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Determination of Carbadox and metabolites of Carbadox and Olaquindox in muscle tissue using high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A sensitive and robust LC–APCI-MS/MS method has been developed for the unambiguous detection and quantitative determination of the antimicrobial agent Carbadox, its metabolite quinoxaline-2-carboxylic acid and methyl-3-quinoxaline-2-carboxylic acid the major metabolite of Olaquindox. The method was aimed for application in the assaying of muscle tissue so the developed sample preparation scheme subjected samples to enzymatic digestion prior to the application of solid phase extraction clean-up. Subsequently the purified extracts were analyzed by reversed-phase LC–MS/MS in positive APCI and multiple reaction monitoring mode. The method was validated at a level of 1 μg/kg. The decision limits CCα and detection capability CCβ ranged from 0.09 μg/kg to 0.24 μg/kg and from 0.12 μg/kg to 0.41 μg/kg, respectively. The accuracy and precision of the method were satisfactory. The recoveries ranged from 92% to 101% for the metabolites and from 60% to 62% for Carbadox, with coefficient of variances (CVs) less than 12%. The developed method proved efficient and straightforward allowing positive identification and quantitation of the target banned analytes and is thus suitable for application in residue control programmes and metabolism studies.

1. Introduction

Quinoxaline-2-carboxylic acid (QCA) and methyl-3-quinoxaline-2-carboxylic acid (MQCA) are the marker residues of Carbadox (CBX) and Olaquindox (OLQ) which have been used as antimicrobial drugs to control swine dysentery and bacterial enteritis in young swine and as growth promoters to improve feed efficiency and to increase the rate of weight gain [1]. Due to health concerns over possible carcinogenic, mutagenic and photoallergenic effects of the drugs and their mono (MCBX) and desoxy (DCBX) metabolites [2,3], the use of CBX and OLQ has been banned in EU since 1998 [4]. QCA is the major remaining detectable residue in animal tissues and thus was considered as the marker compound for monitoring CBX in livestock animal production [5]. Olaquindox is a similar quinoxaline-*N*-dioxide drug used in veterinary medicine, and is metabolized to methyl-3-quinoxaline-2-carboxylic acid (MQCA), which has a structure similar to QCA [6]. MQCA is the last major remaining detectable residue of OLQ, and therefore was considered as the marker compound for the drug [7]. The structure of CBX and OLQ and their marker compounds QCA and MQCA is shown in Fig. 1.

Recently, the health concerns and the ongoing use of these compounds in some countries have been stated at the 18th session of the Codex Committee on Residues of Veterinary Drugs in Foods (2009) [8]. A concentration of 10 μg/kg has been recommended as Minimum Required Performance Limit (MRPL) according to the EURL (European Union Reference Laboratory Fougères-France, responsible for carbadox-B2f residue group) for both QCA and MQCA in meat [9]. Monitoring for use of these compounds is carried out through the National Residue Control Plans of the individual EU-Member States [10]. Therefore, it is necessary to have sensitive, simple and reliable analytical methods applicable to meat samples.

A number of methods have been reported for the markers and metabolites of Carbadox and Olaquindox and the literature has been recently covered by a review paper [11]. Methods utilizing high performance liquid chromatography (HPLC) with UV detection [12–14], gas chromatography coupled to electron capture and mass spectrometry detection (GC–MS) [15–19] and LC–MS/MS detection [20–23], have been published describing the analysis of the metabolites of CBX and OLQ in muscle and/or liver tissues. However, only one HPLC-UV [14] and two LC–MS/MS methods [21,22] describe the simultaneous analysis of both QCA and MQCA.

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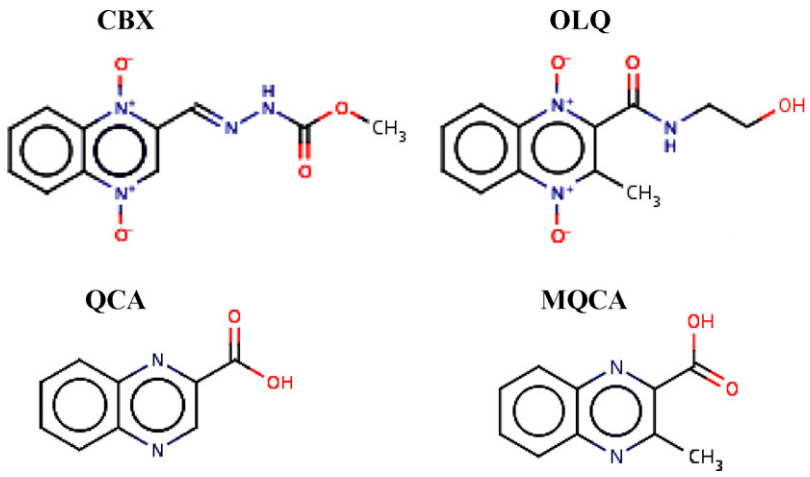

Fig. 1. Structures of CBX and OLQ and their marker compounds QCA and MQCA.

