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Confirmation of carbadox and olaquindox metabolites in porcine liver using liquid chromatography–electrospray, tandem mass spectrometry

M.J. Hutchinson[a], P.B. Young[b], D.G. Kennedy[b,*]

[a] *Queen's University Belfast, Department of Veterinary Science, Belfast, UK*
[b] *Chemical Surveillance Department, Veterinary Sciences Division, Department of Agriculture and Rural Development for Northern Ireland, Stoney Road, Stormont, Belfast BT43SD, UK*

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Abstract

A method is described for the quantitative determination of quinoxaline-2-carboxylic acid (QCA) and methyl-3-quinoxaline-2-carboxylic acid (MQCA), the metabolites that have been designated as the marker residues for the veterinary drugs, carbadox and olaquindox, respectively, in swine tissue. The method is suitable for use as a confirmatory method under EU National Surveillance Schemes. Porcine liver samples were subjected to protease digestion followed by liquid–liquid extraction. Further clean-up was performed by automated solid phase extraction (SPE) and was followed by a final liquid–liquid extraction step. Analysis was performed using a narrow bore column HPLC coupled to electrospray MS/MS, operated in positive ion mode. MS/MS product ions were monitored at *m/z* 102 and 75 amu for QCA, *m/z* 145 and 102 amu for MQCA and at *m/z* 106 and 152 amu for the d_4-QCA and d_7-MQCA internal standards, respectively. The method has been validated at 3.0, 10, 50 and 150 $\mu g\,kg^{-1}$ for both metabolites. The method performance characteristics—the decision limit (CCα) and the detection capability (CCβ) have been determined for QCA at 0.4 and 1.2 $\mu g\,kg^{-1}$, respectively, and for MQCA at 0.7 and 3.6 $\mu g\,kg^{-1}$, respectively.

Keywords: Carbadox; Olaquindox; Quinoxaline-2-carboxylic acid; Methyl-3-quinoxaline-2-carboxylic acid

1. Introduction

Carbadox (methyl-3-(2-quinoxalinylmethylene) carbazate-N^1,N^4-dioxide-CBX) is an anti-microbial drug that has been used as a medicinal feed additive for the prevention of swine dysentery and bacterial enteritis in young swine [1], and as a growth promoter. CBX is rapidly metabolised via mono and desoxy compounds to quinoxaline-2-carboxylic acid (QCA) [2]. QCA is the longest existing detectable metabolite found in tissue and was therefore, designated as the marker substance for CBX use in animals [3]. Olaquindox (2-(*N*-2-hydroxyethylcarbamoyl)-3-methylquinoxaline-N^1,N^4-dioxide-OQX) is a similar quinoxaline-*N*-dioxide drug used in veterinary medicine. Metabolism of the drug, again via mono and desoxy compounds, produces 3-methyl-quinoxaline-2-carboxylic acid (MQCA) [4], a compound structurally similar to QCA. MQCA is the last major remaining detectable metabolite of OQX, and therefore was designated the marker substance for the drug [5].

Within the EU the product licences of both drugs where withdrawn in 1998, due to health concerns over possible carcinogenic, mutagenic and photoallergenic effects of the drugs and their desoxy metabolites [6]. To ensure confidence in the meat industry and to enforce the ban of both compounds, tissues of food-producing animals must be guaranteed free of such residues within the EU. Methods such as high performance liquid chromatography (HPLC) with UV detection [7,8] and gas chromatography–mass spectrometry (GC–MS) [9,10] have been published, describing the analysis of QCA in tissue. No methods have yet been published describing the analysis of MQCA, Published methods describe only the detection of olaquindox or its desoxy metabolites in tissue [11,12]. A sensitive method for the extraction and analysis of

* Corresponding author. Tel.: +44 2890 525 651; fax: +44 2890 525 626.
E-mail address: glenn.kennedy@dardni.gov.uk (D.G. Kennedy).

