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Development and validation of a method for the confirmation of halofuginone in chicken liver and eggs using electrospray tandem mass spectrometry

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Abstract

A method is described for the quantitative confirmation of halofuginone (HFG) residues in chicken liver and eggs. This method is based on LC coupled to positive ion electrospray MS–MS of the tissue extracts, prepared by trypsin digestion of the tissues followed by liquid–liquid extraction and final clean-up using Solid Phase Extraction (SPE). The $[M+H]^+$ ion at m/z 416 is monitored along with four transitions at m/z 398, 138, 120 and 100. The method has been validated according to the draft EU criteria for the analysis of veterinary drug residues at 15, 30 and 45 μg kg^{-1} in liver and 5, 15 and 50 μg kg^{-1} in eggs. The new analytical limits, CCα and CCβ were calculated for liver and were 35.4 and 43.6 μg kg^{-1}, respectively.
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1. Introduction

In poultry, coccidiosis may lead to poor weight gain and reduced egg production [1]. Halofuginone (HFG, Fig. 1) is used world-wide to prevent coccidiosis in commercial poultry production [2]. HFG (DL-*trans*-7 bromo-6-chloro-3-[3-(hydroxy-2-piperidyl)acetonyl]-4(3H)-quinazolinone hydrobromide) is licensed, as a feed additive, for incorporation in

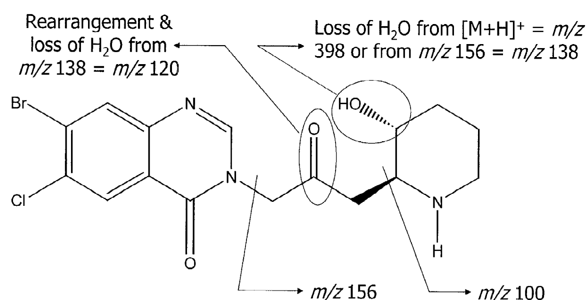

Fig. 1. Structure and proposed fragmentation of HFG.

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poultry feed to permit continuous administration to broiler chickens and turkeys. In the UK, HFG, as the hydrobromide salt, may be added to broiler feedingstuffs at a final concentration of 3 mg kg^{-1} and is the active ingredient of the premix "Stenorol™" (Hoechst–Roussel). Halofuginone hydrobromide has a withdrawal period of 5 days.

As a result of the widespread use of coccidiostats in food producing animals, the potential exists for coccidiostat residues to enter the human food chain. Surveillance schemes are in place in most countries to monitor the occurrence of residues in food of animal origin. The concentration of a residue in food is dependent on a number of factors such as dose, excretion, metabolism, absorption and distribution of the drug. The rate at which these occur determines the concentration and the chemical nature of the drug residue [3]. The European Agency for the Evaluation of Medicinal Products (EMEA) established HFG as the marker residue and has set a MRL (Maximum Residue Limit) of 10 and 30 μg kg^{-1} for bovine muscle and liver, respectively [4]. However, no MRL has yet been established by EMEA for HFG residues in poultry. HFG is not licensed for use in commercial egg-layers, and as a consequence eggs for human consumption should be free from HFG residues. However, other workers have shown that HFG can potentially be transferred to eggs [5].

Several papers have described the determination of HFG in animal feedingstuffs, using a range of analytical techniques, including GC and HPLC [6–10]. Immunoassay techniques for the determination of HFG in chicken plasma [11] and tissue [11,12] have also been described. However, there are relatively few published methods for the chemical determination of HFG in tissue. All of these methods use HPLC with UV detection and have been described for both chicken tissues [12–15] and eggs [15,16]. These methods have been validated at concentrations down to 15 μg kg^{-1} in chicken liver [13] and 5.0 μg kg^{-1} in eggs [16]. To date, no methods have been published for the confirmation of HFG in poultry products using mass spectrometry.