Studies of Carbadox metabolism revealed that DCBX persists much longer in animal tissues and based on these findings an LC–MS/MS method was developed for the determination of DCBX, QCA and MQCA [22]. An enzyme-linked immunosorbent assay was developed for monitoring QCA in edible animal tissues by HPLC-UV [24]. A sensitive method has been developed for the determination of MQCA in fish tissue by UPLC–MS/MS [25]. LC–MS/MS and HPLC-UV techniques have also been applied for the developed of methods in other matrices than meat, such as, animal feed [26–29] and plasma [30,31]. In feeding stuff, mostly Carbadox and Olaquindox are monitored using solid–liquid extraction (SLE) [26], SLE and SPE [27], matrix solid-phase dispersion (MSPD) [28] and recently molecular imprinted solid phase (MISPE) extraction clean-up steps [29]. Cyadox (CYX) and its main metabolites (bisdesoxycyadox (BDCYX) and QCA) were determined in animal plasma by deproteinization with organic solvent and injection to the HPLC system [30,31]. After administration CBX is metabolized in a certain time frame and the drug residues are dispersed in different levels in various body tissues: higher concentration are found in liver (44.7 ± 27.0%), lower in kidney (14.5 ± 4.9%) and muscle (6.7 ± 2.5%) and even lower amounts in fat (<2%) [32]. The clearance of Carbadox from blood, liver, kidney and muscle of pigs after 72 h of withdrawal has been independently confirmed using liquid chromatography (lower than 2 μg/kg), depending on the initial amount of oral administration [33].

To the best of our knowledge there is no published method describing the determination of both CBX and the main markers of CBX and OLQ in animal tissues. So the principle aim of the present study was to develop a very sensitive and specific method for the determination of Carbadox and the key metabolite residues (QCA and MQCA) in animal tissues.

Further to this most of the published methods utilize time consuming and rather tedious extraction schemes such as liquid–liquid extraction (LLE). When processing muscle tissue LLE may often lead to the formation of emulsions, thus increasing error measurement and do not offering potential for automation and the handling of large number of samples for routine analytical purposes. Hence our aim was to develop a simpler and quicker sample preparation procedure which could be amenable to automation. We chose to involve hydrolysis and sample clean-up on two SPE cartridges connected in series. Subsequent sample analysis by LC–MS/MS employing two product ions per one precursor ion facilitated high method specificity and sensitivity as well as excellent reproducibility and linearity allowing use of the method in routine analysis and metabolism studies.

2. Experimental

2.1. Chemicals and materials

Carbadox was obtained from Chiron AS (Trondheim, Norway), quinoxalaline-2-carboxylic acid (QCA) and methyl-3-quinoxaline-2-carboxylic acid (MQCA) were obtained from Dr Ehrenstorfer GmbH (Augsburg, Germany). The Internal standard, deuterated quinoxaline carboxylic acid (QCA-d4) was obtained from Sigma (Sigma–Aldrich, Steinhem, Germany). Methanol (HPLC grade), formic acid (p.a), acetic acid (98–100%) and tris(hydroxymethyl)aminomethane were obtained from Merck (Darmstadt, Germany), protease Subtilisin was from Sigma (Sigma–Aldrich, Steinhem, Germany), ammonia (25%) and hydrochloric acid (37%) were from Panreac (Barcelona, Spain). Purified water was prepared with a Pure Lab system (Sation 9000, Spain). Oasis HLB (60 mg, 3 ml) cartridges were purchased from Waters (Milford, MA, USA) and Amino Supelclean NH_2 (500 mg, 3 ml) cartridges from Supelco (Bellenfonte, IL, USA).