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both QCA and MQCA residues in animals of food origin was required. We aimed to produce a validated method that could readily confirm QCA and MQCA at concentrations below any future minimum performance requirements that would be set by the EU. A previously published method [13] developed by this laboratory was adapted to extract and isolate both MQCA and QCA. Samples were then analysed using an HPLC system coupled to a tandem mass spectrometer equipped with an electrospray interface, operated in positive ionisation mode.

The EU has revised the criteria that must be applied in both the screening and confirmation of veterinary drug residues in animals of food origin [14], replacing those previously used [15]. This paper describes a method for the confirmation of both the carbadox metabolite QCA and the olaquindox metabolite MQCA in porcine liver that meets the new technical criteria. The described method also offers a considerable advantage in terms of turnaround time over previously published methods [7–10] and in the numbers of samples that can be processed by a skilled analyst per batch [13].

2. Experimental

2.1. Materials

All solvents were of HPLC grade and all other chemicals were of analytical reagent grade. De-ionised water was used throughout the study. Deuterated quinoxaline carboxylic acid (d_4-QCA) was obtained from RIVM (European Union Reference Laboratory, Bilthoven, The Netherlands) and deuterated methyl quinoxaline carboxylic acid (d_7-MQCA) was obtained by custom synthesis from CSS (Craigavon, UK) for use as internal standards. QCA was obtained from Sigma-Aldrich (Gillingham, UK). MQCA was obtained by custom synthesis by CSS (Craigavon, UK). Stock standard solutions of QCA (1.0 mg ml^{-1}), d_4-QCA (10.0 μg ml^{-1}), MQCA (1.0 mg ml^{-1}) and d_7-MQCA (1.0 mg ml^{-1}) were prepared by dissolving each in methanol. Working combined mixed standard and combined mixed internal standard solutions (1.0 μg ml^{-1}) were prepared by dilution of the stock standards in methanol. Stock standards were stable for 1 year and working standards were stable for at least 3 months when stored in amber vials below 4 °C.

Protease type XIV for enzymatic digest was obtained from Sigma-Aldrich. Solutions of protease type XIV were prepared in water (50 mg ml^{-1}) and prepared each day, as required. The enzymatic digest solution consisted of 0.2 M Tris (hydroxymethyl) methylamine containing 0.1 M calcium chloride, pH 9.6 ± 0.2. The back extraction solution consisted of 0.1 M sodium phosphate buffer, pH 8.0 (prepared from Na_2HPO_4 and $NaHPO_4$, any pH adjustments necessary being made with 1 M NaOH). Mobile phase (A) containing methanol/acetonitrile/water/acetic acid (10:10:79.6:0.4 (v/v)) and mobile phase (B) containing methanol, were filtered and degassed before use.

2.2. Tissue extraction for LC–MS/MS analysis

Aliquots of control liver or test homogenate (5.00 ± 0.05 g) were weighed out into polyethylene tubes (50 ml). Two known negative samples as well as four recovery samples fortified at 10 μg kg^{-1} QCA and MQCA (50 μl of the relevant 1 μg ml^{-1} working standard) were analysed with each batch. All samples, recoveries, and known negatives were fortified with internal standards d_4-QCA and d_7-MQCA (50 μl of the relevant 1 μg ml^{-1} deuterated working standard) at 10 μg kg^{-1}. The extraction method was a modification of that previously described by Hutchinson et al. [13]. Briefly, 0.2 M Tris/HCl buffer, pH 9.6, (8 ml) and protease type XIV solution (50 mg ml^{-1}, 50 μl) was added to all samples, mixed and incubated (55 °C) overnight. The samples were cooled to room temperature, acidified with concentrated hydrochloric acid (1 ml), centrifuged (2000 × *g*, 4 °C, 5 min) and the supernatant decanted into a clean centrifuge tube. Ethyl acetate (6 ml) was added to the supernatant and the tubes shaken and centrifuged (2000 × *g*, 4 °C, 10 min), with the upper layer transferred into a 50 ml polyethylene tube and the extraction repeated and combined. Back extraction solution was added to the extract, the tubes shaken and centrifuged (2000 × *g*, 4 °C, 10 min). The upper organic layer was aspirated to waste and an aliquot (4 ml) of the aqueous phase transferred to a 10 ml glass tube containing concentrated hydrochloric acid (1 ml). The solid phase extraction (SPE) was carried out on an ASPEC XL4 coupled to a 404-syringe pump (Gilson, Middleton, WI, USA) using non-endcapped benzenesulphonic acid (SCX) cartridges with 1 g sorbent material and 3 ml reservoir capacity (IST, Mid-Glamorgan, UK). Each eluted sample was acidified with concentrated hydrochloric acid (300 μl). Ethyl acetate (2 ml) added, the solutions in the tubes were mixed and centrifuged (2000 × *g*, 4 °C, 10 min). The upper organic layer was transferred into 6 ml tubes. The extraction was repeated a further two times with the extracts being combined. The sample extracts were taken to dryness under a stream of nitrogen at 60 °C, methanol-water solution (5:95, (v/v), 100 μl) added and tube vortexed for 15 s. All the solutions were then transferred to tapered microvials for analysis.