The European Union is currently revising the technical criteria that must be applied in the screening and confirmation of veterinary drug residues in food of animal origin [17]. These criteria will replace those contained in Commission Decision 93/256/EEC [18]. None of the methods described above meet either the current or new criteria for the confirmation of substances, such as HFG, that are contained in Group B of Annex I of Council 96/23/EC [19]. The described method is based on a previously published method [13], which has been modified to remove some of the more cumbersome parts of the extraction procedure. The current paper describes a method for determination of HFG in eggs and liver using liquid chromatography–electrospray mass spectrometry (LC–ESI-MS–MS), suitable for the confirmation of HFG, using the revised EU criteria [17]. HFG is extracted as a free base with ethyl acetate after digestion of the tissues with trypsin and then back-extracted into aqueous ammonium acetate buffer, followed by solid-phase extraction. HFG is analysed by reversed-phase chromatography with detection by ESI-MS–MS. The method has been validated in liver at 15, 30 and 45 μg kg^{-1} (equivalent to 0.5, 1.0 and 1.5 times the EMEA MRL for HFG in the bovine) and at 5, 15 and 50 μg kg^{-1} in eggs.

2. Experimental

2.1. Materials

All solvents used were of HPLC grade and other chemicals were of analytical reagent-grade. Distilled or deionised water was used throughout. Roussel kindly supplied the standard halofuginone hydrobromide sample. Stock standards (1.0 mg ml^{-1}) of HFG were prepared in 0.125 *M* ammonium acetate buffer, pH 4.9, and were stable for 6 months when stored at 4 °C. Dilute standard solutions (0–1.6 μg ml^{-1}) of HFG were prepared by dilution of the stock standard with ammonium acetate buffer and were also stable for 6 months at 4 °C. Working standards (0.2–1.6 μg ml^{-1}) used for matrix calibration were prepared by dilution of stock standards in ammonium acetate buffer. The working standards were stable for at least 6 months when stored at 4 °C.

2.2. Equipment

An ASPEC XL4 V2 (Gilson Medical Electronics, Villiers-le-Bel, France) was used to automate the solid-phase extraction step in this method. Oasis

HLB SPE cartridges (3 cc, 60 mg, Waters Corporation, USA) were used for sample clean-up.

2.3. LC–MS–MS system

The HPLC system was a Hewlett-Packard (Stockport, Cheshire, UK) series 1100 binary pump, autosampler and solvent degasser and a Phenomenex (Macclesfield, Cheshire, UK) Prodigy 5 μm, C_{18} column (25 cm×4.6 mm I.D.). Mobile phase, methanol–water–glacial acetic acid (40:59.5:0.5), was pumped at a rate of 1.0 ml/min. The sample volume injected was 25 μl and the total run time was 10 min. The LC system was coupled via an electrospray interface to the Quattro LC (Micromass, Wythenshawe, UK). The column effluent was split approximately 5:1 before entering the mass spectrometer. The MS source was maintained at 150 °C. Nitrogen was used as the drying and nebulising gas at flow-rates of 500 and 80 l/h, respectively. Spectra for HFG were obtained in positive mode over the range m/z 50–450 with the instrument configured for MS only. The collision cell entrance and exit energies were set to 0 and 2 eV, respectively. The cone voltage was optimised at 30 V for the production of the molecular $[M+H]^+$ ion at m/z 416 for HFG. For multiple reaction monitoring (MRM), quadrupole 1 was set to transmit the molecular ion and quadrupole 2 to transmit the molecular ion and HFG transition ions at m/z 398, 138, 120 and 100. Argon was bled into the cell as the collision gas at 2.3×10^{-3} mbar. The collision energy was optimised at 5 eV for HFG and at 15, 20, 20 and 20 eV for HFG daughter ions at m/z 398, 138, 120 and 100, respectively. The dwell time for each ion was 0.25 s. The system was equilibrated by pumping the mobile phase for 15 min before beginning of the analysis. A matrix calibration curve consisting of a zero and five standards was run at the start and end of each batch. Peak area data for the molecular ions, $[M+H]^+$ of HFG were collected at m/z 416. Data for the transition from the transition product ions of HFG at m/z 398, 138, 120 and 100 were also monitored.

