Chromatographia (2013) 76:651–662
DOI 10.1007/s10337-013-2444-4

ORIGINAL

Investigation of Matrix Effects on the Determination of Carbadox and Olaquindox in Feed by LC–MS/MS

Dorina Bodi · Christiane Ringling · Caroline Schödel · Angelika Preiß-Weigert · Hildburg Fry

Received: 24 October 2012 / Revised: 21 February 2013 / Accepted: 4 March 2013 / Published online: 22 March 2013

Abstract A comprehensive survey of matrix effects on the LC–MS/MS analysis of the banned antibiotic growth promoters carbadox and olaquindox in feed was carried out. Various factors of sample preparation procedure and measurement were systematically investigated by pre- and post-extraction addition and postcolumn infusion experiments. In general, strong signal suppression up to 70 % for carbadox and up to 90 % for olaquindox was observed when using different extraction solvents and techniques as well as different chromatographic conditions. Reduction of matrix effects was achieved by SPE clean-up and dilution of sample extracts. Nevertheless, matrix effect profiles determined by postcolumn infusion revealed, that reduction of signal suppression at a respective retention time cannot guarantee improvement of the methods performance. If high variability of matrix effects is present along the chromatographic run, accuracy might decrease despite reduced signal suppression. Besides method parameters, different feedingstuffs were investigated and showed similar matrix effects.

Keywords LC–MS/MS · Matrix effects · Ion suppression · Feed · Olaquindox · Carbadox

Electronic supplementary material The online version of this article (doi:10.1007/s10337-013-2444-4) contains supplementary material, which is available to authorized users.

D. Bodi (✉) · C. Ringling · C. Schödel · A. Preiß-Weigert · H. Fry
NRL Feed Additives, Federal Institute for Risk Assessment, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany
e-mail: dorina.bodi@bfr.bund.de

Introduction

The antibiotics carbadox (3-(2-quinoxalinylmethylene) carbazic acid methyl ester *N*,*N*′-dioxide) and olaquindox (*N*-(2-hydroxyethyl)-3-methyl-2-quinoxalinecarboxamide 1,4-dioxide) have been registered as growth promoters in feed for piglets and were used for the prevention and treatment of dysenteric diseases in swine 1. The usage of these substances as antibacterial growth promoters had been criticized because of the involved risk of resistant microbial strain formation [1]. Besides, carbadox (CBX, Fig. 1a) and olaquindox (OQX, Fig. 1b) are suspected to be mutagenic, carcinogenic and photoallergenic [1–3]. Due to these harmful effects, the authorization of both quinoxalines for the use as feed additives according to Council Directive 70/524/EEC [4] was withdrawn in 1998 by Commission Regulation (EC) No. 2788/98 [5]. Up to now no acceptable daily intake (ADI) or maximum residue limit (MRL) have been established for the growth promoters [6]. The banning of CBX and OQX as feed additives in the European Union (EU) consequently has led to a zero tolerance of the substances in feed and food. Adequate determination methods for feed and food matrices are required for official control. According to Commission Decision 2002/657/EC [7] requirements for methods for the control of residues of certain substances as CBX and OQX include minimum required performance limits (MRPL). Decision limits (CCα) and detection capabilities (CCβ) or limits of detection (LOD) and limits of quantification (LOQ), respectively, have to be below the MRPL.

Several techniques have been applied for the determination of CBX and OQX in feed and animal tissues. Gas chromatography–mass spectrometry [8, 9], high performance liquid chromatography (HPLC) UV [10–12], and increasingly liquid chromatography–tandem mass spectrometry (LC–MS/MS)

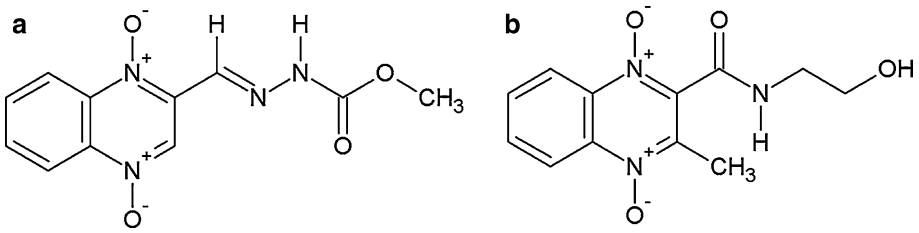

Fig. 1 Structures of **a** CBX and **b** OQX

[13–18] are used for detection. Today, LC–MS/MS can be marked as the method of choice for CBX and OQX analysis, because of its selectivity and sensitivity. Collaborative studies of different analytical methods (microbiological inhibition, high-voltage electrophoresis, thin-layer chromatography, HPLC–UV, and LC–MS/MS) for the control of antimicrobial growth promoters in feed were carried out in the frame of the SIMBAG-FEED project [19]. The appointed MRPL for OQX and CBX in feedingstuffs was 3 and 4 mg kg^{-1}, respectively. The results of the studies revealed the advantages of LC–MS/MS. However, repeatability and reproducibility relative standard deviations of LC–MS/MS methods were higher than the values required according to Commission Decision 2002/657/EC. Furthermore, multi-level standard addition (MLSA) was determined to be necessary for quantitative analysis of CBX and OQX in feed due to variability of the sample composition and resulting matrix effects (ME) [19].

This outcome accorded with the difficulties in LC–MS/MS analysis in complex matrices as feed materials, which have been frequently described as ME [20, 21]. Signal suppression or enhancement as a result of co-elution of matrix compounds and analyte molecules can affect the accuracy, precision and capacity of the method of analysis [20]. These ME are commonly explained by the competition of molecules for ionization in the atmospheric pressure ionization (API) source. The ionization efficiency of analyte molecules depends on the amount of co-eluting matrix and analyte molecules and also on their physicochemical properties. The extent of ME is influenced both by physicochemical properties of the analytes and matrix components, as well as by the type (electrospray ionization, atmospheric pressure chemical ionization) [22] and geometry (z-spray, orthogonal spray and linear spray) of the ion source [23]. Previously published studies concerning ME differentiate between endogenous (originating from the sample material) and exogenous (introduced during sample preparation and clean-up) interfering substances [24]. Examination of ME may help to reveal the source and nature of substances influencing the analysis. This knowledge may support the optimization of sample preparation and measurement.

