of 1 mL were diluted to 20 mL buffer, 2 mL extracts to 40 mL and 300 μL extracts to 6 mL. Larger loading volumes were also studied (up to 9 mL) but they were discarded because method throughput was not viable.

The highest absolute recoveries (data not shown) for TCs were obtained for 1 mL-loadings (54–96%) rather than for the 2 mL (22–70%). For SAs, no statistical differences were found regarding their recovery when loading 1 or 2 mL with and without the previous concentration (p-value >0.05). Although concentrated replicates displayed statistically

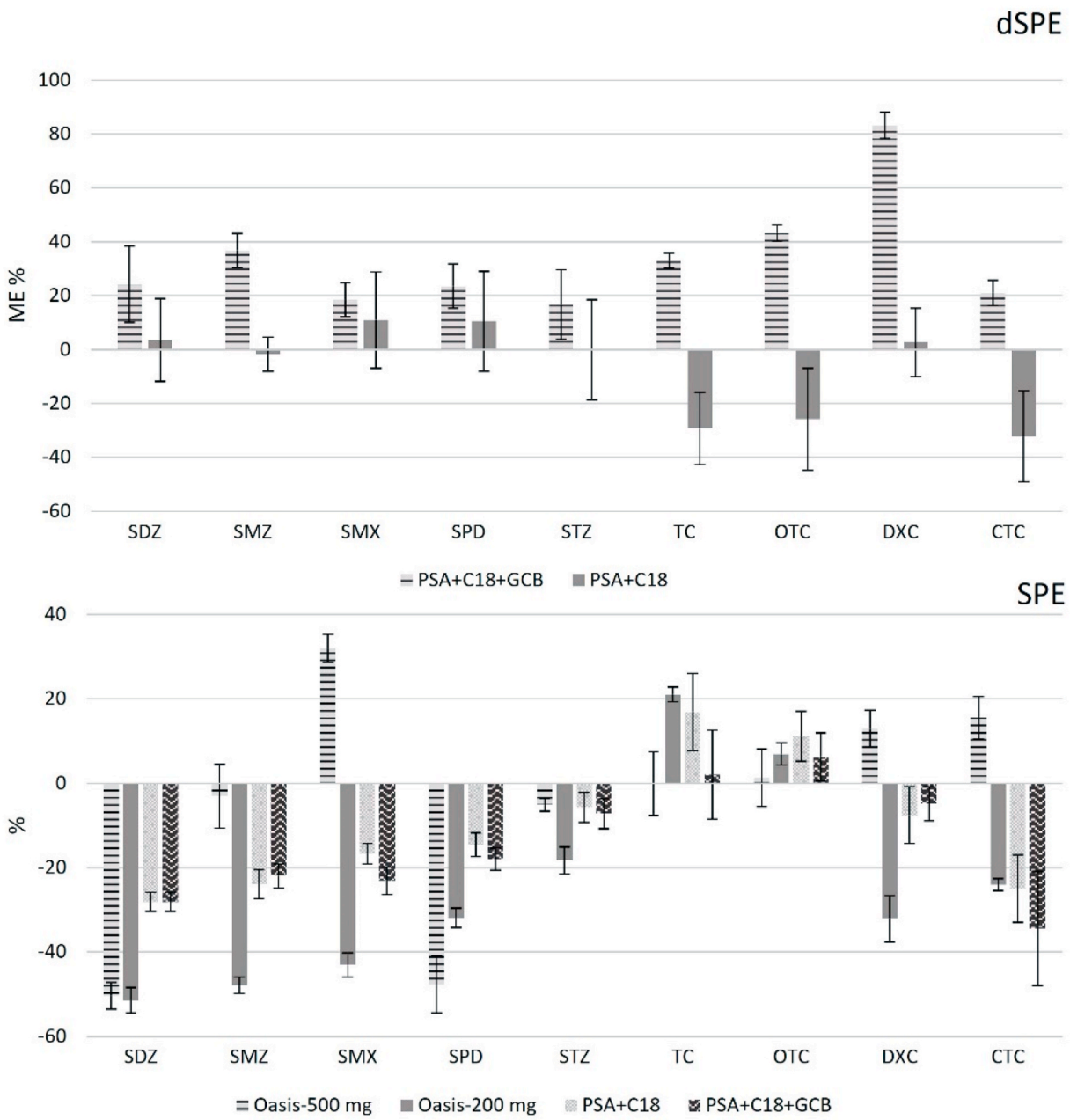

Fig. 2. ME % at the detection for the target analytes in lettuce with dSPE and SPE clean-up approaches (n = 3 for each approach). For dSPE, PSA and C_{18} sorbents were tested with (striped) and without GCB (grey), whereas in the case of SPE, PSA + C_{18} (dotted), PSA + C_{18}+GCB (zigzag) and Oasis HLB 200 mg (grey) and 500 mg (striped) cartridges were studied.

higher recoveries for TCs (75–110% vs. 54–96%), the improvement did not justify the time and nitrogen consumption of the extra evaporation step. Thus, optimal loading conditions were set in 1 mL extract without intermediate concentration.

Once the optimal SPE cartridge and the loading volume were fixed, the analytes' elution profile was studied by adding four separated aliquots of 3 mL of ACN to the cartridges. Elution of all the target analytes was performed using 9 mL of ACN (see Fig. 3a and **b**). Using 12 mL of ACN improved the recoveries of STZ, OTC and DXC only by 4%, which was taken as a negligible improvement taking into account the increased nitrogen consumption for the evaporation of a bigger elution volume.

SPE clean-up protocol is followed by an evaporation step before the UHPLC-MS/MS analysis. Losses of the target analytes during the evaporation step have been reported in the literature, especially for TCs, caused by the binding of the analytes to the silanol groups on glassware [11,27,28]. Hence, in order to evaluate and minimize possible analyte losses in this step, different approaches were evaluated: (i) evaporation to dryness and (ii) evaporation to approx. 1 mL. Besides, and in the case of evaporation to dryness, the use of glass test tubes, silanized glass test tubes and plastic test tubes was studied. For the optimisation of this step, three replicates of spiked ACN (9 mL) were prepared for each studied condition.