2.3. LC–MS/MS analysis

All LC electrospray MS/MS analyses were performed using a Quattro LC (Micromass, Wythenshawe, UK) operating in positive ionisation mode. A Hewlett Packard (Stockport, UK) HPLC system comprising an 1100 Series binary pump, autosampler and solvent degasser were coupled via an electrospray interface to the Quattro LC. Two Luna 3 μm C_{18} 150 mm × 2.0 mm HPLC columns (Phenomenex, Macclesfield, UK) were used with 3 μ 2.0 mm C_{18} guard columns attached. Each were attached to the MS system via a switch valve (Prolab, UK), while one column was in use the other was being re-equilibrated with mobile phase to pre-run condi-

tions. The mobile phase was pumped at a rate of 0.2 ml min^{-1} with all the column effluent entering the mass spectrometer. Before beginning analysis the system was equilibrated by pumping mobile phase through both columns for 25–30 min. At the start of each injection cycle, the mobile phase consisted of solvent A. These conditions were maintained for 10 min. Over the next 5 min the mobile phase was altered to 20:80 (A:B) using a linear gradient. Over the next 0.5 min, the mobile phase mixture was returned to pre-run conditions at 100:0 (mobile phase A:B) and held until the completion of the run at 20 min. The sample volume injected was 15 μl. The MS source temperature was maintained at 150 °C and nitrogen was used as the drying and nebulising gases at flow rates of 600 and 80 l h^{-1}, respectively. Spectra for MQCA and d_7-MQCA (Fig. 1) were obtained over the range *m/z* 50–250 amu with ESI–MS. Spectra for QCA and d_4-QCA were the same as those described and published in an earlier report from our laboratory [13].

Multiple reaction monitoring (MRM) was set for the detection of QCA, d_4-QCA, MQCA and d_7-MQCA. Quadrupole 1 was set to transmit the protonated molecular ions ($[M+H]^+$) of QCA (*m/z* 175 amu), d_4-QCA (*m/z* 179 amu), MQCA (*m/z* 189 amu) and d_7-MQCA (*m/z* 196 amu). Quadrupole 2 was set to transmit the QCA product ions at *m/z* 102 and 75 amu, the MQCA product ions at *m/z* 145 and 102 amu along with the internal standard product ions for d_4-QCA and d_7-MQCA at *m/z* 106 and 152 amu, respectively. Argon was used as the collision gas and it was bled into the cell at a pressure of 2.3×10^{-3} mbar. The energies of the entrance and exit of the collision cell were set to 0 and 2 eV, respectively. The collision energy was optimised at 30 and 45 eV for the QCA product ions at *m/z* 102 and 75 amu, respectively. The collision energy was optimised at 16 and 34 eV for the MQCA product ions at *m/z* 145 and 102 amu, respectively. The internal standard product ions for d_4-QCA (*m/z* 106 amu) and d_7-MQCA (*m/z* 152 amu) were optimised at 30 and 16 eV, respectively. The cone voltage for all QCA and MQCA ions was 30 and 20 V, respectively and the dwell time for each ion was 0.5 s.