The investigation of ME has become a mandatory part of method development and validation, especially for LC–MS/MS methods [25]. Post-extraction addition and postcolumn infusion are two main techniques, which are used to assess the influence of matrix components [26]. The first technique determines the influence of the matrix on the analyte signal at its retention time. Therefore, the responses of the analyte in fortified blank sample extracts (post-extraction) and in solutions in pure solvent are compared to each other. Matuszewski et al. calculated the matrix effect by dividing the response of the spiked extract by the response of the pure solution and multiplying the result with 100. They introduced two more parameters into the discussion of ME: recovery and process efficiency. Results of the post-extraction addition and the analyte in pure solvent are compared to a third spike experiment, where the equivalent amount of analyte is added to a blank sample before the extraction procedure (pre-extraction addition) [27]. The other prominent approach is the post-column infusion technique, which is used to explore the matrix influence on the analyte signal over the whole chromatographic run [20]. A standard solution is infused by a syringe pump or auxiliary pump postcolumn into the chromatographic flow. The injection of a blank matrix extract onto the chromatographic column induces a change in the analyte signal by affecting the ionization efficiency in the ion source. The chromatographic profile of the matrix injection is compared to a reference profile of pure mobile phase. This experiment can provide an image of the ME caused by different extraction procedures, but also of chromatographic columns, mobile phase compositions and additives. Besides the investigation of ME the postcolumn infusion technique can also be used to compensate their influence on the analyte signal. As described by Choi et al. [28], permanent postcolumn infusion of an isotopically labelled internal standard may be adopted to correct signal suppression or enhancement during quantitative analysis.

The current paper describes investigations of ME on LC–MS/MS analysis of the banned antibiotics CBX and OQX in feed. During method development, we observed considerable variability of the analytical results due to ME.

In order to choose an adequate sample clean-up procedure for an in-house determination method, we systematically investigated the influence of the sample material on the measurement. Analyte concentrations were in the mg kg^{-1} range to avoid effects originating from the lack of LC–MS/MS sensitivity. Post-extraction addition and

postcolumn infusion experiments were carried out to compare the influence of different analytical columns, mobile phases, extraction solvents and techniques. Different sample preparation procedures like clean-up and dilution of sample extracts were examined. The ability of the applied measures for the compensation of matrix effect was compared and possible set screws in the analytical process to influence ME were identified. Finally, variability of ME caused by different feedingstuffs was investigated with regard to applicable quantification approaches.

Materials and Methods

Reagents and Materials

LC–MS-grade methanol and acetonitrile, *N*,*N*-dimethylformamide (p.a.), isooctane (p.a.) and formic acid (p.a.) were purchased from Merck (Darmstadt, Germany). We obtained *n*-hexane, *n*-pentane and ethylacetate picograde for residue analysis, isopropanol gradient grade for LC from Promochem (Wesel, Germany) and acetic acid glacial from Roth (Karlsruhe, Germany). All water used was deionized and purified by a TKA Water Purification System (TKA, Niederelbert, Germany). Mobile phase additives ammonium acetate (p.a.) and ammonium formate (p.a.) were from Fluka (Buchs, Switzerland) and VWR (Darmstadt, Germany). Isolute HM-N from Biotage (Uppsala, Sweden) and Sea Sand for analysis from Merck were used as dispersants for the accelerated solvent extraction. Solid phase extraction (SPE) cartridges Alumina N Plus (120 Å, 50–300 μm, 1,720 mg) and Oasis HLB 3 cc (60 mg) were from Waters (Milford, MA, USA). Samples were filtered using centrifuge filters with 0.45 μm nylon membrane from VWR.

The analytical standards carbadox (>99 % purity) and olaquindox (>95.8 % purity) were obtained from Sigma-Aldrich (Seelze, Germany). Standard stock solutions 1 mg mL^{-1} were prepared in brown glass volumetric flasks by dissolving 10 mg of CBX and OQX, respectively, in 10 mL of dimethylformamide by 10-min ultrasonication. Mixed standard solutions and dilutions were prepared in acetonitrile. The postcolumn infusion solution (27.5 ng mL^{-1}) was prepared in mobile phase containing 50 % aqueous buffer and 50 % organic solvent (methanol or acetonitrile) corresponding to the used gradient.

Apparatus

The LC–MS/MS system used consisted of an Agilent 1200 Series HPLC (autosampler, degasser, quaternary pump and column oven) and a 4000 QTrap quadrupole tandem mass spectrometer (ABSciex, Ontario, Canada). A Havard Apparatus syringe pump 11 plus with gastight 1 and 5 mL syringes (Hamilton Bonaduz-AG, Switzerland) was used to introduce standard solutions directly into the mass spectrometer for optimizing mass spectrometric parameters and for postcolumn infusion experiments. Mass spectrometric parameters were optimized by direct infusion of 100 ng mL^{-1} solutions of the analytes in 5 mM ammonium acetate buffer and acetonitrile (50/50 v/v) into the mass spectrometer. Extraction of the feed samples was carried out using an accelerated solvent extractor ASE 200 with solvent controller and compressor model 6-4 (Dionex, Sunnyvale, CA, USA) or an ultrasonic bath.

LC–MS/MS Analysis

The atmospheric pressure ion source was operated in positive electrospray mode. Three transitions of the protonated molecules to their specific product ions were chosen per analyte for multiple reaction monitoring (MRM). The parameters declustering potential (DP), and collision energy (CE) were optimized as follows: CBX1 *m/z* 263.2 → 231.0 (DP = 66 V, CE = 19 V), CBX2 *m/z* 263.2 → 129.0 (DP = 66 V, CE = 45 V), CBX3 *m/z* 263.2 → 103.0 (DP = 66 V, CE = 47 V), OQX1 *m/z* 264.2 → 143.1 (DP = 61 V, CE = 45 V), OQX2 *m/z* 264.2 → 221.0 (DP = 61 V, CE = 21 V) and OQX3 *m/z* 264.2 → 212.1 (DP = 61 V, CE = 31 V). General ion source parameters were optimized by flow injection analysis (without analytical column, at 200 μL min^{-1} flow rate) to the following: entrance potential = 10 V, curtain gas = 20 psi, collision gas = medium, ion spray voltage = 4,500 V, source temperature = 650 °C, gas 1 (nebulizer gas) = 30 psi, gas 2 (heating gas) = 55 psi. All gas supplies are nitrogen of 5.0 purity. LC–MS/MS data were acquired and analysed with Analyst 1.4.2/1.5 software. Three different chromatographic columns were used, an Ascentis Express C18 150 × 4.6 mm, 2.7 μm particle size (Supelco, Bellefonte, PA, USA), an Aqua 3 u C18 125 A 75 × 2.0 mm, 3.0 μm particle size and a Luna 3 u HILIC 150 × 3.0 mm, 3 μm particle size (Phenomenex, Aschaffenburg, Germany), each column with a respective pre-column filter or guard cartridge. Experiments for comparison of sample preparation procedures and sample material were measured using the Ascentis Express column at 50 °C with ammonium formate buffer (5 mM) as mobile phase A and methanol as mobile phase B. The injection volume was 5 μL. Separation was achieved at 50 °C column oven temperature and a flow rate of 200 μL min^{-1} using the following gradient: 0–2 min 50 % B, 2–6 min increased to 90 % B and maintained for 4 min, from 10 to 12 min B was returned to initial conditions and kept for further 6 min for equilibration of the column. Other mobile phases and gradient programs were used to examine the influence of chromatographic separation on ME.