The results concluded that plastic test tubes (66–79% for TCs and 61–73% for SAs) were the best alternative to perform eluates' evaporation, which is in concordance with the literature [48,49], followed by the evaporation to 1 mL in glass test tubes (54–57% for TCs and 49–56% for SAs). Nonetheless, even if recoveries improved, longer evaporation times and, in consequence, higher nitrogen consumption was required for evaporation in plastic test tubes (120 min in plastic test tubes versus 50 min in glass test tubes) and evaporation can be expected to be even

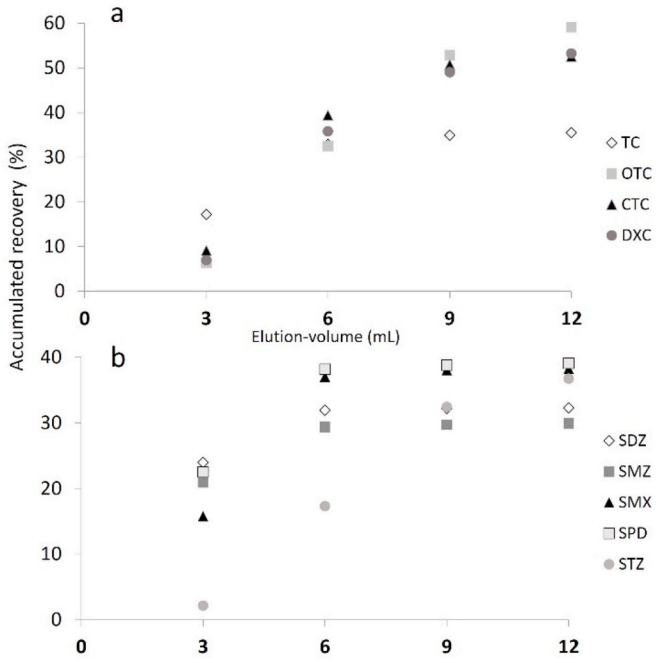

Fig. 3. Elution profile (accumulated absolute recoveries, n = 3) for TCs (**a**) and SAs (**b**) in each elution volume.

longer in the presence of matrix. Thus, evaporation to approx. 1 mL and the use of glass test tubes were selected as the optimal and used in further experiments.

3.4. Method validation

3.4.1. Figures of merit of UHPLC-MS/MS

The main figures of merit of the UHPLC-MS/MS method are summarized in Table S2. External calibration curves showed good linearity over a wide concentration range (i.e., 0.2–86.0 μg kg^{-1}) with determination coefficients (r^2) between 0.9984 and 0.9999. The repeatability and intermediate repeatability of the measurements with the UHPLC-MS/MS system was assessed by injecting in triplicate the external calibration solutions (0.25–25 μg kg^{-1}) in the same day and different days (n = 3), respectively. Adequate repeatability and intermediate repeatability were obtained with RSD values below 15% and 14%, respectively, for all the analytes and surrogates at all concentration levels except in the case of CTC at 0.25 μg kg^{-1} (32% and 20%, respectively). LOQ_{INS} were below 0.6 μg kg^{-1} for the compounds of both antibiotic families.

3.4.2. Figures of merit of QuEChERS-based extraction followed by SPE-UHPLC-MS/MS and dSPE-UHPLC-MS/MS methods

The figures of merit of the QuEChERS-based extraction followed by SPE-UHPLC-MS/MS and dSPE-UHPLC-MS/MS methods were determined for all target antibiotics in the four vegetable matrices (i.e., lettuce, onion, tomato and carrot).

First, the matrix effect at detection was evaluated for all tested matrices under the optimum conditions of both methods (see Fig. 4, and Figs. S4 and S5 in the supplementary). In fact, although LC-MS is one of the most sensitive and selective analytical techniques, it could suffer from matrix effect especially when ESI is used as ionisation source for the analysis of complex matrices due to the co-elution of analytes and matrix components that may alter the ionisation efficiency of the target analytes causing a loss (ion suppression) or an increase (ion enhancement) in its chromatographic signal and hence, directly affecting method accuracy [50]. Based on the results summarized in Fig. 4, ion suppression was mainly observed, especially for SAs, when SPE clean-up was applied regardless of the vegetable matrix. However, signal enhancement was observed, especially for TCs, when the dSPE clean-up protocol was used. Hence, a lower matrix effect affecting the detection was observed for TCs after SPE clean-up, while in the case of SAs, dSPE clean-up retrieved better results, except for SMZ and STZ in tomato and onion. For these last cases, SPE clean-up would be more appropriate. Since both analytical approaches showed complementary responses in terms of matrix effect, the rest of figures of merit were accurately determined in order to figure out the adequacy of methods for the simultaneous analysis of antibiotics with different physicochemical parameters.

Table 3 summarizes the main figures of merit of QuEChERS-based extraction followed by SPE-HPLC-MS/MS and dSPE-UHPLC-MS/MS, respectively. Exemplarily the total ion chromatograms of the four vegetables spiked at 5 μg kg^{-1} and 50 μg kg^{-1} after the treatment of both analytical methods are shown in Figs. S6 and S7.

LOQ_{PRO} values were calculated for both procedures, QuEChERS-based extraction followed by SPE and dSPE clean-ups. In the case of SPE, LOQ_{PRO} values remained below 4.4 μg kg^{-1} for TCs in all vegetables except for CTC in lettuce (11.3 μg kg^{-1}) and 3.0 μg kg^{-1} for SAs, with exception of SDZ in onion (3.9 μg kg^{-1}) and STZ in carrot (5.0 μg kg^{-1}). Lower LOQ_{PRO} values (0.1–1.9 μg kg^{-1}) were obtained when the dSPE clean-up was applied, being 3.7 μg kg^{-1} the highest value determined for DXC in carrot. Comparing with other QuEChERS-based methods reported in the literature, where the dSPE clean-up approach was applied, the methodological approach proposed in this work allows getting lower LOQ_{PRO} than the ones reported by He et al. for TC (5 μg kg^{-1}), SMX, SDZ and STZ (2 μg kg^{-1}) [5] and the ones published by Yu et al. for both SAs (1.10–3.90 μg kg^{-1}) and TCs (2.00–9.73 μg kg^{-1}) [10]. Those values were in the same range than the ones calculated in this work using SPE clean-up, especially the above-mentioned 9.73 μg kg^{-1} LOQ_{PRO} value determined for CTC in leafy vegetables, which concurs with what it has been observed in this work for CTC in lettuce. However, Feng and co-workers obtained lower LOQ_{PRO} values with UAE combined with SPE protocol (0.05–0.76 μg kg^{-1} for TCs and 0.02–0.05 μg kg^{-1} for SAs) [12]. It should be highlighted, that those values were calculated as ten times the standard deviation of the measurements of control samples divided by the slope of the calibration curve, whereas in this case LOQ_{PRO} were calculated taking into account the signal of the procedural blanks (n = 3) plus ten times their standard deviations, and converted to concentration units using external calibration. The different calculation approaches in the literature to get LOQ_{PRO} values make difficult their comparison.