The concentration of QCA in a sample was calculated by comparing the ratio of the *m/z* 175 → *m/z* 102 amu (QCA base peak) response to the *m/z* 179 → *m/z* 106 amu response (d_4-QCA) in the sample with those in the standards (10 μg kg^{-1}) within the run. Concentrations for MQCA were calculated by comparing the ratio of the *m/z* 189 → *m/z* 102 amu (MQCA base peak) response to the *m/z* 196 → *m/z* 152 amu re-

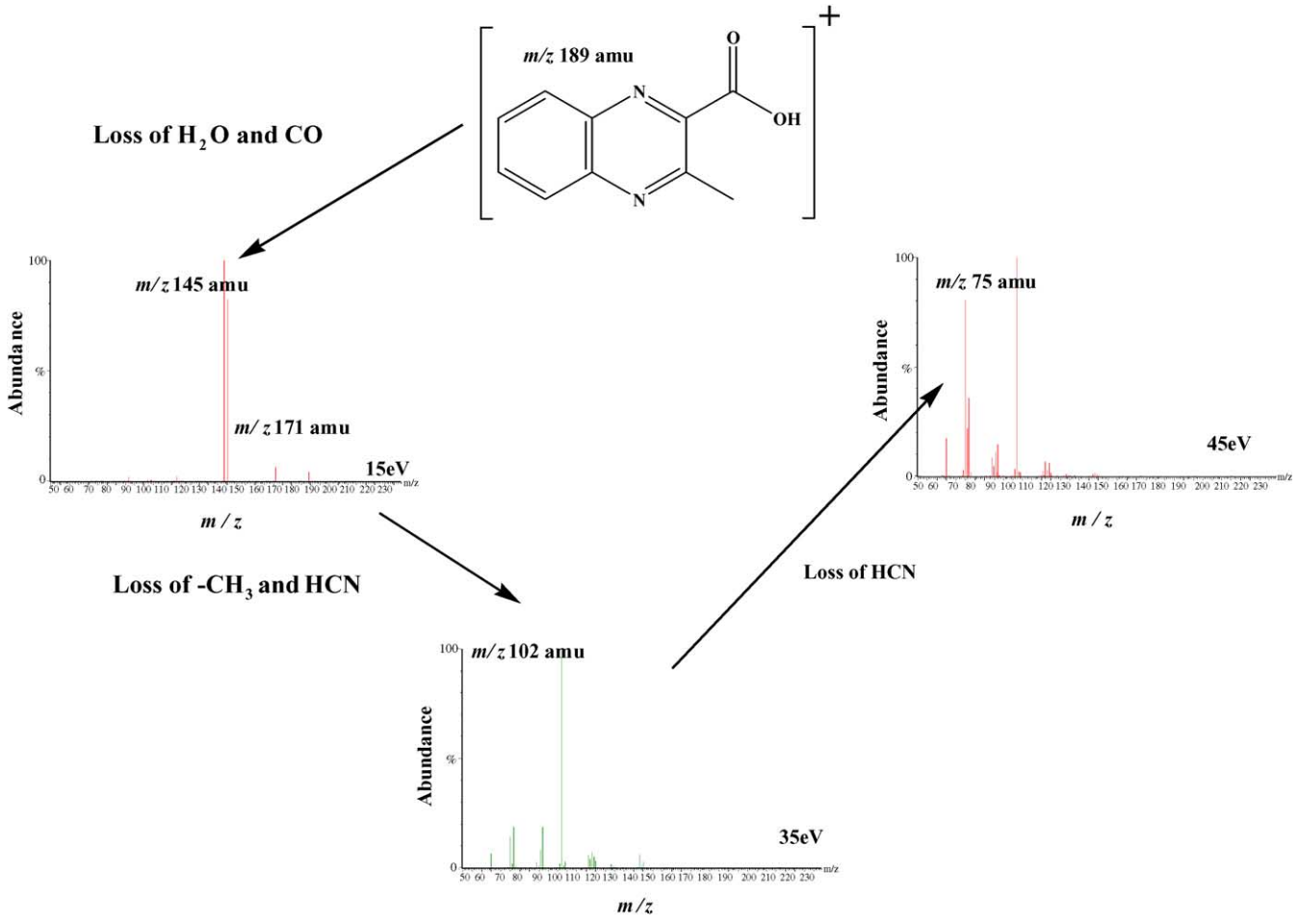

Fig. 1. Structure and MS/MS of MQCA.

sponse (d_7-MQCA) in the sample with those in the standards (10 μg kg^{-1}) within the run.