Sample Material

The main part of experiments was carried out using a CBX and OQX-free complete feed for fattening pigs (blank material). In addition, a complementary feed for dairy cows, a compound feed for weaning piglets, and a complete feed for laying hens were examined. Prior to extraction, the feed samples were ground to a particle diameter of 1 mm. The ground feed was homogenized by shaking for at least 1 h.

Sample Preparation

The following extraction solvents were used and compared for accelerated solvent extraction (ASE) and ultrasonic extraction (USE): acetonitrile (ACN), ACN containing 2 % formic acid, methanol (MeOH), ACN/MeOH (3/7) (v/v), acetonitrile/methanol (1/1), ACN/MeOH (7/3), ACN/MeOH (7/3) (v/v) containing 2 % formic acid, and ACN/MeOH/H_2O (1/1/1) (v/v/v). For ASE, 7.5 g of feed sample was weighed in a beaker. To avoid aggregation of the sample during ASE, 2.5 g Isolute HM-N was added and the mixture was filled into an extraction cell which was sealed by a glass fibre filter. The applied amount of dispersant was adjusted to fill the extraction cell completely. ASE was performed at 80 °C and 103 bar with 5 min preheating time and 5 min extraction time. One and two extraction cycles were tested. The resulting extraction solutions were centrifuged at 3,000×*g* and 10 °C for 10 min. Supernatants were brought to a standardized volume of 25 mL by evaporation under a nitrogen stream. Aliquots of 500 µL were evaporated to dryness and reconstituted in the same volume of mobile phase [ammonium formate/MeOH (50/50) (v/v)]. Finally, the sample solution was centrifuge filtered at 14,000×*g* (1 min) for LC–MS/MS measurement.

For ultrasonic extraction, 7.5 g of feed sample was weighed in a beaker and 20 mL of extraction solvent (solvents according to ASE) was added. The suspension was sonicated for 10 min and transferred into a polyethylene centrifuge tube afterwards. The beaker was flushed with 5 mL extraction solvent, and combined solutions were centrifuged at 3,000×*g* and 10 °C for 10 min. The residue was re-extracted with 4 mL extraction solvent and also centrifuged. Combined supernatants were made up to 25 mL. Aliquots were prepared for measurement as described for pre- and post-extraction addition experiments with ASE.

Clean-Up by LLE

Several nonpolar organic solvents (*n*-hexane, *n*-pentane and isooctane) were used for LLE to remove fat from the raw ASE extracts. For LLE, equivalent volumes (2.5 mL) of the ASE extract and defattening solvent were combined and vortex mixed for 15 s. Separation of the two phases was achieved by centrifugation at 470×*g* and 10 °C for 5 min. The phase of defattening solvent was removed and LLE was repeated. The remaining solution was prepared for LC–MS/MS as described above.

Clean-Up by SPE

Two solid phase materials, Oasis HLB and Alumina N, were compared for sample clean-up. Oasis HLB cartridges were conditioned with 2 mL of MeOH and MeOH/H_2O [5/95 (v/v)]. Aliquots (500 µL) of ASE extracts were evaporated to dryness under a nitrogen stream (45 °C; heated metal block) and reconstituted in 500 µL MeOH/H_2O (5/95 (v/v)). Reconstituted sample extracts were loaded onto the SPE cartridge. Test tubes were rinsed with 500 µL MeOH/H_2O (5/95 (v/v)), which was also added to the cartridge followed by a washing step with 2 mL of MeOH/H_2O (5/95 (v/v)). OQX and CBX were eluted from the SPE column separately, using 5 mL of MeOH/H_2O ((20/80) (v/v)) for OQX and 5 mL of MeOH/H_2O (50/50 (v/v)) for CBX. Eluates were evaporated to dryness, dissolved in 500 µL HPLC mobile phase (ammonium formate/MeOH (50/50) (v/v)), and centrifuge filtered (14,000×*g*, 1 min) before analysis.

Alumina N SPE cartridges were conditioned using 5 mL of MeOH before loading the sample, which was prepared as described for the Oasis HLB cartridges. Both analytes were eluted from the cartridge with 5 mL of MeOH/H_2O (50/50 (v/v)). The eluate was treated and prepared for analysis as described for Oasis HLB SPE.

Dilute and Shoot

The dilute-and-shoot approach is based on the theory that dilution of a sample extract and thereby reduction of the matrix concentration has a beneficial effect on the analyte signal despite the reduced analyte concentration. Sample extracts of both pre- and post-extraction addition experiments were diluted by factor 2, 5 and 10 with pure mobile phase [MeOH/H_2O (50/50 (v/v))] resulting in analyte concentrations of 0.5, 0.2, and 0.1 µg mL^{-1}. Standard solutions in pure mobile phase containing the respective analyte concentration were prepared as well.

Pre- and Post-Extraction Addition Experiments

For spiking of samples for pre-extraction addition experiments, standard solution was added directly onto the feed sample before the extraction procedure. Samples were vortex mixed and were allowed to stand for 10 min. For post-extraction addition the blank sample extracts were fortified. Therefore, the standard solution was added to the aliquots of blank feed extraction solution. Aliquots of blank

extract were added to the respective amount of standard solution to receive the intended fortification levels in final solutions. Pre- and post-extraction addition experiments were carried out for accelerated solvent extracts without clean-up and after liquid–liquid extraction (LLE) or solid phase extraction (SPE).

According to Matuszewski et al. [27], three analytical sets have to be prepared, including a standard line in pure mobile phase, a standard line in blank matrix extract (post-extraction addition) and a standard line of blank samples spiked pre-extraction. Comparison of the slopes of these standard lines can indicate the effect of matrix and sample preparation on the analyte response. The approach can also show a possible dependence of ME from the analyte concentration. This entire experimental approach of pre- and post-extraction addition was carried out exemplarily for ASE with acetonitrile/methanol 1:1. Samples were fortified at six concentration levels, which were 0.05, 0.5, 1.0, 1.5, 2.0 and 2.5 μg mL^{-1} in solution according to 0.17, 0.33, 3.33, 5.0, 6.67, and 8.33 mg kg^{-1} in the feed sample. Other extraction techniques and solvents were examined by means of one spiking concentration of 1.0 μg mL^{-1} (3.33 mg kg^{-1}) for pre- and post-extraction addition. Further experiments were performed with a single fortification level of 1.0 μg mL^{-1} (3.33 mg kg^{-1}). Standard solutions and samples spiked pre- and post-extraction were prepared and measured in triplicate for each experiment. Results from pre- and post-extraction experiments were used to calculate the parameters recovery (RE) and matrix effect (ME) by means of Eqs. (1) and (2).

$$\text{RE}\ (\%) = \frac{\text{Response pre-extraction}}{\text{Response post-extraction}} \times 100\,\% \quad [27] \qquad (1)$$

$$\text{ME}\ (\%) = \left(\frac{\text{Response post-extraction}}{\text{Response pure standard solution}} \times 100\,\%\right) - 100\,\% \quad [29] \qquad (2)$$

For calculation of the ME, we adopted the approach of Stahnke et al. [29]. Therefore, Matuszewski's ME [27] is subtracted by 100 % resulting in a bipolar term with negative values for signal suppression and positive values for enhancement.