Two different approaches were used to determine analytes concentrations in spiked samples: (i) external calibration and deuterated analogues to correct absolute recoveries, and (ii) matrix-matched calibration approach using the corresponding vegetable matrix. Before using matrix-matched calibration curve to get concentrations, the adequacy of the target lines was assured (r^2 between 0.9921 and 0.9972, and repeatability, expressed as RSD, less than 28% with QuEChERS-SPE-UHPLC-MS/MS (except for DXC and TC, 33%) and 24% with QuEChERS-dSPE-UHPLC-MS/MS for all target analytes at 25 μg kg^{-1} level). Overall, the minimum trueness requirements established by the Regulation (EU) 2021/808 of March 22, 2021 (i.e., 70–120% for 1–10 μg kg^{-1} concentrations and 80–120% for concentrations higher than 10 μg kg^{-1} with precision, expressed as RSD, ≤30%) [39] were obtained using both analytical methods (i.e., QuEChERS-SPE-UHPLC-MS/MS and QuEChERS-dSPE-UHPLC-MS/MS) at the three validation concentration levels by using either surrogate correction or, alternatively, matrix-matched calibration approaches (see Table 3), with some exceptions. Irrespective of the clean-up method used, apparent recoveries for some analytes, especially for SAs, fell outside the established ranged by the guideline, however, according to the standard SANTE/11813/2017, lower recoveries values (30–70%) are acceptable in case of their proven consistency (RSD <20%).

Going deeper into the results, when SPE clean-up was used, surrogate

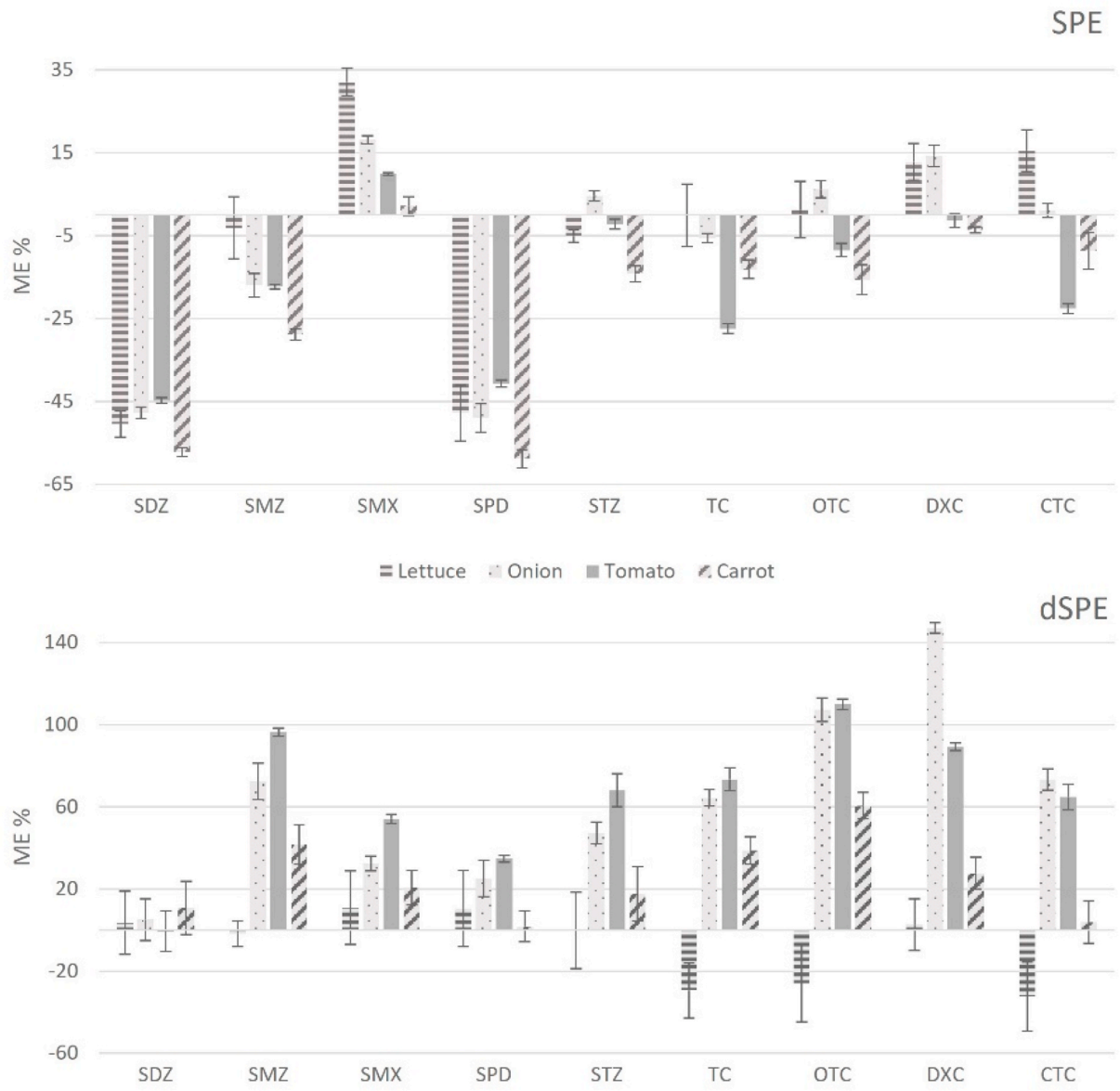

Fig. 4. ME % (n = 3) at the detection for the target analytes in the four vegetable matrices with SPE and dSPE clean-up approaches.

correction approach retrieved more adequate apparent recoveries (trueness) than matrix-matched calibration, especially in onion and carrot matrices. Those vegetables together with tomato resulted to be the most complex for STZ determination. STZ showed a different behaviour in comparison with the rest of the SAs (except in lettuce), thus, in order to fit the requirements that enabled the analysis of STZ by this method, another surrogate should be used. Furthermore, when the SPE approach was applied, no recovery could be given for CTC at the lowest validation level due to the high LOQ_{PRO} attributed to this compound. The same issue was reported by Yu et al. [10], who justified it as an extraction efficiency problem owing to TC antibiotics complexation with organic matter and metallic ions commonly present in vegetables. Determination of CTC at low levels was possible accurately even at low concentration levels (5 μg kg^{-1}) using dSPE clean-up (see Table 3). As a matter of fact, when dSPE was used for clean-up purposes, adequate trueness values were obtained for all the target analytes in all vegetables when matrix-matched quantification approach was used.