3. Results and discussion

3.1. LC–MS/MS of QCA and MQCA

Both QCA and MQCA show similar fragmentation patterns, the fragmentation for QCA having been described previously [13]. The MS/MS of the molecular ion of QCA (*m/z* 175 amu) produces a prominent product ion at *m/z* 175 → 129 amu, resulting from the successive loss of water and carbon monoxide (supported by the observation of a small peak at *m/z* 175 → 157 amu—the loss of H_2O; data not shown). Fig. 1 shows the MS/MS of the molecular ion of MQCA (*m/z* 189 amu), which produces a similar prominent product ion at *m/z* 189 → 145 amu, thought to result from the successive loss of water and carbon monoxide. This again is supported by the observation of a small peak resulting from the loss of H_2O (*m/z* 189 → 171 amu). An identical product ion for QCA and MQCA is formed at *m/z* 102 amu. In the case of QCA it may be due to the further loss of HCN from the *m/z* 175 → 129 amu product ion, but for MQCA it may be due to the loss from the *m/z* 189 → 145 amu product ion of a methyl group and HCN. QCA produces another prominent product ion at *m/z* 175 → 75 amu, this may be attributed to the successive loss of another HCN from the *m/z* 175 → 102 amu product, MQCA again shows a similar loss of HCN that is supported by the observation of a small peak at *m/z* 189 → 75 amu.

According to current technical criteria for residue identification in food of animal origin, a minimum of four identification points are required to confirm unauthorised substances [14]. The criteria score 1.5 identification points for each MS/MS product ion measured, plus 1 identification point for the precursor ion (whether it is separately measured or not). The described method therefore, scores four identification points for QCA and MQCA through the measurement of two product ions plus the precursor ion, thus fulfilling the identification criteria. In this method, ion ratios were measured for the purposes of analyte identification from the following transition products: *m/z* 75:102 (from the protonated molecular ion at *m/z* 175 for QCA) and *m/z* 145:102 (from the protonated molecular ion at *m/z* 189 for MQCA). However, for unambiguous identification the ion ratios of unknown samples must correspond to those in the standards within the run, within predefined limits. The tolerances allowed for the ion ratios vary relative to the intensity of the product ion to the base peak ion. These ratios had to meet pre-set tolerances before they were considered acceptable for inclusion in the validation data [14]. Results were only included after the application of all the identification criteria described above. All results were calculated by comparison of the ratio of the analyte base peak area to the corresponding deuterated internal standard peak area in the sample with those in the bracketing standards (10 μg kg^{-1}) within the run.

3.2. Method performance characteristics

The QCA confirmatory method previously described by this laboratory, has a major advantage over older GC–MS methods, in that it permits the processing of 16 samples in duplicate (excluding negatives, controls and check samples) in 1.5 days by a skilled analyst [13]. A minor modification of this method has allowed the additional extraction and analysis of MQCA using this procedure. The absolute recovery, based on the analysis of four negative liver samples fortified with both QCA and MQCA at 10 μg kg^{-1} and carried though the method in the absence of internal standard, achieved by the described method is 54.2 ± 6.1% and 48.0 ± 2.9% for QCA and MQCA, respectively. The recovery achieved for analysis of QCA using the present method is similar to the value previously reported by us for the analysis of QCA in porcine liver (57.6 ± 5.1) [13]. Fig. 2 shows MRM chromatograms for a negative liver fortified with QCA (3.0 μg kg^{-1}), a negative liver, and a QCA standard (10 μg kg^{-1}) at *m/z* 175 → 102 amu, 175 → 75 amu and *m/z* 179 → 106 amu (internal standard). Fig. 3 shows MRM chromatograms for a negative liver fortified with MQCA (3.0 μg kg^{-1}), a negative liver, and a MQCA standard (10 μg kg^{-1}) at *m/z* 189 → 145 amu, *m/z* 189 → 102 amu and *m/z* 196 → 152 amu (internal standard). Both sets of chromatograms showed no spurious peaks resulting from matrix interferences.