Postcolumn Infusion Experiments

Postcolumn infusion was performed according to King et al. [30]. The standard solution contained 0.275 μg mL^{-1} CBX and OQX and was introduced via a T-piece at a flow rate of 20 μL min^{-1} into the 200 μL min^{-1} HPLC-flow after the analytical column. Figure 2 shows the instrumental setup to generate infusion chromatograms and matrix effect profiles.

The analyte concentration in the postcolumn infusion solution was prepared to correspond to the absolute amount of analyte resulting from the injection of 5 μL of a 1 μg mL^{-1} standard mix pre-column into the HPLC. Two measurements were performed to calculate the matrix effect profile for each sample material. The first step was the injection of pure mobile phase representing the reference signal and was followed by injection of the blank matrix extract obtained using different extraction techniques and solvents, clean-up procedures and sample material. According to Stahnke et al., the response at each retention time i of sample and reference chromatograms were smoothed using Eq. (3) with a smoothing factor f of 0.7. To generate the ME profiles the ME of each measured data point (ME-PCI_i) of the quantifier transitions (CBX1 and OQX1) was calculated using the smoothed responses as described in Eq. (4).

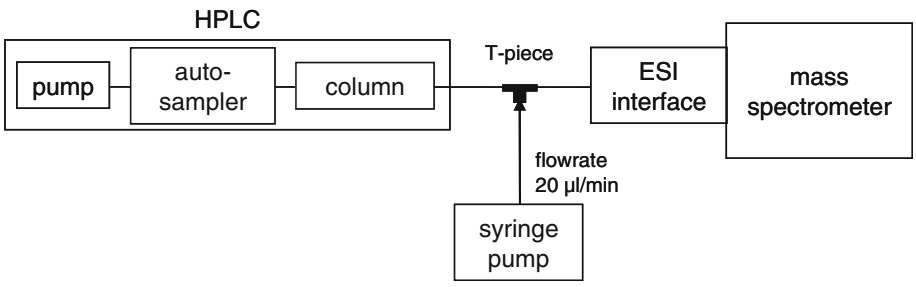

Fig. 2 Schematic diagram of the postcolumn infusion system

$$\text{Smoothed response}_i = (1-f)\cdot \text{response}_i + f \cdot \text{response}_{i-1} \quad [29] \tag{3}$$

$$\text{ME-PCI}_i\,(\%) = \left(\frac{\text{Smoothed response}_i \text{ blank matrix extract}}{\text{Smoothed response}_i \text{ solvent (reference)}} \times 100\,\%\right) - 100\,\% \quad [29]. \tag{4}$$

Three matrix effect profiles were generated for each experiment, the resulting mean ME profiles are presented in the respective figures. For comparison to post-extraction addition ME the mean postcolumn infusion ME at the retention times (±0.2 min) of CBX and OQX were calculated.

Results and Discussion

ME and RE of Different Extraction Procedures and Solvents

ME and RE for the set of 6 concentration levels using ASE with ACN/MeOH (50/50, v/v) were calculated according to Eqs. (1) and (2) are shown in Table 1. RE of the extraction method was satisfying for both analytes with mean values of 84.9 % (CBX) and 89.2 % (OQX). In general, both CBX and OQX responses are strongly affected by suppression, though OQX more than CBX. A slight dependence of ME on the analyte concentration in the extracts was demonstrated, as signal suppression decreased for CBX from −82 % (0.05 μg mL^{-1}) to −61 % (2.5 μg mL^{-1}) and for OQX from −92 % (0.05 μg mL^{-1}) to 86 % (2.5 μg mL^{-1}) with increasing concentrations. According to proposed mechanisms of ME, analyte molecules and co-eluting matrix molecules are competing for ionization in the electrospray [21]. With increasing analyte concentrations, the ratio of analyte molecules to other molecules increases, which is likely to cause higher ionization rates of the analyte. However, detection of the analytes in pure solvent is highly sensitive causing responses which may reach the saturation threshold of the detector. A lack of linearity of the standard lines in pure a solvent may simulate a decrease of matrix effect.

Table 1 Matrix effect (ME) and recovery (RE) for samples after ASE with acetonitrile/methanol 1:1 ($n = 3$ for each level)

Concentration (μg mL^{-1})	CBX		OQX	
	ME (%)	RE (%)	ME (%)	RE (%)
0.05	−81.6	86.6	−92.3	78.0
0.5	−74.0	84.4	−91.1	101.7
1.0	−69.1	81.8	−91.0	104.1
1.5	−65.9	82.3	−88.1	87.3
2.0	−63.5	85.0	−86.9	81.1
2.5	−60.6	89.2	−86.2	83.0
Mean		84.9		89.2

ME and RE of samples from other extraction solvents, techniques and clean-up procedures were examined at only one fortification level (1 μg mL^{-1}).

Accelerated Solvent Extraction

Application of one and two extraction cycles with the same total volume of extraction solvent was tested. RE and ME slightly increased (up to 6 % higher RE and higher signal suppression) from single to double extraction (data not shown). Further tests were performed with two extraction cycles. Different extraction solvents for ASE were compared concerning ME and RE. Results for selected extraction solvents are shown in Fig. 3a for CBX and Fig. 3b for OQX.

RE ranged from 78 to 92 % for CBX and from 60 to 95 % for OQX. Highest RE were achieved using acetonitrile–methanol mixtures for ASE, but strong signal suppression was observed. In general, signal suppression for OQX was about 20–30 % higher than for CBX. MeOH, ACN/MeOH and ACN/MeOH/H_2O caused strong ME for CBX (−55 to −73 %) and OQX (−80 to −89 %). Lowest ME were observed with pure ACN extraction and ACN/MeOH 7/3 v/v (only with USE) for both analytes. One reason might be the reduced protein concentration in ACN extracts, which is due to the protein precipitating properties of ACN [31]. In order to increase protein precipitation and extraction efficiency, additional tests were carried out with acidified extraction solvents [ACN and ACN/MeOH (7/3) containing 2 % formic acid]. Compared to the plain ACN, RE was improved (>80 % for CBX, >75 % for OQX) and ME increased by 15 % for CBX and by 12 % for OQX with acidified ACN (Table 2). Extraction efficiency may have improved generally with formic acid and therefore extracted amounts of both, analytes and matrix compounds, have increased. Addition of formic acid to ACN/MeOH 7/3 did not improve RE and only ME for CBX was reduced from −53 to −41 %. Overall, addition of 2 % formic acid to the extraction solvents did not result in significant reduction of ME.