The Tris buffer was prepared as follows: 12.1 g of Tris was dissolved in 800 ml of water. The pH was adjusted to 9.5 by using a solution of HCl acid (1 M) and water was added to a final volume of 1000 ml. Stock solutions for all standard compounds were prepared in methanol at a concentration of 1 mg/ml. Working standard solutions were prepared by diluting the stock solutions with methanol. All standard solutions were stored at −20 °C.

2.2. Samples

Porcine and bovine muscle as well as porcine liver samples, 100 g from each sample, were obtained from slaughterhouses, were simultaneously cut and mixed (homogenized) with a B-400 homogenization mixer (BUCHI, Flawil, Switzerland) and stored at −20 °C. Samples were analyzed for the presence of the analytes and were used as negative and quality controls.

2.3. Equipment

A Surveyor MS pump Plus coupled with a Finnigan Surveyor autosampler plus was used for separation. For detection a triple-quadruple Thermo Electron TSQ Quantum AM mass spectrometer was used (Thermo Electron, San Jose, CA, USA). Xcalibur software data system enabled instrument control and efficient data acquisition, data processing and results delivery.

2.4. LC–MS/MS analysis conditions

Chromatographic separation was achieved on a 5 μm Hypersil ODS column (150 mm × 4.6 mm, Thermo Electron) protected with a 5 μm Prevail C18 guard column (7.5 mm × 4.6 mm, Alltech) and maintained at a constant column temperature of 30 °C. The mobile phase consisted of a mixture of water with 1% acetic acid as solvent A and methanol with 1% acetic acid as solvent B. Gradient elution was performed at a flow rate of 0.7 ml/min. At the start, the mobile phase was composed of 90% solvent A and 10% of solvent B (v/v). These conditions were kept for 8 min. Over the 5 next min the mobile phase composition was changed in a linear mode to 10% A:90% B (v/v), where the conditions were kept isocratic for 3 min. For the equilibration of the column the initial conditions were run for 4 min between each injection cycle. Injection volume was 20 μl. The ionization of the analytes was carried out using positive ion Atmospheric Pressure Chemical Ionization (APCI) mode. The Multiple Reaction Monitoring (MRM) scheme involved transitions of the precursor ions to two selected products ions.

The capillary temperature was maintained at 320 °C, the vaporizer temperature at 450 °C, the discharge current at 7 μA, the nitrogen sheath gas flow rate at 50 and the auxiliary gas at 0 (both arbitrary units). The argon collision gas pressure was set at 1.5 mTorr. For the unit resolution, the mass peak full width at half its maximum height was set to 0.7 for both quadrupoles Q1 and Q3. The scan time was set at 500 ms for each MRM channel.

2.5. Sample preparation

Aliquots of homogenate meat sample (5.0 g) were weighed out into polypropylene tubes (50 ml) and were fortified with internal standard QCA-d4 at the concentration of 4 μg/kg. To this, 10 ml of 0.1 M Tris buffer (pH 9.5), containing 5 mg of Subtilisin-A were added. The samples were incubated for 2 h at 52 °C and, after cooling down to room temperature, were acidified with 1.5 ml 1.2 M hydrochloric acid (pH 1.8), centrifuged (4000 rpm, 5 min) and the supernatant was decanted into a clean 10 ml glass. The supernatant was loaded on an Oasis HLB cartridge, which was previously conditioned with 3 ml of methanol and 3 ml of water. After a washing step with 3 ml water, the analytes were eluted with 5 ml acetic acid/methanol (2/98, v/v). The extract was directly loaded on an amino SPE cartridge, which was previously conditioned with 5 ml acetic acid/methanol (2/98, v/v) and the first fraction (A) containing Carbadox was collected into a 10 ml glass tube containing 20 μl QCA-d4 (1 ng/μl). Then the remaining analytes QCA and MQCA were eluted (second fraction) with 3.5 ml of ammonia/methanol (1/10, v/v). The two fractions were then separately evaporated to dryness in a water bath at 55 °C under a stream of nitrogen. The two dried residues were then reconstituted with methanol (80 μl) and transferred to LC inserts in injection vials for further analysis on LC–MS/MS by separate injections. The sample preparation procedure is schematically presented in Fig. SI1 in the Electronic Supplementary material.