2.4. Extraction procedure

Samples of minced liver or homogenised egg (2.00±0.02 g) for analysis were weighed into polypropylene centrifuge tubes. Known negative tissue samples were analysed in every batch. Additional negative samples were taken through the analytical procedure to serve as matrix standards. After extraction, aliquots (160 μl) of these sample extracts were fortified with either buffer or HFG (40 μl) at a range of concentrations (0.0, 0.2, 0.4, 0.8, 1.2 and 1.6 μg ml^{-1}) to yield a series of matrix standards (equivalent to 0, 20, 40, 60 and 80 μg kg^{-1}), respectively. Fortified liver and egg samples were prepared by addition of appropriate amounts of the 1.0 μg ml^{-1} HFG working standard, to the known negative samples (e.g. 60 μl standard into 2.00 g tissue is equivalent to a fortification level of 30 μg kg^{-1}). The samples were allowed to stand for 15 min prior to extraction. Trypsin (25 mg ml^{-1} in water, 2.0 ml) was added to each tube. The contents of the tubes were vortex mixed and pH adjusted to between 7 and 8 using sodium carbonate solution (10% w/v). The samples were then incubated overnight at 40 °C in an orbital shaker incubator (Gallenkamp, UK). Thereafter, the tubes were removed and cooled for 20 min. About 1 ml of sodium carbonate solution (10% w/v) was added to each tube along with ethyl acetate (10 ml for liver and 15 ml for egg samples). The contents of the tubes were shaken thoroughly on a mechanical shaker for 3 min. The tubes were then centrifuged for 2 min at 600 *g* at 4 °C. The organic layer was transferred into a clean tube and the aqueous phase was re-extracted with 10 ml of ethyl acetate, as described above. The combined ethyl acetate extracts were extracted twice with 0.125 *M* ammonium acetate buffer, pH 4.9 (5 ml) using a mechanical shaker for 1 min. The combined aqueous layers were shaken gently with hexane (5 ml) for 20 s to remove any residual ethyl acetate. The hexane layer was then aspirated to waste.

2.5. Sample clean-up

The extracts were cleaned up using an automated solid-phase extraction system (ASPEC). Oasis cartridges were conditioned using methanol (3 ml) followed by water (3 ml) and 0.125 *M* ammonium acetate buffer, pH 4.9 (3 ml). Sample extracts were then applied to the cartridges, which were then washed with toluene (2 ml) to remove any non-polar fatty compounds present in the extract. The tubes were dried by pushing air through them at 19 ml min^{-1}. The analyte was eluted into 3-ml glass tubes

using 2 ml of methanol. The methanol was evaporated to dryness at 40 °C under a stream of nitrogen. The dried extracts were reconstituted by the addition of 0.125 *M* ammonium acetate buffer, pH 4.9 (500 μl) to each tube. The tubes were vortexed for 30 s and 200 μl was transferred to microvials for LC–MS–MS analysis. Blank samples intended for use as matrix-matched standards were treated in a different manner, as described below.

2.6. Matrix calibration curve

Matrix standards were prepared by reconstituting blank tissue extracts in 400 μl ammonium acetate buffer. Aliquots (100 μl) of HFG (0, 0.2, 0.4, 0.8, 1.2 and 1.6 μg ml^{-1}) were added to the reconstituted extracts to give matrix standards equivalent to 0, 10, 20, 40, 60 and 80 μg HFG per kg. The tubes were vortexed for 30 s and 200 μl was transferred to microvials for LC–MS–MS analysis, as described above. Quantification was achieved by interpolation of the abundance of the precursor ion (m/z 416) in samples against a matrix standard curve (abundance at m/z 416 vs. concentration).

3. Results and discussion

3.1. Fragmentation of HFG

MS–MS of the $[M+H]^+$ molecular ion of HFG m/z 416 (Fig. 1) produced four prominent transitions at m/z 398, 138, 120 and 100. The transition formed at m/z 398 resulted from loss of H_2O. The transition formed at m/z 100 is due to the cleavage between aliphatic-CH_2 group and the hydroxy piperidyl ring as shown in Fig. 1. A weak ion at m/z 156, resulting from cleavage in the centre of the molecule between the quinazolinone moiety and the remainder of the molecule (as indicated in Fig. 1), rearranged and lost water to form a transition product at m/z 138. The transition product at m/z 120 is formed by further loss of water (from the OH group attached to the piperidyl ring).