Comparing with published scientific works, the recoveries calculated for SAs in this study after SPE clean-up are similar to the ones presented by Yu et al. [10], except for STZ. For this antibiotic, the dSPE protocol validated in this work offered the highest recoveries. Furthermore, Yu et al. [10] could not recover CTC at the lowest validation level (5 μg kg^{-1}) while in this work apparent recoveries above 98% were calculated by the dSPE approach and SPE protocol except for lettuce. Higher recoveries (88–107% *vs* 57%) were also calculated in this work for OTC at 5 μg kg^{-1} concentration. In the case of He et al. [5], similar recoveries are given in tomato for SAs and TCs, though the recoveries calculated for OTC are higher in this work (SPE: 93% and 79% and dSPE: 92% and 112% for 5 and 50 μg kg^{-1}, respectively) *vs* (66% and 55% for 5 and 50 μg kg^{-1}, respectively). The recovery values published by Chuang et al. [4] calculated with matrix-matched calibration -after dSPE clean-up- for SDZ, SMX and OTC in lettuce at 200 μg kg^{-1} validation level (74%, 74% and 72%, respectively) are also akin to the ones determined in this work at the highest validation level evaluated here, 50 μg kg^{-1} (86%, 92% and 91% for SDZ, SMX and OTC, respectively).

Regarding precision, it was determined in terms of repeatability at all concentration levels tested and intermediate repeatability (same method processed at different days) at the highest concentration level tested (i.e., 50 μg kg^{-1}). According to the results shown in Table 3, both analytical methods provided adequate repeatability values (RSD % <

Table 3
Apparent recoveries (intra-day repeatability %, intermediate repeatability %) with QuEChERS-SPE-UHPLC-MS/MS and QuEChERS-dSPE-UHPLC-MS/MS methods for the target analytes in the four different vegetable matrices at the three studied concentration levels.

Matrix	Analyte	QuEChERS-SPE-UHPLC-MS/MS						QuEChERS-dSPE-UHPLC-MS/MS						
		LOQ proc ($\mu g \cdot kg^{-1}$)	R% using surrogates			R% using matrix-matched		LOQ proc ($\mu g \cdot kg^{-1}$)	R% using surrogates			R% using matrix-matched		
			5 $\mu g\ kg^{-1}$	25 $\mu g\ kg^{-1}$	50 $\mu g\ kg^{-1}$	5 $\mu g\ kg^{-1}$	25 $\mu g\ kg^{-1}$		5 $\mu g\ kg^{-1}$	25 $\mu g\ kg^{-1}$	50 $\mu g\ kg^{-1}$	5 $\mu g\ kg^{-1}$	25 $\mu g\ kg^{-1}$	50 $\mu g\ kg^{-1}$
Lettuce	CTC[a]	11.3	–	102 (10%)	105 (4%, 9%)	–	74 (17%)	0.3	124 (6%)	149 (7%)	155 (6%, 5%)	105 (23%)	102 (7%)	98 (4%)
	DXC[a]	2.5	97 (21%)	103 (6%)	125 (11%, 12%)	98 (1%)	88 (33%)	0.6	134 (12%)	164 (6%)	178 (3%, 4%)	108 (10%)	98 (9%)	102 (2%)
	OTC[a]	2.0	107 (9%)	80 (10%)	109 (11%, 15%)	107 (3%)	96 (14%)	0.5	98 (6%)	116 (2%)	113 (4%, 3%)	104 (2%)	110 (15%)	91 (5%)
	TC[a]	4.4	77 (26%)	75 (15%)	99 (8%, 11%)	118 (23%)	78 (33%)	0.6	109 (1%)	119 (4%)	118 (7%, 5%)	111 (4%)	108 (16%)	92 (6%)
	SDZ[b]	1.0	73 (8%)	91 (1%)	84 (6%, 6%)	73 (15%)	129 (7%)	0.1	92 (6%)	86 (8%)	79 (7%, 4%)	122 (30%)	117 (24%)	86 (1%)
	SMZ[b]	1.6	68 (14%)	88 (4%)	95 (2%, 6%)	102 (16%)	122 (7%)	0.3	109 (10%)	104 (5%)	100 (8%, 5%)	121 (13%)	111 (15%)	90 (3%)
	SMX[c]	0.5	91 (6%)	101 (3%)	92 (1%, 4%)	86 (23%)	137 (3%)	1.2	95 (10%)	99 (3%)	97 (1%, 2%)	117 (15%)	109 (13%)	92 (4%)
	SPD[b]	0.4	94 (21%)	94 (5%)	77 (<1%, 5%)	57 (17%)	109 (25%)	0.2	54 (11%)	55 (11%)	56 (6%, 3%)	108 (10%)	104 (6%)	96 (2%)
	STZ[b]	2.1	70 (7%)	89 (6%)	71 (2%, 8%)	81 (21%)	128 (2%)	0.6	120 (23%)	83 (1%)	76 (9%, 5%)	251 (9%)	105 (3%)	67 (4%)
Onion	CTC[a]	3.1	108 (15%)	100 (9%)	81 (8%, 5%)	107 (3%)	77 (3%)	0.5	123 (4%)	131 (3%)	126 (<1%, 2%)	100 (4%)	93 (6%)	108 (10%)
	DXC[a]	1.6	94 (4%)	113 (3%)	112 (7%, 5%)	96 (10%)	91 (12%)	1.0	126 (6%)	178 (6%)	188 (4%, 4%)	82 (4%)	88 (3%)	114 (8%)
	OTC[a]	1.4	90 (14%)	74 (9%)	73 (1%, 2%)	122 (5%)	79 (22%)	0.4	97 (8%)	116 (3%)	118 (11%, 7%)	82 (16%)	89 (4%)	113 (17%)
	TC[a]	2.3	43 (13%)	108 (<1%)	107 (5%, 4%)	185 (33%)	94 (22%)	0.5	107 (12%)	120 (7%)	117 (2%, 3%)	96 (12%)	97 (2%)	104 (11%)
	SDZ[b]	3.9	74 (7%)	70 (6%)	56 (7%, 9%)	198 (5%)	50 (28%)	0.1	51 (3%)	52 (2%)	50 (3%, 3%)	99 (6%)	105 (2%)	96 (7%)
	SMZ[b]	0.8	84 (4%)	87 (3%)	88 (3%, 8%)	255 (23%)	39 (22%)	0.2	93 (9%)	93 (2%)	87 (1%, 1%)	91 (7%)	99 (4%)	101 (10%)
	SMX[c]	2.2	92 (15%)	116 (8%)	98 (1%, 3%)	136 (3%)	73 (15%)	1.3	91 (2%)	96 (3%)	94 (3%, 4%)	96 (10%)	103 (5%)	97 (6%)
	SPD[b]	0.3	91 (3%)	107 (4%)	102 (11%, 10%)	124 (9%)	80 (28%)	0.2	54 (6%)	57 (3%)	50 (8%, 9%)	93 (14%)	104 (<1%)	96 (2%)
	STZ[b]	0.3	80 (11%)	47 (5%)	46 (6%, 10%)	261 (9%)	38 (25%)	0.2	104 (20%)	105 (14%)	96 (2%, 3%)	97 (22%)	92 (15%)	86 (1%)
Tomato	CTC[a]	2.8	99 (15%)	89 (6%)	121 (7%, 7%)	87 (11%)	97 (24%)	0.8	103 (22%)	132 (2%)	116 (1%, 4%)	88 (25%)	114 (8%)	88 (5%)
	DXC[a]	1.2	101 (10%)	104 (3%)	135 (9%, 9%)	91 (12%)	94 (24%)	1.0	153 (3%)	167 (5%)	139 (15%, 11%)	118 (7%)	122 (11%)	82 (8%)
	OTC[a]	1.0	93 (14%)	70 (15%)	79 (6%, 8%)	109 (14%)	106 (17%)	1.1	92 (15%)	108 (8%)	112 (4%, 4%)	84 (11%)	98 (15%)	102 (5%)
	TC[a]	2.4	115 (17%)	90 (18%)	102 (7%, 7%)	53 (23%)	113 (18%)	1.0	111 (2%)	121 (2%)	127 (3%, 2%)	91 (5%)	96 (9%)	104 (4%)
	SDZ[b]	2.0	109 (24%)	59 (5%)	84 (3%, 5%)	107 (16%)	96 (19%)	0.2	42 (11%)	40 (4%)	41 (5%, 4%)	105 (6%)	98 (3%)	102 (2%)
	SMZ[b]	1.9	88 (5%)	85 (1%)	121 (6%, 6%)	137 (1%)	84 (24%)	0.1	96 (12%)	102 (2%)	101 (1%, 2%)	93 (6%)	91 (6%)	109 (5%)
	SMX[c]	1.4	91 (12%)	100 (7%)	95 (4%, 4%)	89 (5%)	87 (19%)	0.5	99 (4%)	105 (5%)	104 (4%, 3%)	104 (6%)	103 (9%)	97 (2%)
	SPD[b]	0.3	100 (7%)	93 (4%)	127 (4%, 5%)	91 (4%)	91 (25%)	0.2	58 (7%)	61 (2%)	56 (5%, 3%)	100 (3%)	97 (2%)	103 (2%)
	STZ[b]	0.5	53 (9%)	48 (6%)	47 (1%, 2%)	103 (6%)	78 (17%)	0.5	89 (13%)	85 (<1%)	82 (2%, 2%)	105 (7%)	93 (5%)	108 (4%)
Carrot	CTC[a]	1.9	98 (12%)	91 (2%)	91 (10%, 10%)	94 (13%)	58 (7%)	0.3	107 (8%)	109 (11%)	101 (12%, 9%)	108 (18%)	108 (4%)	93 (9%)
	DXC[a]	1.5	100 (15%)	119 (6%)	120 (8%, 5%)	61 (11%)	79 (1%)	3.7	106 (3%)	124 (3%)	123 (4%, 2%)	102 (9%)	101 (9%)	99 (3%)
	OTC[a]	3.5	88 (13%)	81 (3%)	80 (3%, 3%)	73 (12%)	64 (7%)	1.2	109 (5%)	123 (3%)	126 (4%, 4%)	89 (11%)	95 (11%)	105 (6%)