The accuracy and precision of the method was determined over the concentration range (3.0–150 μg kg^{-1}) on three separate occasions, which reflects QCA concentrations encountered in Northern Ireland statutory control schemes, and also provides a wide concentration range over which to assess MQCA method performance. The results are summarised in Table 1 for QCA and MQCA. All samples included in this study met the identification criteria described above.

Table 1
Accuracy and precision of LC–MS/MS method for QCA and MQCA in fortified porcine liver (n = 6 at each concentration, on each of three separate days)

Compound	Fortification level (μg/kg)	Overall mean (μg/kg)	Overall recovery (%)	Within day CV	Between day CV
QCA	3.0	3.2	107	7.1	6.6
QCA	10.0	10.2	102	3.2	5.4
QCA	50.0	48.7	97	3.0	3.9
QCA	150.0	141.8	95	2.0	3.0
MQCA	3.0	3.3	111	7.4	13.1
MQCA	10.0	10.7	107	5.6	5.3
MQCA	50.0	49.9	100	5.9	6.3
MQCA	150.0	139.3	93	3.9	5.5

Recovery correction was applied using the deuterated internal standards for QCA and MeQCA.

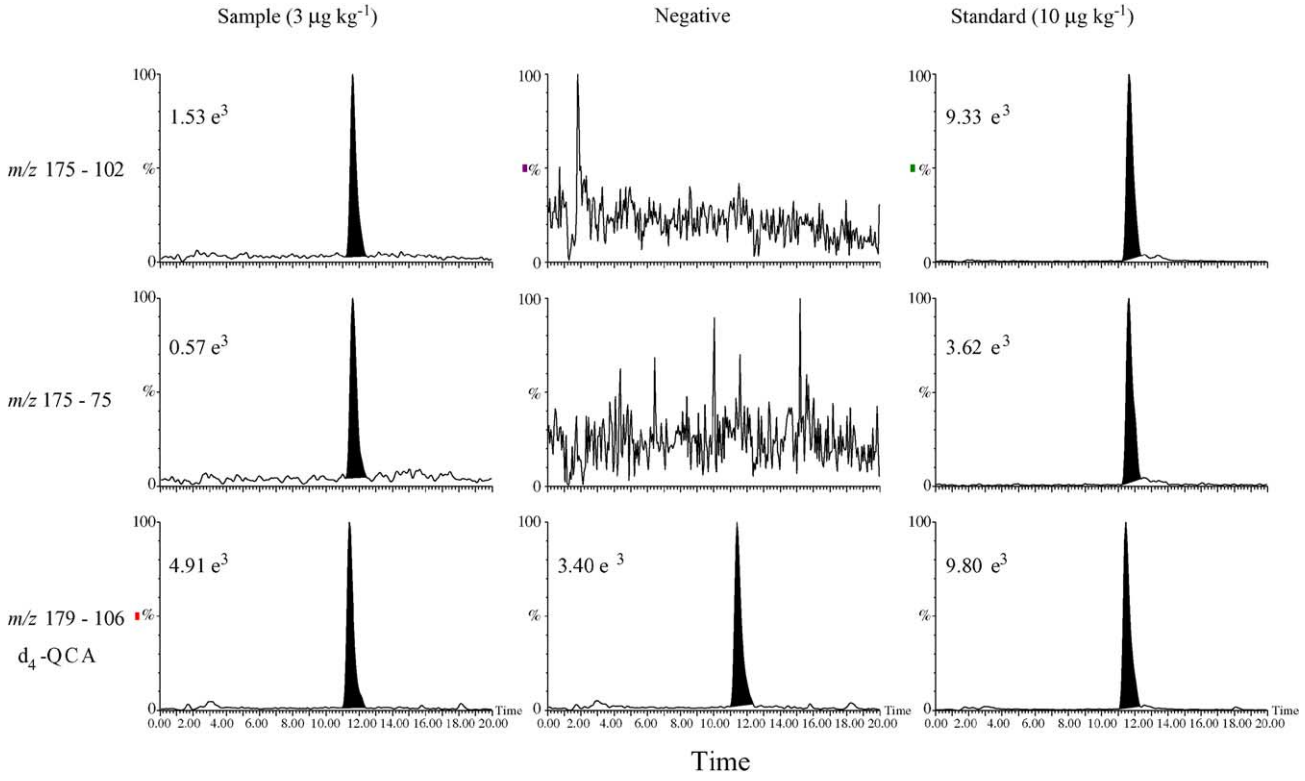

Fig. 2. MRM chromatograms of QCA (*m*/*z* 175 → *m*/*z* 102 and 75 amu) and d_4-QCA (internal standard; *m*/*z* 179 → *m*/*z* 106 amu) in a negative liver sample fortified with QCA at a concentration of 3.0 μg kg^{-1} (left column), a known negative liver sample (centre column), and a standard solution (10 μg kg^{-1}) of QCA (right column). *Y*-axis normalised to 100% of the largest peak.