3.2. Identification of HFG

The revised criteria introduce a number of new concepts into EU legislation for the confirmation of veterinary drug residues. A system of Identification Points (IPs) has been introduced to define the number of ions and their corresponding ratios that must be measured for both authorised and unauthorised substances when mass spectroscopic analysis is employed [17]. For HFG, a Group B substance, a minimum of three IPs must be earned. According to the criteria, measurement of the $[M+H]^+$ ion at m/z 416 earns one IP. The measurement of the transitions at m/z 398, 138, 120 and 100 each earn 1.5 IPs. This method earns a total of seven IPs and thus meets the revised criteria. In addition, for unambiguous identification, at least one ion ratio must be measured and must correspond to that in standards to within pre-set tolerances [17]. In this method, four ion ratios were measured (m/z 398:416; 138:416; 120:416 and 100:416), all of which had to meet the pre-set tolerances [17] before unambiguous identification of HFG was deemed to have occurred.

3.3. Analysis of HFG in liver

Fig. 2 shows MRM chromatograms for HFG standard (10 μg kg^{-1}), a negative liver fortified with HFG (15 μg kg^{-1}) and a negative liver, for the precursor ion at m/z 416, and for the transitions at m/z 398, 138, 120 and 100. No interfering peaks were observed in the negative liver sample. We found evidence of modulation of ionisation as a result of the presence of sample matrix in the extracts. Accordingly, standard curves (0–80 μg kg^{-1}) prepared by fortifying blank matrix, which had been taken through the extraction procedure, were used for the quantification of HFG in liver. These standard curves were linear over the concentration range studied (typically: $r^2=0.9941$).

The recovery and repeatability of the analytical method were measured by the analysis of six blank poultry liver samples fortified with HFG at each of three concentrations (15, 30 and 45 μg kg^{-1} equivalent to 0.5, 1.0 and 1.5 times the MRL for HFG in bovines) on three separate occasions. All the results were calculated after the application of the identification criteria described above and are summarised in Table 1. The data show that the described method is capable of quantifying HFG in liver within the guidelines laid down in the revised criteria.

The revised criteria also introduce two analytical limits CCα (Decision Limit) and CCβ (Detection