(*continued on next page*)

Table 3 (*continued*)

Matrix	Analyte	QuEChERS-SPE-UHPLC-MS/MS						QuEChERS-dSPE-UHPLC-MS/MS					
		LOQ proc ($\mu g \cdot kg^{-1}$)	R% using surrogates			R% using matrix-matched		LOQ proc ($\mu g \cdot kg^{-1}$)	R% using surrogates			R% using matrix-matched	
			5 μg kg^{-1}	25 μg kg^{-1}	50 μg kg^{-1}	5 μg kg^{-1}	25 μg kg^{-1}		5 μg kg^{-1}	25 μg kg^{-1}	50 μg kg^{-1}	5 μg kg^{-1}	25 μg kg^{-1}	50 μg kg^{-1}
	TC[a]	2.6	55 (15%)	113 (15%)	121 (2%, 3%)	142 (9%)	51 (1%)	1.9	113 (4%)	121 (1%)	127 (4%, 3%)	98 (11%)	98 (9%)	102 (3%)
	SDZ[b]	0.1	114 (13%)	62 (4%)	62 (4%, 4%)	82 (18%)	54 (1%)	0.1	86 (8%)	81 (7%)	84 (9%, 6%)	101 (6%)	93 (5%)	108 (13%)
	SMZ[c]	0.9	77 (3%)	93 (11%)	99 (2%, 4%)	164 (31%)	47 (10%)	0.3	91 (6%)	93 (6%)	96 (7%, 5%)	103 (5%)	98 (7%)	103 (4%)
	SMX[b]	1.2	86 (19%)	109 (11%)	103 (3%, 3%)	69 (11%)	69 (7%)	1.0	114 (6%)	111 (6%)	106 (3%, 2%)	123 (10%)	106 (4%)	94 (7%)
	SPD[b]	0.5	89 (2%)	89 (7%)	92 (1%, 5%)	67 (7%)	69 (1%)	0.4	65 (5%)	62 (2%)	62 (3%, 4%)	106 (8%)	98 (10%)	103 (2%)
	STZ[b]	5.0	53 (16%)	50 (19%)	53 (4%, 4%)	77 (24%)	65 (16%)	0.7	90 (5%)	87 (4%)	101 (9%, 10%)	90 (9%)	84 (11%)	119 (12%)

Analyte concentration in sample corrected with [a][$^2H_6$]-TC, [b][$^2H_4$]-SMZ and [c][$^{13}C_6$]-SMX.

30%) regardless of the quantification method used for all the target compounds in all the evaluated vegetable matrixes. The RSD values ranged from 2% to 26% and 1%–11% at low and high concentration tested levels, respectively, using surrogates calibration method and SPE clean-up, whereas the RSD values found with the matrix-matched calibration method were a little bit higher but still adequate (between 1-24% and 1–28%, with two exceptions of 33%, at low and high concentration tested levels, respectively). In the case of using d-SPE all RSD % values were lower than 30%, concretely, between 1-23% and 1–15% for low and high concentration levels using surrogates for quantification, respectively, and between 2-30% and 1–17% for low and high concentration levels using matrix-matched calibration approach. Regarding the intermediate repeatability, it was calculated for the highest concentration level (using surrogates for quantification) and values ranged from 2% to 15% and 1%–11% for QuEChERS-SPE-UHPLC-MS/MS and QuEChERS-dSPE-UHPLC-MS/MS methods, respectively, were obtained.