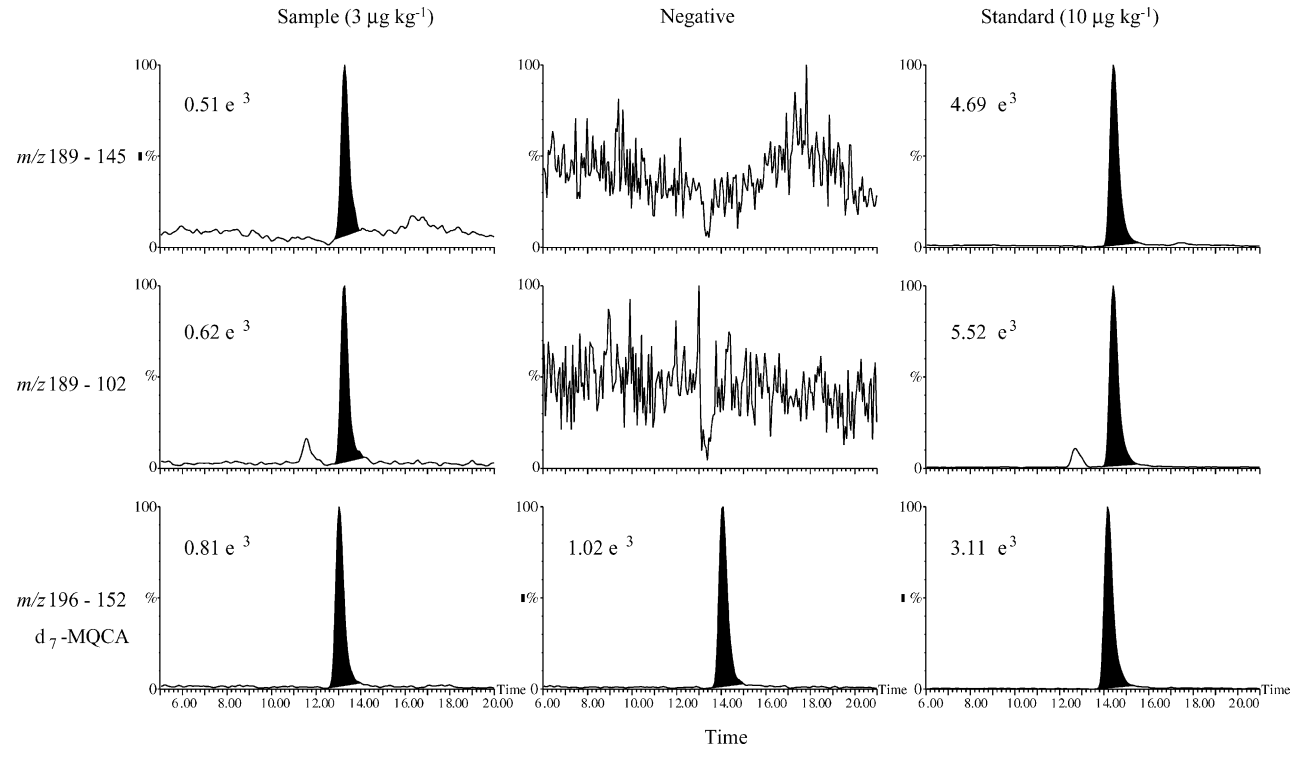

Fig. 3. MRM chromatograms of MQCA (*m*/*z* 189 → *m*/*z* 145 and 102 amu) and d_7-MQCA (internal standard; *m*/*z* 196 → *m*/*z* 152 amu) in a negative liver sample fortified with MQCA at a concentration of 3 μg kg^{-1} (left column), a known negative liver sample (centre column), and a standard solution (10 μg kg^{-1}) of MQCA (right column). *Y*-axis normalised to 100% of the largest peak.

The performance characteristics CCα and CCβ, introduced by Commission Decision 2002/657/EC [14] were calculated following the analysis of six negative liver samples, fortified with QCA and MQCA at each of three concentrations (2.0, 3.0 and 4.0 $\mu g\,kg^{-1}$). As yet, no Minimum Required Performance Limit (MRPL) has been established for either compound. As a consequence, the concentrations chosen for this study were somewhat arbitrary—the Commission Decision specifies the concentrations to be chosen for the determination of these parameters as a proportion of the MRPL. However, since these are well below previous EU MRLs for carbadox and olaquindox and are close to the detraction limit obtainable using the most advanced equipment that is routinely available in residues monitoring laboratories within the EU—we believe that the values of CCα and CCβ reported here are realistic given probable future EU decisions on the MRPLs for these compounds. In all of the samples, the identity of QCA and MQCA was confirmed using the identification criteria described above.

The decision limit (CCα) is defined as the limit at which it can be decided that a result is non-compliant with an error probability of α ($\alpha = 1\%$ for unauthorised substances). CCα was calculated as described in ISO Guide 11843 [16], as recommended by Commission Decision 2002/657/EC [14]. CCα was calculated on the basis of the more intense product ion. Thus for QCA, the ratio of the abundance of the product ion at m/z 175 → 102 to that of the d_4 QCA internal standard, was used to calculate a value of 0.4 $\mu g\,kg^{-1}$ for CCα. Similarly, a value of 0.7 $\mu g\,kg^{-1}$ was calculated for MQCA using the ratio of the abundance of the product ion at m/z 189 → 102 to that of the d_7 MQCA internal standard.