Ultrasonic Extraction

RE of CBX and OQX using ultrasonic extraction with different solvents did not exceed 60 % (see Fig. 3),

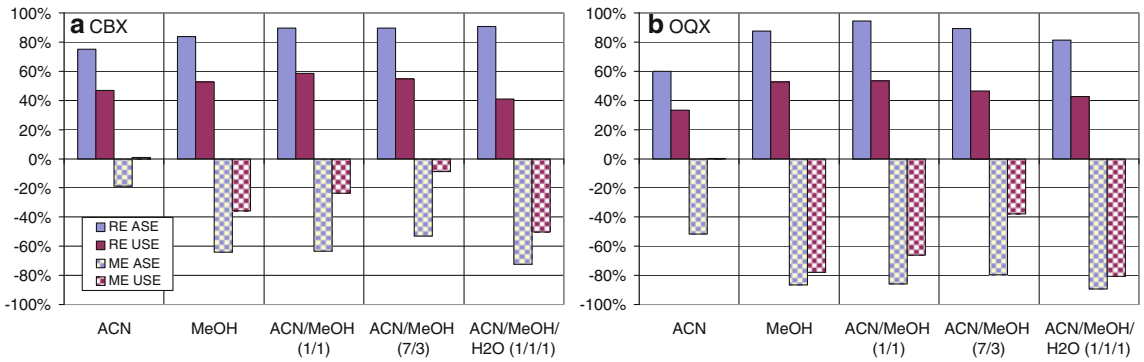

Fig. 3 Recovery (*RE*) and matrix effect (*ME*) in % for **a** CBX and **b** OQX in feed samples after accelerated solvent extraction (ASE *blue columns*) and ultrasonic extraction (USE *purple columns*) using different extraction solvents. Pre- and post-extraction addition at 1.0 µg mL^{-1} (3.33 mg kg^{-1}) ($n = 3$)

Table 2 Matrix effect (ME) and recovery (RE) of samples after ASE: comparison of extraction solvents with and without formic acid (FA), $n = 3$

Extraction solvent	CBX		OQX	
	ME ± RSD (%)	RE (%)	ME (%)	RE (%)
ACN	−18.8	75.2	−51.5	59.8
ACN + 2 % FA	−34.2	83.2	−63.5	78.6
ACN/MeOH 7:3	−53.1	89.8	−79.8	89.3
ACN/MeOH 7:3 + 2 % FA	−40.7	91.1	−79.6	90.2

indicating that extraction at room temperature and standard pressure was less effective than at high temperature and/or high pressure (ASE). Especially high extraction temperatures proved to be essential for extraction efficiency with regard to unwanted matrix components when comparing different methods pressurized liquid extraction for environmental and biological matrices [32]. However, ME observed with USE were similar to or lower than those with ASE. Strongest ME occurred with water containing solvent mixtures (< −50 % for CBX, < −80 % for OQX). USE with ACN induced no ME for both analytes in contrast to ASE with −20 % for CBX and −50 % for OQX. Weak ion suppression of less than −10 % for CBX and less than −40 % for OQX occurred with USE and ACN/MeOH 7/3, which was considerably low compared to ASE using this solvent mixture (−50 % for CBX; −80 % for OQX). ME for OQX was generally higher than for CBX for using USE, which is the same as with ASE.

ME Profiles

ME profiles of CBX and OQX were nearly congruent in all experiments. Figure 4a shows the infusion chromatograms of the quantifier transitions of CBX and OQX of pure ACN/MeOH 1/1 (reference) and a blank sample extract (ASE with ACN/MeOH 1/1) injection. Matrix effect profiles of CBX and OQX derived from these chromatograms are shown in Fig. 4b. A summary of the infusion chromatograms and ME profiles of all transitions of CBX and OQX for this extraction (ASE using ACN/MeOH 1/1) is provided in the supplementary material.

Presentation of the profiles of both analytes for the following results is therefore not necessary, and profiles of CBX are shown exemplarily. Similarity of matrix effect profiles, even of structurally different substances, in the same sample matrix was already described by Stahnke et al. [29]. Although matrix effect values varied between extraction techniques (Fig. 5a, b) and solvents, the relative course of the profiles was similar.

During the first 6 min of each run the profiles ranged from −40 to 25 % matrix effect. At 7.2 min retention time a characteristic drop in the profile up to −100 % ME appeared. This strong signal suppression is due to the increased percentage of methanol in the mobile phase which causes elution of high amounts of interfering matrix compounds from the column. Afterwards the profile returned to lower degrees of signal suppression. In the last section of the run again several distinct peaks of signal suppression occurred.

Although both analytes elute from the column at a mobile phase composition of 90 % methanol and 10 % buffer, the signal of olaquindox is more affected than the CBX signal. This confirmed results from post-extraction addition.

Reduction of ME by Modified Chromatography?

The potential of changing chromatographic conditions to reduce ME should be verified. Therefore, methanol and acetonitrile mobile phases and HPLC columns of different polarity were compared. Postcolumn infusion and post-extraction addition experiments were carried out exemplarily for one blank matrix extract (ASE using ACN containing 2 % FA). Due to the different properties of MeOH and ACN, retention of the analytes from the same column

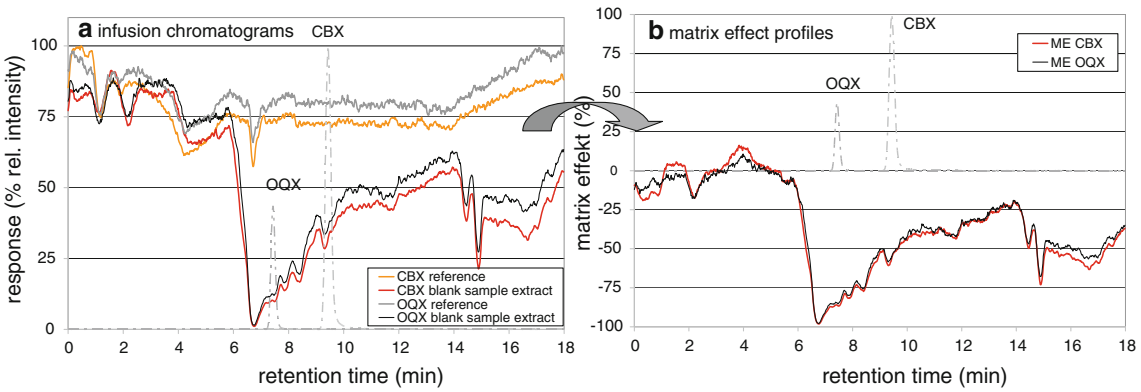

Fig. 4 Generation of matrix effect profiles **a** infusion chromatograms of reference and blank matrix injection of CBX and OQX, **b** resulting matrix effect profiles of CBX and OQX in feed extracted with ACN/MeOH 1/1 (v/v) using ASE