3.5. Method application

The methods were applied to twenty-five vegetable (lettuce, onion, tomato, and carrot) samples from both ecological and non-ecological agriculture (see section 2.1) obtained in local markets. Target compounds were not detected or concentrations detected were below the LOQ_{PRO} values in the case of all the samples, except two SAs which were only detected in a lettuce sample from non-ecological agriculture at 1.2 μg kg^{-1} for SMZ and 0.5 μg kg^{-1} for SPD, with the protocol including dSPE clean-up and with both analytical procedures, respectively. As found concentrations were close to LOQ_{PRO} values, standard additions were carried out to guarantee quantification. The occurrence of both SAs in vegetable samples was already reported by other authors, appearing in similar concentrations to the ones obtained in the present work [10, 12]. No antibiotics were found in ecological vegetable samples, which could be attributed to the reduction of antibiotics in stockbreeding -in comparison to conventional system-by promoting phytotherapeutical or homeopathic treatments [51].

Unlike in foodstuff of animal origin, there is no maximum residue limit (MRL) set for antibiotics in crops intended for human consumption in the EU [17]. Instead, acceptable daily intake (ADI) values are established by the World Health Organization (WHO), which can be employed to assess the safety of agricultural products. SMZ was the only SA stipulated by the committee of WHO, for which the maximum intake per day is set at 50 μg kg^{-1} body weight [52]. Hence, considering that the antibiotic concentrations detected in this work are below the threshold, the vegetables acquired in local markets and department stores of the Basque Country do not seem to be an important direct source of antibiotics.

4. Conclusions

In this work, two accurate sample preparation procedures were developed for the analysis of multiclass antibiotics, including four TCs and five SAs, in four vegetable matrices (lettuce, onion, tomato, and carrot) after evaluation of the critical steps involved in the analytical procedures. In this sense, pure ACN was the optimal solvent to perform both methods' extraction of the target analytes and showed that the extraction efficiency with respect to the addition of QuEChERS salts was matrix-dependent. It should be noted that, as far as we are concerned, this is the first time that a comparison between the extraction recoveries obtained using both salts (Na_2SO_4 or $MgSO_4$) is reported, despite of being Mg a known bias in the determination of antibiotics in environmental samples. Regarding the clean-up step, the efficiency of two clean-up strategies was compared, SPE *vs* dSPE, concluding that SPE was the most suitable clean-up procedure to control matrix effect affecting the detection for TCs, while dSPE retrieved better results for most of the SAs. Overall, the validated methods showed good performance in terms of LOQs, linearity, precision and trueness for the trace determination of the target analytes in different vegetable matrices, which suppose a step forward to ensure food safety and human health. Although, taking into account the lower number of steps involved in the QuEChERS-dSPE-UHPLC-MS/MS method and the higher overall accuracy of the results (using matrix-matched quantification) compared to QuEChERS-SPE-UHPLC-MS/MS, it could be considered the most appropriate method for the simultaneous detection of these compounds in vegetables. However, both methods were applied to twenty-five real samples of organic and traditional lettuce, onion, tomato, and carrots, with positive findings of 1.2 μg kg^{-1} of SMZ and 0.5 μg kg^{-1} of SPD in a lettuce sample from non-ecological agriculture. Therefore, the methods optimised in this study are reliable to assess the presence of antibiotics in raw vegetables at much lower levels than those regulated for daily intake.

Credit author statement

IVL Investigation, Data curation, Validation, Formal analysis, Methodology, Writing – original draft, Visualization. JCBM. Investigation, Data curation, Validation. IB Investigation, Data curation. BGG Investigation, Supervision. MO Methodology, Conceptualization, Formal analysis, Funding acquisition. OZ Supervision, Methodology, Conceptualization, Formal analysis, Writing – review & editing. AP Supervision, Methodology, Conceptualization, Formal analysis, Writing – review & editing, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.124192.

References

[1] J. García, M.J. García-Galán, J.W. Day, R. Boopathy, J.R. White, S. Wallace, R. G. Hunter, A review of emerging organic contaminants (EOCs), antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in the environment: increasing removal with wetlands and reducing environmental impacts, Bioresour. Technol. 307 (2020), 123228, https://doi.org/10.1016/j.biortech.2020.123228.

[2] B.J.A. Berendsen, R.S. Wegh, J. Memelink, T. Zuidema, L.A.M. Stolker, The analysis of animal faeces as a tool to monitor antibiotic usage, Talanta 132 (2015) 258–268, https://doi.org/10.1016/j.talanta.2014.09.022.

[3] S. Quaik, A. Embrandiri, B. Ravindran, K. Hossain, N.A. Al-Dhabi, M.V. Arasu, S. Ignacimuthu, N. Ismail, Veterinary antibiotics in animal manure and manure laden soil: scenario and challenges in asian countries, J. King Saud Univ. Sci. 32 (2) (2020) 1300–1305, https://doi.org/10.1016/j.jksus.2019.11.015.

[4] Y.-H. Chuang, Y. Zhang, W. Zhang, S.A. Boyd, H. Li, Comparison of accelerated solvent extraction and quick, Easy, Cheap, effective, rugged and Safe method for extraction and determination of pharmaceuticals in vegetables, J. Chromatogr. A 1404 (2015) 1–9, https://doi.org/10.1016/j.chroma.2015.05.022.

[5] Z. He, Y. Wang, Y. Xu, X. Liu, Determination of antibiotics in vegetables using QuEChERS-based method and liquid chromatography-quadrupole linear ion trap mass spectrometry, Food Anal. Methods 11 (10) (2018) 2857–2864, https://doi.org/10.1007/s12161-018-1252-8.

[6] C. Zhang, Y. Deng, J. Zheng, Y. Zhang, L. Yang, C. Liao, L. Su, Y. Zhou, D. Gong, L. Chen, A. Luo, The application of the QuEChERS methodology in the determination of antibiotics in food: a review, TrAC, Trends Anal. Chem. 118 (2019) 517–537, https://doi.org/10.1016/j.trac.2019.06.012.

[7] H.R. Gaskins, C.T. Collier, D.B. Anderson, Antibiotics as growth PROMOTANTS: mode of action, Anim. Biotechnol. 13 (1) (2002) 29–42, https://doi.org/10.1081/ABIO-120005768.

[8] E.C. Regulation (, No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on Additives for Use in Animal Nutrition (Text with EEA Relevance), vol. 268, 2003. http://data.europa.eu/eli/reg/2003/1831/oj/eng. accessed 2021-10-13.