3. Results and discussion

3.1. Development of the LC–MS/MS methodology

Precursor-ion spectrum of each compound was acquired by direct infusion of analyte solutions (concentration of 5 ng/μl) through a syringe pump operating at a speed of 10 μl/min into the atmospheric pressure ionization source of the mass spectrometer. From the main precursor ions produced, fragmentation occurred in the second quadrupole and the suitable product ions for each analyte were selected. Furthermore, all related mass spectrometer parameters including capillary temperature, vaporizer temperature, discharge current, nitrogen gas flow, capillary voltage, collision gas pressure, collision energy were optimized by flow injection analysis to improve sensitivity. One precursor ion and two product ions were selected for each analyte in order to comply with criteria needed for a confirmatory method. The selected precursor ions, product ions and collision energy (CE) for each compound are shown at Table 1. In the published works on LC–MS analysis of CBX and its metabolites, electrospray ionization (ESI) mode is applied. We found that both ESI and APCI can be used for the ionization of the target analytes. However, APCI resulted in higher efficiency for CBX and for the acidic metabolites QCA and MQCA and was thus selected as the ionization mode for this study.

The composition of the mobile phase can also influence the performance of the ionization process. The use of acetic acid in the LC solvent improved the signal and the chromatography of the analytes compared to then utilization of formic acid or no acid at all. Furthermore with regard to the selection of organic modifier methanol and acetonitrile were tested. Methanol provided better chromatographic separation of the target analytes and was selected for the rest of the study. The gradient elution program was selected as it provided baseline resolution of the analytes with high detection signal and improved peak shapes. The selected program facilitated efficient removal of matrix constituents and as a result reduced noise level and reduced risk for carry-over effects and column deterioration.

3.2. Sample preparation

In previous studies the most commonly applied procedure for the extraction of the analytes from the muscle tissue was by acidic or alkaline hydrolysis [14,19,21–23]. Enzymatic digestion was also applied [20]. Acidic and alkaline hydrolysis procedures require special caution and eventually can lead to the generation of additional interference from matrix substances and as a result often both LLE and SPE clean-up. Boison et al. [22] used acidic hydrolysis clean-up and SPE utilizing rather large solvent volumes for the washing and eluting steps. For our work enzymatic hydrolysis with protease was preferred as an effective hydrolysis procedure rendering cleaner products.

SPE was deemed necessary for clean-up of the muscle tissue samples in order to effectively purify the tissue extracts. Because of the different structure of the compounds, a compromise was deemed necessary in order to effectively combine esters (CBX) and acids (QCA, MQCA) which by nature differ in polarity and chemical properties. Due to the presence of substituted electron withdrawing carboxylic acid in the quinoxaline ring of QCA and MQCA, an amount of 1.2 M hydrochloric acid for pH adjustment to 1.8 was added to the muscle tissue samples in order to suppress their ionization and enhance retention on the SPE. Solid phase extraction cartridges, including mix-mode Discovery DSC-18 (500 mg, 3 ml, Supelco) and Oasis HLB (60 mg, 3 ml, Waters) were tested. For the washing step different combinations of methanol and water were tested. The best choice was found to be the application of pure water as such a washing solvent was found to remove interferences and provide sample clean-up without eluting the analytes. For the elution of the analytes methanol, acetic acid and combinations of them with water were tested. When using the Oasis cartridge, 5 ml of the mixture acetic acid:methanol (2/98, v/v) as the elution solvent provided the highest recovery. Overall the Oasis cartridge gave better recoveries and more satisfactory peak shapes at the final chromatogram and was thus selected to be used for this study.

The eluate from the Oasis SPE cartridge was directly loaded on an anion-exchange SPE amino cartridge. The ion-exchange mechanism is based mainly on the electrostatic attraction of the charged functional groups of the analytes to oppositely charged

Table 1
Precursor and most abundant product ions with their collision energy.

Compound	Rt (min)	Precursor ion $(M+H)^+$	Product ions (m/z)	CE (eV)
CBX	13.44	263.06	231.05	14
			129.07[a]	30
QCA	13.01	175.06	129.05[a]	16
			102.07	29
MQCA	12.97	189.09	102.10[a]	30
			143.96	15
QCA-d4	12.95	179.10	133.09[a]	18
			106.05	31

[a] The most abundant ion (also used for quantification).

group on the amino sorbent. In order for electrostatic retention to occur, both analytes and sorbent functional groups should be in their ionized form. This was achieved through strict pH control of the sample matrix which was ranged from 7 to 8. Prior to their use the amino cartridges had been conditioned with 5 ml of a mixture of acetic acid/methanol (2/98, v/v). Flow rate control during all SPE steps was found to be important for obtaining high recovery and repeatability. Too fast a flow rate can result in low recoveries due to analyte breakthrough during sample loading step or inadequate elution during the elution step. In the washing step too high a flow rate was found to be less effective in removing interferences and thus result to "dirty" extracts. The extract from Oasis cartridge was applied at a consistent flow rate of ca. 0.5 ml/min to ensure optimal analyte retention as it was found that this flow rate was critical for consistent recovery. To elute QCA and MQCA, a solution of ammonia/methanol (1/10, v/v) pH of 11.6 was used to neutralize the functional group on the sorbent surface and to disrupt the electrostatic forces between the analytes functional groups and the functional group of the sorbent.