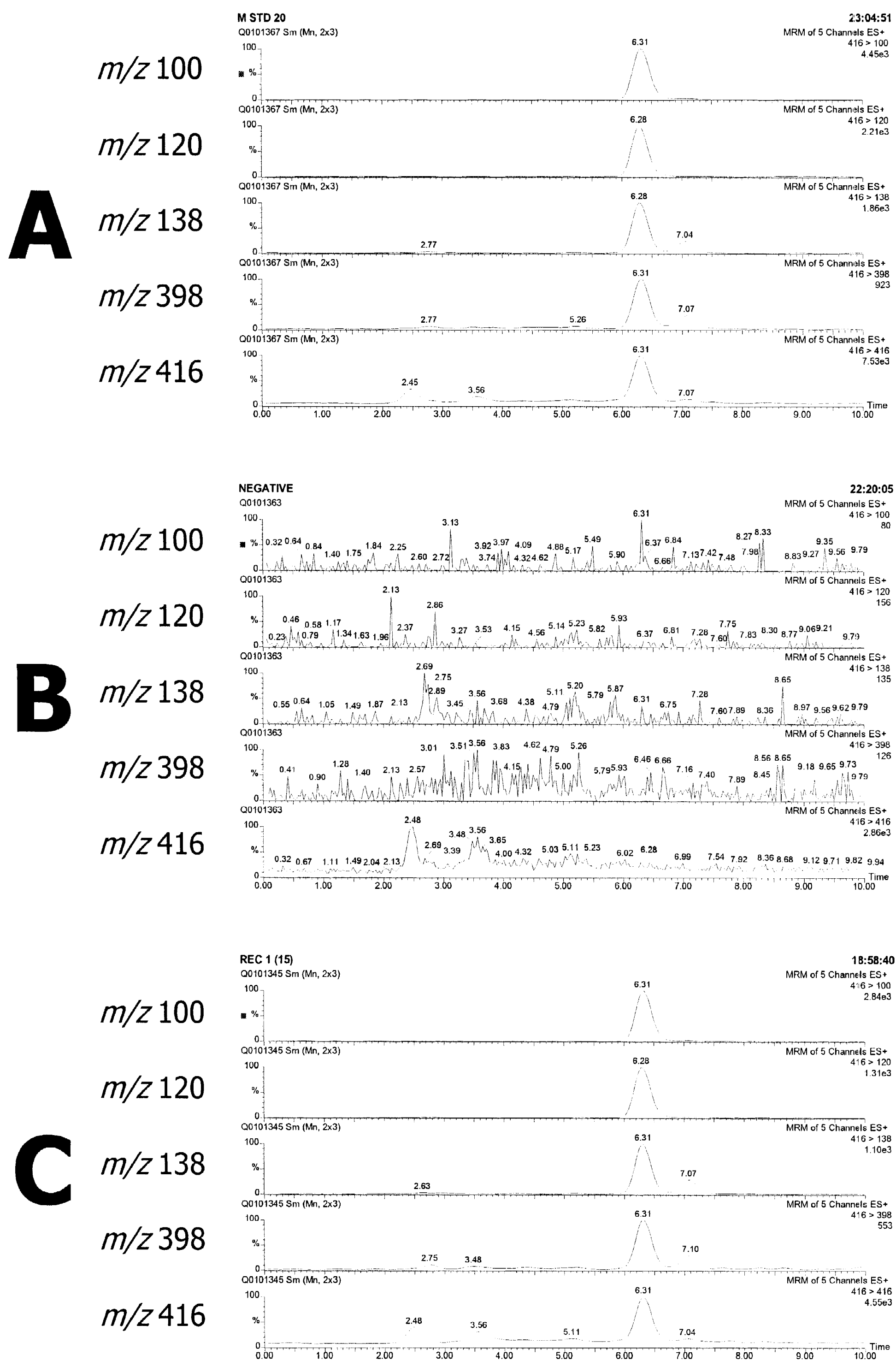

Fig. 2. Chromatograms obtained for a matrix standard (10 μg kg^{-1}, A), a blank liver sample (B) and a blank liver fortified with HFG at 15 μg kg^{-1} (C).

Table 1
Recovery and repeatability for the determination of HFG in poultry liver and eggs, based on the analysis of HFG-free liver and eggs fortified with HFG

Matrix	Fortification level (μg/kg)	Mean recovery (μg/kg)	Recovery (%RSD)	Repeatability (%RSD)
Liver	15	11.4	7.1	4.5
	30	22.9	8.6	9.5
	45	35.5	4.1	13.2
Egg	5	3.9	9.1	13.7
	15	11.5	6.2	5.6
	50	37.4	5.6	7.6

For each matrix, six samples were analysed at each concentration on each of 3 days ($n=18$ analyses for each matrix/concentration combination).

Capability) to replace the limits of detection and quantification, respectively. These new limits are based on the critical value of the net state variable (CCα) and the minimum detectable value of the net state variable (CCβ) defined by ISO [20]. CCα, the Decision Limit, is the limit at which it can be decided that a sample is truly violative (greater than the MRL for a Group B substance) with an error probability of α. For Group B substances, $\alpha=5\%$. CCα is determined in such a way that it fulfils the criteria for identification and quantification for the analyte under investigation. CCβ, the Detection Capability, is the smallest content of analyte that may be detected, identified and quantified in a sample with an error probability of β. In the case of Group B substances, the β error should be less than or equal to 5%. For these substances, the detection capability is the concentration at which the method is able to detect MRL violations with a statistical certainty of $1-\beta$. For MRL compounds, the analytical limits CCα and CCβ were determined graphically, as required by Commission Decision 2002/657/EC [17], using the data generated during validation of the method in liver (Table 1). Using this procedure, the values for CCα and CCβ were shown to be 35.4 and 43.6 μg kg^{-1}, respectively.