[9] F. Zhao, L. Chen, L. Yang, S. Li, L. Sun, X. Yu, Distribution, dynamics and determinants of antibiotics in soils in a peri-urban area of yangtze river delta, eastern China, Chemosphere 211 (2018) 261–270, https://doi.org/10.1016/j.chemosphere.2018.07.162.

[10] X. Yu, H. Liu, C. Pu, J. Chen, Y. Sun, L. Hu, Determination of multiple antibiotics in leafy vegetables using QuEChERS-UHPLC-MS/MS, J. Separ. Sci. 41 (3) (2018) 713–722, https://doi.org/10.1002/jssc.201700798.

[11] J. Scaria, K.V. Anupama, P.V. Nidheesh, Tetracyclines in the environment: an overview on the occurrence, fate, toxicity, detection, removal methods, and sludge management, Sci. Total Environ. 771 (2021), 145291, https://doi.org/10.1016/j.scitotenv.2021.145291.

[12] Y. Feng, W.-J. Zhang, Y.-W. Liu, J.-M. Xue, S.-Q. Zhang, Z.-J. Li, A simple, sensitive, and reliable method for the simultaneous determination of multiple antibiotics in vegetables through SPE-HPLC-MS/MS, Molecules 13 (2018).

[13] B. Halling-Sørensen, S. Nors Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Holten Lützhøft, S.E. Jørgensen, Occurrence, fate and effects of pharmaceutical substances in the environment- A review, Chemosphere 36 (2) (1998) 357–393, https://doi.org/10.1016/S0045-6535(97)00354-8.

[14] K.J. Greenlees, L.G. Friedlander, A. Boxall, Antibiotic residues in food and drinking water, and food safety regulations, in: Chemical Analysis of Antibiotic Residues in Food, John Wiley & Sons, Ltd, 2011, pp. 111–123, https://doi.org/10.1002/9781118067208.ch3.

[15] Z. Li, X. Xie, S. Zhang, Y. Liang, Negative effects of oxytetracycline on wheat (Triticum aestivum L.) growth, root activity, photosynthesis, and chlorophyll contents, Agric. Sci. China 10 (10) (2011) 1545–1553, https://doi.org/10.1016/S1671-2927(11)60150-8.

[16] A. Van Epps, L. Blaney, Antibiotic residues in animal waste: occurrence and degradation in conventional agricultural waste management practices, Curr. Pollut. Rep. 2 (3) (2016) 135–155, https://doi.org/10.1007/s40726-016-0037-1.

[17] M.B.M. Ahmed, A.U. Rajapaksha, J.E. Lim, N.T. Vu, I.S. Kim, H.M. Kang, S.S. Lee, Y.S. Ok, Distribution and accumulative pattern of tetracyclines and sulfonamides in edible vegetables of cucumber, tomato, and lettuce, J. Agric. Food Chem. 8 (2015).

[18] S.-C. Kim, K. Carlson, Quantification of human and veterinary antibiotics in water and sediment using SPE/LC/MS/MS, Anal. Bioanal. Chem. 387 (4) (2007) 1301–1315, https://doi.org/10.1007/s00216-006-0613-0.

[19] A.B.A. Boxall, P. Johnson, E.J. Smith, C.J. Sinclair, E. Stutt, L.S. Levy, Uptake of veterinary medicines from soils into plants, J. Agric. Food Chem. 54 (6) (2006) 2288–2297, https://doi.org/10.1021/jf053041t.

[20] M. Pan, L.M. Chu, Transfer of antibiotics from wastewater or animal manure to soil and edible crops, Environ. Pollut. 231 (2017) 829–836, https://doi.org/10.1016/j.envpol.2017.08.051.

[21] K. Kumar, S.C. Gupta, S.K. Baidoo, Y. Chander, C.J. Rosen, Antibiotic uptake by plants from soil fertilized with animal manure, J. Environ. Qual. 34 (6) (2005) 2082–2085, https://doi.org/10.2134/jeq2005.0026.

[22] D.H. Kang, S. Gupta, C. Rosen, V. Fritz, A. Singh, Y. Chander, H. Murray, C. Rohwer, Antibiotic uptake by vegetable crops from manure-applied soils, J. Agric. Food Chem. 61 (42) (2013) 9992–10001, https://doi.org/10.1021/jf404045m.

[23] X. Hu, Q. Zhou, Y. Luo, Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China, Environ. Pollut. 158 (9) (2010) 2992–2998, https://doi.org/10.1016/j.envpol.2010.05.023.

[24] A.R. Batchelder, Chlortetracycline and oxytetracycline effects on plant growth and development in soil systems, J. Environ. Qual. 11 (4) (1982) 675–678, https://doi.org/10.2134/jeq1982.00472425001100040023x.

[25] E. Marinou, V. Samanidou, I. Papadoyannis, Development of a high pressure liquid chromatography with diode array detection method for the determination of four tetracycline residues in milk by using QuEChERS dispersive extraction, Separations 6 (2) (2019) 21, https://doi.org/10.3390/separations6020021.

[26] J. Chen, G.-G. Ying, W.-J. Deng, Antibiotic residues in food: extraction, analysis, and human health concerns, J. Agric. Food Chem. 67 (27) (2019) 7569–7586, https://doi.org/10.1021/acs.jafc.9b01334.

[27] C.R. Anderson, H.S. Rupp, W.-H. Wu, Complexities in tetracycline analysis—chemistry, matrix extraction, cleanup, and liquid chromatography, J. Chromatogr. A 1075 (1–2) (2005) 23–32, https://doi.org/10.1016/j.chroma.2005.04.013.

[28] L.-J. Zhou, G.-G. Ying, S. Liu, J.-L. Zhao, F. Chen, R.-Q. Zhang, F.-Q. Peng, Q.-Q. Zhang, Simultaneous determination of human and veterinary antibiotics in various environmental matrices by rapid resolution liquid chromatography–electrospray ionization tandem mass spectrometry, J. Chromatogr. A 1244 (2012) 123–138, https://doi.org/10.1016/j.chroma.2012.04.076.

[29] A. Gajda, A. Posyniak, Tetracyclines and their epimers in animal tissues by high-performance liquid chromatography, Bulletin Veterin. Inst. Pulawy 53 (2) (2009) 263–367.

[30] Z. Ye, H.S. Weinberg, M.T. Meyer, Trace analysis of trimethoprim and sulfonamide, macrolide, quinolone, and tetracycline antibiotics in chlorinated drinking water using liquid chromatography electrospray tandem mass spectrometry, Anal. Chem. 79 (3) (2007) 1135–1144, https://doi.org/10.1021/ac060972a.