3.3. Validation procedure

The validation of the developed method was according to the European Commission Decision 2002/657/EC [34]. The criteria concerning the relative retention time of the analyte which shall correspond to that of the calibration solution at a tolerance of ±2.5%, was fulfilled. Moreover, all relevant ion ratios of the product ions met the criteria needed as shown in Table 2. During the validation study parameters such as accuracy, repeatability, linearity, decision limit (CCα), detection capability (CCβ), specificity and measurement of uncertainty (U) were evaluated.

The accuracy was determined by spiking the porcine muscle samples with 1, 1.5 and 2 μg/kg from each compound. Six replicates from each concentration were analyzed on 3 different occasions: Days 1, 2 and 3. The mean recovery and the coefficients of variation (CV) were calculated. For the QCA and MQCA the recovery ranged between 92% and 101% with the CVs ranging between 1.4% and 6.4%. For CBX the recovery ranged between 60% and 62% with the CVs ranging between 3.8% and 11.7%. The QCA-d4 corrected very well for any losses or matrix effect for the 2 metabolites, but did not have an essential effect on CBX. This was somewhat expected as the chemical properties of CBX and the main metabolite markers are different: CBX is a basic molecule whereas the metabolites are of acidic nature; hence they can be expected to have different ionization performance while they are eluted in a different SPE fraction during sample preparation. To overcome these issues, a second internal standard should be added and the ideal candidate would be a deuterated analog of CBX; however this is not commercially available. Nevertheless, the methods performance with regard to CBX was acceptable with CVs being satisfactory and recoveries unwavering. The results with the estimated low repeatability are given in Table 3 showing that they are satisfactory.

To study method's linearity a solvent based calibration curve was used with 12 calibration points at concentrations corresponding to 0, 0.2, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 10 and 20 μg/kg in tissue with the internal standards at a concentration of 4 μg/kg of tissue. This dynamic range is wider than the concentration ranges reported in

Table 2
Confirmation based on the ion ratio and RRT of the analytes for 6 spiked meat samples at a concentration of 1 μg/kg.

	Reference		Sample					
Sample identification	Standard		1	2	3	4	5	6
Analyte: CBX				Matrix: meat				
Ion ratio	0.783		0.724	0.756	0.703	0.725	0.776	0.792
Tolerance ion ratio ± 20%	0.627	0.940	IN	IN	IN	IN	IN	IN
RRT analyte	0.991		0.991	0.991	0.991	0.991	0.991	0.991
Tolerance RRT ± 2.5%	0.966	1.016	IN	IN	IN	IN	IN	IN
Analyte: QCA				Matrix: meat				
Ion ratio	0.449		0.522	0.502	0.505	0.462	0.518	0.518
Tolerance Ion ratio ± 20%	0.359	0.538	IN	IN	IN	IN	IN	IN
RRT analyte	0.999		0.999	1.004	1.004	0.999	1.004	1.004
Tolerance RRT ± 2.5%	0.974	1.023	IN	IN	IN	IN	IN	IN
Analyte: MQCA				Matrix: meat				
Ion ratio	0.340		0.336	0.320	0.308	0.349	0.332	0.313
Tolerance ion ratio ± 20%	0.272	0.408	IN	IN	IN	IN	IN	IN
RRT analyte	0.996		0.996	0.996	0.996	0.996	0.996	0.996
Tolerance RRT ± 2.5%	0.971	1.020	IN	IN	IN	IN	IN	IN

Table 3
Precision and accuracy for the analytes on Days 1, 2 and 3.