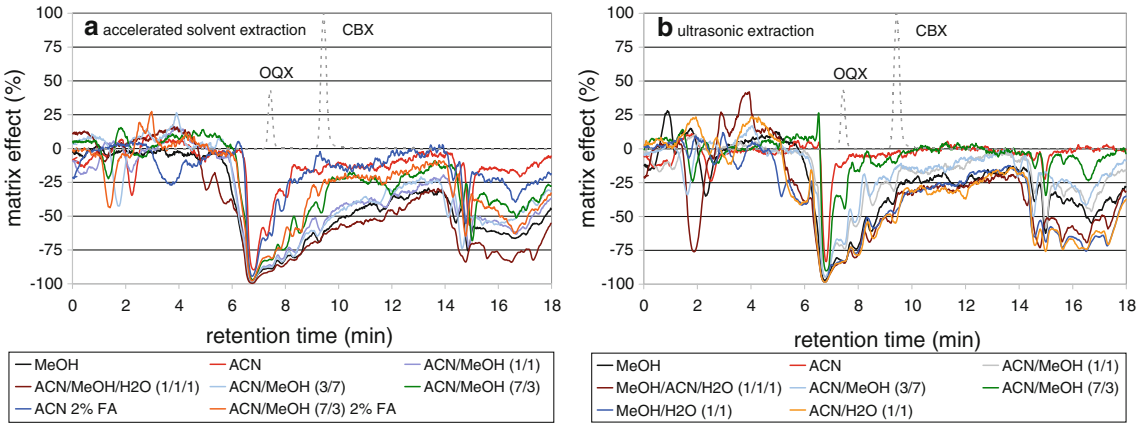

Fig. 5 ME profiles (exemplarily for CBX) in feed for fattening piglets using different extraction solvents for **a** ASE and **b** USE

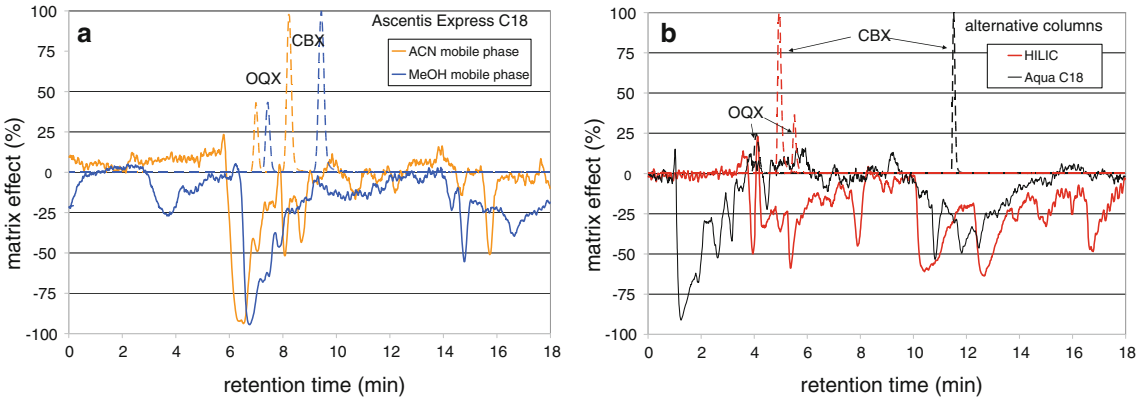

Fig. 6 ME profiles of CBX in feed at modified chromatography conditions **a** mobile phase with methanol and acetonitrile and **b** stationary phase: HILIC and Aqua C18 (mobile phase: ammonium formate buffer and methanol)

varied strongly when using the same composition of organic solvent and buffer. The gradient program with ACN was optimized to receive similar retention times for the analytes as with the MeOH mobile phase system. Matrix effect profiles for both mobile phases (Fig. 6a) were very similar besides the remaining shift of retention time. Furthermore, with ACN mobile phase several distinct peaks of signal suppression occurred in addition to the main ME peak between 6 and 8 min retention time.

Resulting alternation of ME from peak-shaped suppression areas to those with no suppression might be disadvantageous for the methods precision. If slight

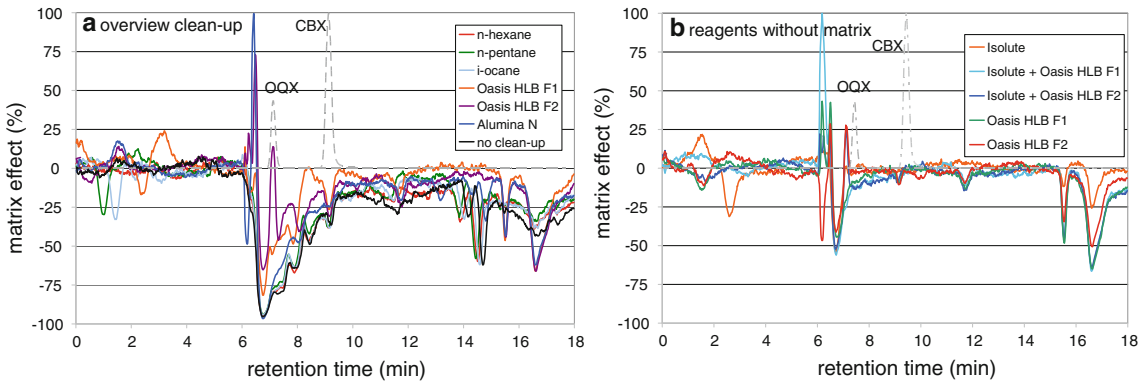

Fig. 7 ME profiles of CBX **a** in feed extracts after LLE, SPE and without clean-up and **b** in pure solvent after ASE (Isoluth) and/or SPE Oasis HLB [elution fractions F1 (OQX) and F2 (CBX)]

chromatographic changes can cause considerable loss or gain of signal intensity, analytical results might not meet required quality criteria. With regard to robustness the methanolic mobile phase should be preferred. ME determined by post-extraction addition corresponded to those determined in postcolumn infusion experiments.

Two more stationary phases were tested concerning their ability to separate matrix components from the analytes. A reversed phase C18 column (Luna Aqua) with silica-based particles of conventional technology and a HILIC (hydrophilic interaction liquid chromatography) column were compared to the Ascentis Express C18. As demonstrated by Fig. 6b, matrix components and analytes were at least separated differently on the Aqua C18 column. Both C18 columns showed similar matrix effect profiles with a 6-min shift of retention time. On the Aqua C18 the main broad band of signal suppression occurred at 1–4 min retention time. OQX was eluted after this suppression area, which was in contrast to the retention on Ascentis Express. CBX eluted during a second matrix effect interval, which occurred in the last third of the HPLC run. ME for CBX and OQX using the Aqua C18 were therefore opposed to those using Ascentis Express.