[31] A.A.M. Stolker, M. Danaher, Sample preparation: extraction and clean-up, in: Chemical Analysis of Antibiotic Residues in Food, John Wiley & Sons, Ltd, 2011, pp. 125–152, https://doi.org/10.1002/9781118067208.ch4.

[32] M. Anastassiades, S.J. Lehotay, D. Štajnbaher, F.J. Schenck, Fast and Easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce, J. AOAC Int. 86 (2) (2003) 412–431, https://doi.org/10.1093/jaoac/86.2.412.

[33] T. Rejczak, T. Tuzimski, A review of recent developments and trendsin the QuEChERS sample preparation approach, Open Chem. 13 (1) (2015), https://doi.org/10.1515/chem-2015-0109.

[34] T. Tuzimski, A QuEChERS-based sample preparation method for the analysis of 5-nitroimidazoles in bovine milk by HPLC–DAD, J. AOAC Int. 100.6 (2017) 1671–1680, https://doi.org/10.5740/jaoacint.17-0213.

[35] Y.B. Ho, M.P. Zakaria, P.A. Latif, N. Saari, Simultaneous determination of veterinary antibiotics and hormone in broiler manure, soil and manure compost by liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1262 (2012) 160–168, https://doi.org/10.1016/j.chroma.2012.09.024.

[36] W.H. Tsai, T.C. Huang, J.J. Huang, Y.H. Hsue, Dispersive solid-phase microextraction method for sample extraction in the analysis of four tetracyclines in water and milk samples by high-performance liquid chromatography with diode-array detection, J. Chromatogr. A 1216 (12) (2009) 2263–2269, https://doi.org/10.1016/j.chroma.2009.01.034.

[37] A. Wilkowska, M. Biziuk, Determination of pesticide residues in food matrices using the QuEChERS methodology, Food Chem. 125 (3) (2011) 803–812, https://doi.org/10.1016/j.foodchem.2010.09.094.

[38] Z. He, Y. Wang, Y. Xu, X. Liu, Determination of antibiotics in vegetables using QuEChERS-based method and liquid chromatography-quadrupole linear ion trap mass spectrometry, Food Anal. Methods 11 (10) (2018) 2857–2864, https://doi.org/10.1007/s12161-018-1252-8.

[39] Commission Implementing Regulation (EU), 2021/808 of 22 March 2021 on the Performance of Analytical Methods for Residues of Pharmacologically Active Substances Used in Food-Producing Animals and on the Interpretation of Results as Well as on the Methods to Be Used for Sampling and Repealing Decisions 2002/657/EC and 98/179/EC (Text with EEA Relevance, vol. 180, 2021. http://data.europa.eu/eli/reg_impl/2021/808/oj/eng. accessed 2022-03-17.

[40] A. Kruve, R. Rebane, K. Kipper, M.-L. Oldekop, H. Evard, K. Herodes, P. Ravio, I. Leito, Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part II, Anal. Chim. Acta 870 (2015) 8–28, https://doi.org/10.1016/j.aca.2015.02.016.

[41] F. Merlo, D. Centenaro, F. Maraschi, A. Profumo, A. Speltini, Green and efficient determination of fluoroquinolone residues in edible green fruits and leafy vegetables by ultrasound-assisted extraction followed by HPLC-MS/MS, Molecules 27 (19) (2022) 6595, https://doi.org/10.3390/molecules27196595.

[42] S. Kurbanoglu, B. Uslu, S.A. Ozkan, Chapter 4 - validation of analytical methods for the assessment of hazards in food, in: A.M. Grumezescu, A.M. Holban (Eds.), Food Safety and Preservation, Academic Press, 2018, pp. 59–90, https://doi.org/10.1016/B978-0-12-814956-0.00004-4.

[43] The FEM Method Validation Team, Validation and peer review of U.S. Environmental protection agency chemical methods of analysis, The EPA Forum on Environmental Measurements (FEM), https://www.epa.gov/sites/default/files/2016-02/documents/chemical_method_guide_revised_020316.pdf, 2016.

[44] B. Magnusson, U. Örnemark, Eurachem Guide: the Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, second ed., 2014.

[45] Y. Liang, M.B. Denton, R.B. Bates, Stability studies of tetracycline in methanol solution, J. Chromatogr. A 827 (1) (1998) 45–55, https://doi.org/10.1016/S0021-9673(98)00755-9.

[46] A. Sunyer-Caldú, M.S. Diaz-Cruz, Development of a QuEChERS-based method for the analysis of pharmaceuticals and personal care products in lettuces grown in field-scale agricultural plots irrigated with reclaimed water, Talanta 230 (2021), 122302, https://doi.org/10.1016/j.talanta.2021.122302.

[47] M. Hashmi, B. Escher, M. Krauss, I. Teodorović, W. Brack, Effect-directed analysis (EDA) of danube river water sample receiving untreated municipal wastewater from novi sad, Serbia, Sci. Total Environ. 624C (2018) 1072–1081, https://doi.org/10.1016/j.scitotenv.2017.12.187.

[48] A.R. Long, L.C. Hsieh, M.S. Malbrouh, C.R. Short, S.A. Barker, Matrix solid-phase dispersion (MSPD) isolation and liquid chromatographic determination of oxytetracycline, tetracycline, and chlortetracycline in milk, J. AOAC Int. 73 (3) (1990) 379–384, https://doi.org/10.1093/jaoac/73.3.379.

[49] B. González-Gaya, L. Cherta, L. Nozal, A. Rico, An optimized sample treatment method for the determination of antibiotics in seawater, marine sediments and biological samples using LC-TOF/MS, Sci. Total Environ. 643 (2018) 994–1004, https://doi.org/10.1016/j.scitotenv.2018.06.079.

[50] W. Zhou, S. Yang, P.G. Wang, Matrix effects and application of matrix effect factor, Bioanalysis 9 (23) (2017) 1839–1844, https://doi.org/10.4155/bio-2017-0214.

[51] B.O.E. es, L. Doue, *Reglamento (CE) nº 889/2008 de la Comisión, de 5 de septiembre de 2008, por el que se establecen disposiciones de aplicación del Reglamento (CE) nº 834/2007 del Consejo sobre producción y etiquetado de los productos ecológicos, con respecto a la producción ecológica, su etiquetado y su control*, 2008, 81848, https://www.boe.es/buscar/doc.php?id=DOUE-L-2008-81848. accessed 2022-09-22.

[52] Residue Evaluation of Certain Veterinary Drugs, Joint FAO/WHO Expert Committee on Food Additives, vol. 357, 2018. Rome.