Compound	Spiked (μg/kg)	Day 1		Day 2		Day 3	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
CBX	1	61.6	3.8	60.8	11.7	62.1	10.1
	1.5	60.9	10.9	60.5	7.5	61.4	6.7
	2	60.3	8.7	62.4	6.5	62.3	8.9
QCA	1	99.9	6.4	98.4	3.1	99.8	2.7
	1.5	100.3	4.0	101.3	3.1	101.4	1.8
	2	100.3	3.1	98.7	3.4	100.9	2.9
MQCA	1	93.5	3.3	92.7	2.2	93.8	2.7
	1.5	92.3	1.4	95.7	3.2	94.9	2.6
	2	92.4	1.8	99.4	3.3	97.5	2.0

the literature. The correlation coefficients (r^2) were greater than 0.994 for all compounds.

Matrix-matched calibration curves of 6 points (blank, 1, 1.5, 2, 3, 5 μg/kg) from the spiked samples were constructed on three different days and standard deviations of the *y*-intercept were calculated. The CCα and CCβ values were calculated based on the procedure described in the ISO 11843 [35]. The CCα equals to the corresponding concentration at the *y*-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept. CCβ equals the corresponding concentration at the decision limit plus 1.64 times the standard deviation of the within-laboratory reproducibility of the mean measured content. The calculated values of CCα and CCβ for all analytes are shown in Table 4. The CCα ranged from 0.09 to 0.24 and the CCβ from 0.12 to 0.41 and are lower than the values already reported in the literature for LC–MS/MS methods. Hutchinson et al. [20] report CCα and CCβ values for QCA 0.16 and 0.27 μg/kg and in a more recent method [21], CCα and CCβ values at 0.4 and 1.2 μg/kg for QCA and 0.7 and 3.6 μg/kg for MQCA. Boison et al. [22] developed a method with the limit of detection (LOD) at 0.3 μg/kg and the limit of quantification (LOQ) at 0.5 μg/kg for both QCA and MQCA. Horie et al. [23] achieved limit of detection at 1 μg/kg for QCA and DCBX. This fact signifies the high sensitivity and the capability of the developed method in detecting trace levels of the compounds.

An important feature of a newly developed method is the possibility to discriminate between the target analyte and closely eluted substances that can potentially interfere in the ion trace and result in false identification and/or inaccurate quantification. The specificity of the method was evaluated through the analysis of different meat samples form the 3 experiments on Days 1, 2 and 3 and also by a fourth experiment on Day 4, including a variety of meat samples from 20 different sources. The chromatograms did not show any interference or ion traces near the retention time of the product ions for all analytes. The 20 samples were also spiked at 1 μg/kg of tissue; the developed method was found able to determine the analytes with satisfactory intensities and accuracy. Fig. 2 shows the chromatogram of a porcine meat sample spiked at a concentration of 1 μg/kg of QCA and MQCA, with the internal standard QCA-d4 at a concentration of 4 μg/kg. Fig. 3 shows the chromatogram of a spiked porcine meat sample containing CBX (1 μg/kg) with the internal standard QCA-d_4 at a concentration of 4 μg/kg.

Table 4
CCα, CCβ values and measurement of uncertainty (*U*%) for the analytes.

Compound	CCα (μg/kg)	CCβ (μg/kg)	*U* (%)
CBX	0.24	0.41	9.32
QCA	0.09	0.15	7.67
MQCA	0.12	0.20	11.96

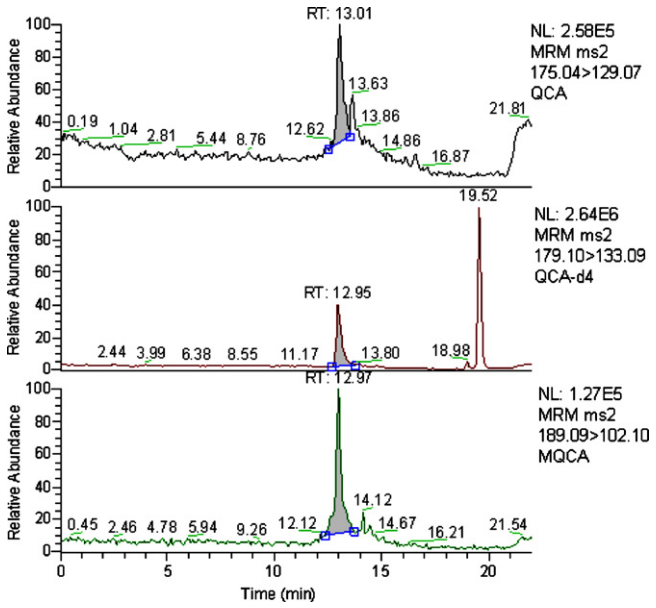

Fig. 2. MRM chromatogram (MS/MS) in APCI of a spiked porcine meat sample containing, QCA and MQCA at a concentration of 1 μg/kg, with QCA-d4 at a concentration of 4 μg/kg.