Using the HILIC column elution order of CBX and OQX was reversed and the matrix effect profile was affected by numerous broad and distinct matrix effect peaks of signal suppression. Due to the change of ME in the interval of elution of the analytes, deviating ME may even more affect the methods precision. Overall, the Ascentis Express C18 column with a methanolic mobile phase is to prefer over the other tested columns.

Reduction of ME by Sample Clean-Up: Defattening

As lipid components are known to cause ME [24], three nonpolar organic solvents were tested to remove fat from the feed sample extracts. *n*-Hexane, *n*-pentane, and isooctane were used for LLE of the crude extracts.

Table 3 SPE recovery of CBX and OQX in feed extracts after SPE (Alumina N or Oasis HLB), total RE for extraction and Oasis HLB SPE and matrix effect (ME) in feed extracts without and after SPE clean-up (ASE extraction with ACN containing 2 % formic acid), $n = 3$

SPE cartridge	RE SPE (%)		RE total (%)		ME (%)	
	CBX	OQX	CBX	OQX	CBX	OQX
Alumina N	77.9	56.4	–	–	−32.8	−73.4
Oasis HLB	73.3	78.3	68.2	73.2	−27.2	−62.9
No SPE	–	–	83.2	78.6	−34.2	−63.5

Pre- and post-extraction addition experiments (data not presented) showed that LLE with these solvents lead to reduction of analyte RE. ME were slightly reduced (CBX) or remained constant (OQX) in comparison to the crude extracts. With *n*-hexane ME for CBX even increased. ME profiles of defatted feed extracts are shown in Fig. 7a compared to those of without clean-up samples and after solid phase extraction (SPE). As there was no difference observed between profiles of untreated and defatted samples, LLE with nonpolar solvents proved to be ineffective for reduction of ME.

Reduction of ME by Sample Clean-Up: Solid Phase Extraction

Two SPE materials (Alumina N and Oasis HLB) were tested to clean-up sample extracts. Pre- and post-extraction experiments were modified in order to investigate RE of the SPE step and ME of the entire sample preparation procedure. Blank sample extracts were spiked directly before SPE an compared to samples spiked post-extraction to evaluate the RE of the clean-up step exclusively. Loss of the analytes occurred resulting in reduced RE, but ME was reduced by 11.3–34.0 % compared to untreated samples, dependent on solid phase and analyte (Table 3). Alumina N material was rejected for further tests, because of poor RE

for OQX (54.6 %). RE of the total sample preparation procedure was determined for the Oasis HLB cartridge. For OQX RE was similar to extraction without SPE, while RE of CBX was 15 % lower with SPE. Signal suppression was only reduced for CBX (7 % less suppression) by the clean-up step.

Blank samples cleaned-up by SPE (Alumina N, Oasis HLB) were investigated by postcolumn infusion to generate ME profiles. As CBX and OQX were eluted separately from the Oasis cartridge using different methanol/water compositions (20 and 50 % methanol), one ME profile was determined for each elution fraction (Fig. 5a). The SPE-profiles differ from the crude extract's profile in two aspects. The central ion suppression area is of lower intensity (Oasis HLB) or returns faster to the normal level (Alumina N). Second, several narrow peaks of signal enhancement emerged, reaching 75 up to 100 % positive matrix effect. Results achieved by postcolumn infusion were in the same range as those from post-extraction addition (Table 3) for CBX, but differed about 20 % ME for OQX. In sum, sample clean-up by Oasis HLB SPE did not result in significant reduction of ME.

Exogenous ME

Sample preparation steps are performed to extract the analyte from the sample material and to remove possible interfering substances before analysis. Thus, it is known that the used reagents can be the source of compounds affecting the analyte signal. To identify ME originating from sample preparation in the present study, the sample preparation procedure was carried out with pure solvent and examined by means of postcolumn infusion. The determined ME profiles (Fig. 7b) revealed, that exogenous ME were present causing both signal suppression and enhancement. Figure 7b demonstrates that these ME did not affect the signals of CBX and OQX at their respective retention times. By means of the ME profiles SPE cartridges can be identified as a possible source of interfering substances compared to reagents from the extraction procedure including the dispersant Isolute HM-N.

Reduction of ME by Dilution of Extracts?

Due to the sensitivity of LC–MS/MS detection, dilution of the crude sample extracts is a possible approach to reduce ME [33]. Application of this technique, which is referred to as dilute-and-shoot, is reasonable, at least if an analyte concentration well above the method's LOQ is present in the sample. To evaluate this method for reduction of ME 2-, 5- and 10-fold diluted ASE (ACN with 2 % formic acid) extracts spiked pre- and post-extraction (1 ng mL^{-1} or 3.3 mg kg^{-1}, respectively) were analysed and compared to respective standard solutions. Table 4 shows the decrease of matrix effect and constant recoveries with increasing dilution factor. Dilution by a factor of 10 reduced ME by 20 % for CBX and by 29 % for OQX. The dilute-and-shoot approach is therefore applicable for quantification of OQX and CBX in feed samples in the concentration range of the MRPL (CBX, 4 mg kg^{-1}; OQX, 3 mg kg^{-1}). In addition, Table 4 shows stronger signal suppression (20–30 %) compared to the respective ME values in Tables 2 and 3 although sample preparation was the same. Deviation of RE values is within an acceptable range (±10 %). This day-to-day variation can be due to variability of the chromatographic system and the daily condition of the LC–MS/MS instrument.

Table 4 Matrix effect (ME) and recovery (RE) of the dilute-and-shoot approach of ASE extracts (extraction solvent acetonitrile with 2 % FA), $n = 3$

Dilution factor	CBX		OQX	
	ME (%)	RE (%)	ME (%)	RE (%)
–	−61.2	88.9	−84.7	88.6
2	−53.3	89.9	−80.7	90.1
5	−47.7	90.7	−71.5	89.2
10	−41.5	91.3	−56.5	88.8

ME in Different Feedingstuffs

Composition of the sample material is an important factor influencing the measurement. Three additional feedingstuffs, including a complementary feed for dairy cows, a compound feed for weaning piglets, and a complete feed for laying hens were examined in comparison to the feed for fattening pigs, which was used for the ME studies. ME