3.4. Analysis of HFG in eggs

Fig. 3 shows MRM chromatograms for HFG standard (10 μg kg^{-1}), a negative egg fortified with HFG (5 μg kg^{-1}) and a negative egg sample, for the precursor ion at m/z 416, and for the transitions at m/z 398, 138, 120 and 100. As was the case with liver, no interfering peaks were observed in the negative egg sample. Halofuginone is not licensed for use in egg-laying birds. Accordingly, eggs should be free from HFG residues. However, the possibility of cross-contamination of HFG-medicated and HFG-free feeds during the feed manufacturing process could lead to commercial egg layers being exposed to low dietary concentrations of HFG. This, in turn, could lead to the accumulation of unwanted HFG residues in eggs. However, since HFG is not licensed for use in egg-laying birds it does not and will not have an established MRL. Therefore, CCα and CCβ cannot be calculated using the same method for liver. However, HFG cannot be regarded as a substance the use of which is prohibited in food-producing animals, for which separate criteria for the determination of CCα and CCβ exist. We therefore adopted a ‘‘common sense’’ approach and have validated the described method concentrations of 50, 15 and 5 μg kg^{-1}, the lowest level being close to the lowest level at which the assay could be validated with satisfactory accuracy and precision. The recovery and repeatability of the method were measured by the analysis of six negative egg samples fortified with HFG at these three concentrations on three separate occasions. All results, which were calculated after the application of the identification criteria described above, are shown in Table 1. The data show that the method is capable of quantifying HFG in eggs within the guidelines laid down in the revised criteria.

In conclusion, this quantitative confirmatory method for HFG has been validated according to the new EU criteria in chicken liver and eggs. The described method has now been applied in this laboratory for