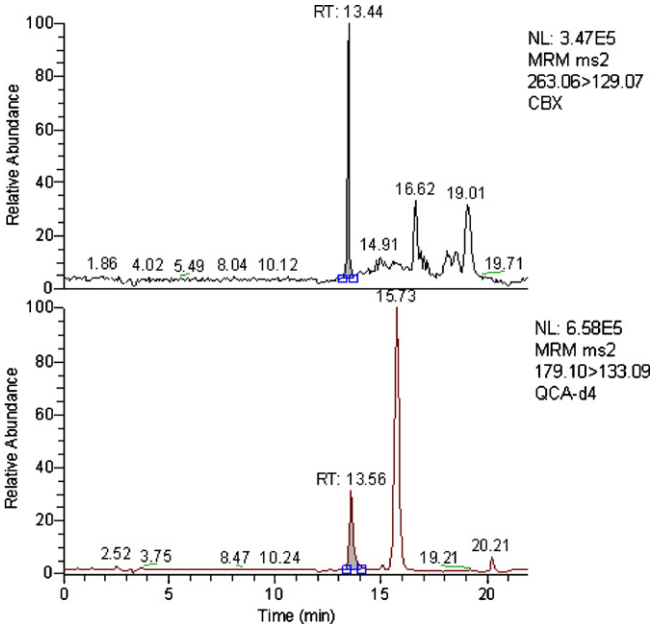

Fig. 3. MRM chromatogram (MS/MS) in APCI of a spiked porcine meat sample containing, CBX at a concentration of 1 μg/kg, with QCA-d4 at a concentration of 4 μg/kg.

In practice, the fitness for purpose of an analytical method applied for routine testing is most commonly assessed through method validation studies. Such studies can also produce data on overall performance and on individual influence factors which can be applied to the estimation of uncertainty associated with the results of the method. In order to define whether a result is compliant or non-compliant, it is necessary to take into account the measurement of uncertainty related to the calculated result. The expanded uncertainty U was measured and calculated by multiplying the combined standard uncertainty u with the coverage factor k. The combined standard uncertainty was determined based on the reproducibility variance (s_R^2) from the three experiments on Days 1, 2 and 3 [36]. The repeatability from the fourth experiment on Day 4 was also included to express the variance of the matrix effect (s_{matrix}^2) of the different animals. For a confidence level of 95% and a coverage factor of 2 the estimated measure uncertainties ($U = k \times \sqrt{s_R^2 + s_{\text{matrix}}^2}$) are shown in Table 4 [37].

3.4. Analysis of real samples

80 samples from porcine muscle and liver and bovine muscle were collected from several meat markets and directly from slaughterhouses through the Greek National Residue Control Program. The samples were analyzed by the developed method and no analytes were detected. Each batch of analysis included spiked samples at a concentration of 1 μg/kg of tissue. QC control charts were constructed containing all fortified samples, showing no results exciding two times the standard deviation (±2SD) of the mean concentration (X) and no inaccurate tensions in the graphs. The first 10 spiked samples were from porcine muscle, the next 5 from bovine muscle and the last 5 from porcine liver. The QC charts for each compound are shown in detail in Figs. SI2–SI4 in the Electronic Supplementary material. Hence the method proves to be robust and applicable to additional specimen (matrices such as bovine muscle and porcine liver).

4. Conclusions

An LC–MS/MS method allowing the determination of Carbadox, its major metabolite (QCA) and the major metabolite of Olaquindox (MQCA) in animal muscle was developed and validated. The validation was done according to predefined analytical criteria specified in the 2002/657/EC Commission Decision. The method performed satisfactorily: the accuracy and repeatability of the method were evaluated over a wide dynamic range 0.2–20 μg/kg. The CCα and CCβ values determined for each analyte were very low indicating the high sensitivity of the method. The sample preparation can be considered straightforward and efficient providing high sample throughput and automation capabilities. Application of the method to test samples showed no false negative or false positive results even after the analysis of a significant number of samples from different matrices. The method can be used for the effective routine analysis of samples under the National Monitoring Plan among the EU member states.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.12.007.

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