The detection capability (CCβ) is defined as the smallest concentration of analyte that can be identified and quantified in a sample with a statistical certainty of $1-\beta$ ($\beta \leq 5\%$ for unauthorised substances). CCβ was calculated as described in ISO Guide 11843 [16], as recommended by Commission Decision 2002/657/EC [14]. CCβ was calculated on the basis of the less intense product ion. The approach of using the more intense ion for the calculation of CCα and the less intense ion for the calculation of CCβ has recently been advocated by Antignac et al. [17]. Thus for QCA, the ratio of the abundance of the product ion at m/z 175 → 75 to that of the d_4 QCA internal standard, was used to calculate a value of 1.2 $\mu g\,kg^{-1}$ for CCβ. Similarly, a value of 3.6 $\mu g\,kg^{-1}$ was calculated for MQCA using the ratio of the abundance of the product ion at m/z 189 → 145 to that of the d_7 MQCA internal standard.

4. Conclusions

A method has been developed for the detection, quantification and confirmation of QCA and MQCA, the marker substances for the use of carbadox and olaquindox, respectively, in porcine tissue. The linearity, accuracy, and precision of the method have been demonstrated over the concentration range 3.0–150 $\mu g\,kg^{-1}$. The method performance characteristics (CCα and CCβ) have been calculated for the described method, these are lower than any expected MRPL that may be set by European Commission and Community Reference Laboratories. The method has been applied to the analysis of incurred liver samples, and is now in routine use in this laboratory to monitor pigs produced in Northern Ireland for the possible misuse of carbadox and olaquindox.

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