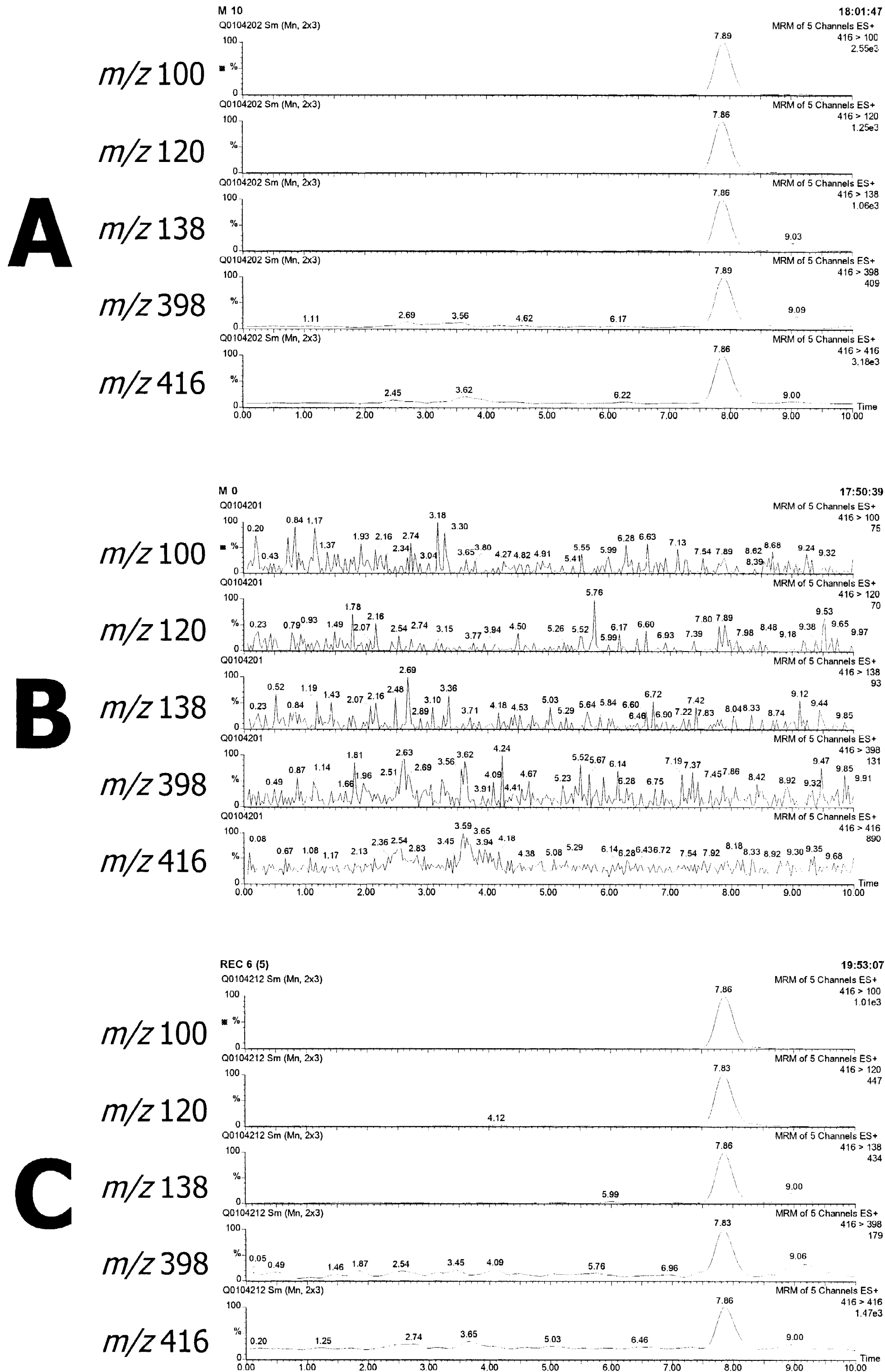

Fig. 3. Chromatograms obtained for a matrix standard (10 μg kg^{-1}, A), a blank egg sample (B) and a blank egg fortified with HFG at 5 μg kg^{-1} (C).

the quantitative confirmation of halofuginone in samples collected as a part of our residues control schemes.

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References

[1] L.R. McDougald, Control of coccidiosis chemotherapy, in: P.L. Long (Ed.), Coccidiosis of Man and Domestic Animals, CRC Press, Boca Raton FL, 1990, p. 307.
[2] M. Kennedy, in: Coccidiosis in Chickens, Alberta Agriculture and Rural Development, Edmonton, Alberta, 1996.
[3] S.B. Turnipseed, Analysis of animal residues, in: S.B. Turnipseed, A.R. Long (Eds.), Analytical Procedures for Drug Residues in Food of Animal Origin, Science Technology System, West Sacramento, CA, 1998, p. 1.
[4] The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines and Information Technology, EMEA/MRL/741/00-Final, 2000.
[5] P.L. Long, K. Sheridan, L.R. McDougald, Poult. Sci. 60 (1981) 2342.
[6] A. Anderson, D.H. Christopher, R.N. Woodhouse, J. Chromatogr. 168 (1979) 471.
[7] Analytical Methods Committee, Analyst 108 (1983) 1252.
[8] C. Tillier, E. Cagniant, Ph. Devaux, J. Chromatogr. 441 (1988) 406.
[9] L. Krivankova, F. Foret, P. Bocek, J. Chromatogr. 545 (1991) 307.
[10] A. Anderson, E. Goodall, G.W. Bliss, R.N. Woodhouse, J. Chromatogr. 212 (1981) 347.
[11] R.C. Beier, L.D. Rowe, M.I.A. El-Aziz Nasr, M.H. Elissalde, B.G. Rose, L.H. Stanker, Food Agric. Immunol. 8 (1996) 11.
[12] R.C. Beier, T.J. Dutko, S.A. Buckley, M.T. Muldoon, C.K. Holtzapple, L.H. Stanker, J. Agric. Food Chem. 46 (1998) 1049.
[13] Analytical Methods Committee, Analyst 109 (1984) 171.
[14] Y. Yamamoto, R. Hashiguchi, K. Araki, H. Kushima, J. Food Hyg. Soc. Jpn. 32 (1991) 444.
[15] Y. Yamamoto, F. Kondo, J. Assoc. Off. Anal. Chem. Int. 84 (2001) 43.
[16] D.C. Holland, R.K. Munns, J.E. Roybal, J.A. Hurlbut, A.R. Long, J. Assoc. Off. Anal. Chem. Int. 78 (1995) 37.
[17] Commission Decision 2002/657/EC of 12th August 2002. Off. J. Eur. Commun. L221 (2002) 8.
[18] Commission Decision 93/256/EEC of 14th May 1993. Off. J. Eur. Commun. L118 (1993) 64.
[19] Commission Decision 96/23/EEC of 23rd May 1996. Off. J. Eur. Commun. L125 (1996) 10.
[20] ISO Guide 11843-1. International Organisation for Standardisation, Geneva, Switzerland, 1997, p. 1.