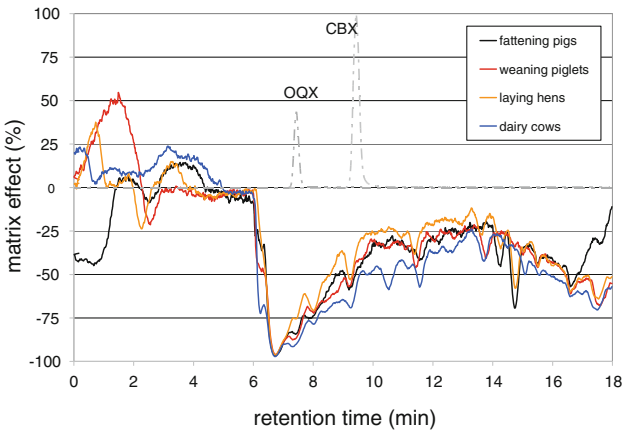

Fig. 8 ME profiles (exemplarily for CBX) of different feedingstuffs

profiles of the four feedingstuffs are shown in Fig. 8. The feed for dairy cows caused highest signal suppression although ME profiles of all feed materials were similar. Determination of ME profiles in different feedingstuffs should also give information about the possibility for the correction of ME in quantitative analysis. Quantification via matrix calibration or matrix-matched standards requires not only a blank feed, but also a feed causing similar ME as the analysed sample. As a matter of fact, not every examined feed is also available as a blank feed and an individual matrix calibration cannot be prepared for each sample. Analytes have to be quantified in similar feed matrices using a representative blank feed for calibration to reduce the analytical effort. As ME profiles in Fig. 6 are quite similar, correction of ME during analysis of CBX and OQX might be possible. Each of these feed samples could be a potential blank feed for preparation of a matrix calibration curve for the other feedingstuffs.

Conclusion

In this study, ME during LC–MS/MS analysis of CBX and OQX in feed were investigated comprehensively. The results revealed to which extent ME may be influenced by the change of certain steps of the sample preparation procedure and chromatographic conditions. Variation of the extraction technique and the extraction solvent, both had considerable impact on the degree of signal suppression. ASE using ACN containing 2 % formic acid gave best results concerning ME and RE in our study. Sample clean-up using SPE and LLE did not lead to significant reduction of ME. SPE clean-up is nevertheless recommendable for analysis of the substances in low concentration levels, especially if enrichment of the analytes is necessary. As CBX and OQX are banned for use in animal feed, resulting in a zero tolerance in feed, very low levels have to be detected for monitoring and official feed control in the EU. The method described by Wu et al. [18] using Oasis HLB cartridges for clean-up achieved LOQs of 10 and 20 μg kg^{-1} for CBX and OQX, respectively. However, concentrations of CBX and OQX in the range of the substances MRPL (CBX, 4 mg kg^{-1}; OQX, 3 mg kg^{-1}) can be quantified without difficulty when using modern LC–MS/MS technique. Dilution of sample extracts is therefore an adequate method to reduce ME.

For the quantification of CBX and OQX in feed samples matrix calibration is obligatory, as correction of ME is needed. Appropriate blank matrices of similar composition as the investigated sample are therefore required. ME determined in different feedingstuffs were similar. This demonstrates that quantification via matrix calibration using different compound or complete feedingstuffs as a blank material is possible. Some complementary feedingstuffs, for example, mineral feed, have to be regarded separately. In this case, quantification could be performed using the standard addition approach.

ME profiles of feed samples after different sample preparation demonstrated that ME may change strongly during a short period of retention time. This deviation of ME is more likely to cause poor accuracy of a determination method than constantly strong ion suppression.

References

1. JECFA (FAO/WHO) (1991) WHO Technical Report Series 799
2. JECFA (FAO/WHO) (1995) WHO Technical Report Series 851
3. JECFA (FAO/WHO) (2003) WHO Technical Report Series 918
4. European Council (1970) Off J Eur Commun L270:1–17
5. European Commission (1998) Off J Eur Commun L347:31–32
6. JECFA (FAO/WHO) and Codex Committee on Residues of Veterinary Drugs in Foods (2011) Agenda Item 10
7. European Commission (2002) Off J Eur Commun L221:8–36
8. Lynch MJ, Mosher FR, Schneider RP, Fouda HG, Risk JE (1991) J Assoc Off Anal Chem 74:611–618
9. Lynch MJ, Bartolucci SR (1982) J Assoc Off Anal Chem 65:66–70
10. Gizzi G, Vincent U, Von Holst C, De Jong J, Genouel C (2007) Food Addit Contam 24:1226–1235
11. Song JM, Qiao XG, Chen HH, Zhao DY, Zhang Y, Xu ZX (2011) J Sci Food Agric 91:2378–2385
12. Wu YJ, Wang YL, Huang L, Tao YF, Yuan ZH, Chen DM (2006) Anal Chim Acta 569:97–102
13. Boison JO, Lee SC, Gedir RG (2009) Anal Chim Acta 637:128–134
14. Fuh MRS, Chan SA, Wang HL, Lin CY (2000) Talanta 52:141–151
15. Hutchinson MJ, Young PB, Kennedy DG (2005) Food Addit Contam 22:113–119
16. Hutchinson MJ, Young PY, Hewitt SA, Faulkner D, Kennedy DG (2002) Analyst 127:342–346
17. Miao XS, March RE, Metcalfe CD (2003) Int J Mass Spectrom 230:123–133
18. Wu CM, Li Y, Shen JZ, Cheng LL, Li YS, Yang CY, Feng PS, Zhang SX (2009) Chromatographia 70:1605–1611
19. Vincent U, Gizzi G, von Hoist C, Zuidema T, De Jong J (2005) SIMBAG FEED Project Report 1-91
20. Taylor PJ (2005) Clin Biochem 38:328–334
21. Gosetti F, Mazzucco E, Zampieri D, Gennaro MC (2010) J Chromatogr A 1217:3929–3937
22. Dams R, Huestis MA, Lambert WE, Murphy CM (2003) J Am Soc Mass Spectrom 14:1290–1294
23. Mei H, Hsieh YS, Nardo C, Xu XY, Wang SY, Ng K, Korfmacher WA (2003) Rapid Commun Mass Spectrom 17:97–103
24. Antignac JP, de Wasch K, Monteau F, De Brabander H, Andre F, Le Bizec B (2005) Anal Chim Acta 529:129–136
25. Annesley TM (2003) Clin Chem 49:1041–1044
26. Cappiello A, Famiglini G, Palma P, Pierini E, Termopoli V, Trufelli H (2008) Anal Chem 80:9343–9348
27. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Anal Chem 75:3019–3030
28. Choi BK, Gusev AI, Hercules DM (1999) Anal Chem 71:4107–4110
29. Stahnke H, Reemtsma T, Alder L (2009) Anal Chem 81:2185–2192

30. King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T (2000) J Am Soc Mass Spectrom 11:942–950
31. Souverain S, Rudaz S, Veuthey JL (2004) J Pharm Biomed Anal 35:913–920
32. Runnqvist H, Bak SA, Hansen M, Styrishave B, Halling-Sorensen B, Bjorklund E (2010) J Chromatogr A 1217:2447–2470
33. Stahnke H, Kittlaus S, Kempe G, Alder L (2012) Anal Chem 84:1474–1482