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Quantitative analysis of clenbuterol in meat products using liquid chromatography–electrospray ionisation tandem mass spectrometry

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Abstract

A method is presented that allows quantitation of clenbuterol in meat and liver products at the ng/kg level by liquid chromatography–electrospray ionisation tandem mass spectrometry (LC–ESIMS–MS) using a stable isotopically labeled internal standard. The practical procedure involves acid extraction followed by two solid-phase clean-up steps with C_{18} and strong cation-exchange (SCX) resins. The typical recovery of the analyte spiked at 0.4 μg/kg in meat and liver samples was at 63±7%. Mass spectral acquisition was done in multiple reaction monitoring (MRM) to provide a high degree of sensitivity, achieving a limit of detection and quantitation at 10 and 15 ng/kg, respectively. Two precursor ions at m/z 277 and 279, corresponding to the characteristic isotopic cluster of the two chlorine atoms of clenbuterol, were monitored by LC–ESIMS–MS to provide unambiguous identity of the analyte. Samples of meat and liver of various origins with either incurred residues or spiked with known amounts of clenbuterol were used to validate the method. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Clenbuterol; Growth hormones

1. Introduction

Clenbuterol is a synthetic drug belonging to the class of the β_2-adrenergic agonists. Due to its β_2-sympathomimetic activity, clenbuterol is often used in human and veterinary medicines as a bronchodilator, cardiotonic and tocolytic agent [1,2]. However this compound may be used illegally as a growth promotor (anabolic) in meat-producing livestock, with concomitant reduction of fat tissues, giving the farmers an economic benefit. Notably, the use of β-agonists for growth promoting purposes in farm animals is not permitted in the European Community (EC) and the maximum residue limits (MRL's) recommended by WHO and *Codex Alimentarius* are 0.2 μg/kg for muscle and 0.6 μg/kg for liver [3,4].

Several analytical methods already exist to monitor clenbuterol at the μg/kg level in tissues and biological fluids by immunoassays [5,6] or by HPLC with UV, electrochemical or fluorescence detection [7–9]. However, most of the classical methodological approaches are not adequately sensitive and do not provide structural confirmation of the analyte. Gas chromatography coupled to a mass selective detector can be employed and provide structural information, but only after derivatisation of the bifunctional polar groups of the molecule [10,11]. Moreover, chemical derivatisation may introduce

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unwanted variability or sample loss, and also reduce the speed of the analytical procedure.

Recent developments in the field of liquid chromatography coupled to mass spectrometry detection (LC–MS) have enabled the analysis of polar and thermolabile chemicals without chemical derivatisation, which has resulted in significantly lower limits of detection. Consequently, LC–MS is often the method of choice in the analysis of trace levels of polar contaminants. In the case of β-agonists, quantitation and confirmation by LC–MS in several matrices such as retina [12], liver [13,14], plasma [15], urine [16–18], and serum [19] with detection limits of 0.01–0.5 μg/kg have been reported. Most of the analyses described for β-agonists have used a one-step solid-phase extraction clean-up which, however, may lead to accelerated column deterioration and more rapid contamination of the MS interface if the method is used as a routine quality control tool. Moreover, in many cases, mass spectral quantitation was performed by monitoring only the precursor ion m/z 277 of clenbuterol (^{35}Cl), which may lead to equivocal, i.e. false positive results.

LC–MS methods using extraction procedures for β-agonists with analyte-specific immobilized antibodies, either off-line [20] or in more complex on-line systems with fully automated valve switching devices [16], have been described. The employment of immunoaffinity columns provides extracts of great purity, but is very costly in routine operations since many of the commercially available columns are for single-use only and analyte binding affinity deteriorates over periodic use. Thus, the aim of this study was to develop a routine method for the detection of trace levels of clenbuterol in meat and liver products based on conventional solid-phase extraction techniques followed by LC–ESIMS–MS for analyte quantification.

2. Experimental

2.1. Chemicals

Perchloric acid, sodium hydroxide, ammonia, ethanol, potassium phosphate, ethyl acetate, ammonium acetate and acetic acid were purchased from Merck (Geneva, Switzerland). Acetonitrile was purchased from Fluka (Buchs, Switzerland). The C_{18} Bond Elut (500 mg, 6 ml) and AccuBond SCX (500 mg, 6 ml with benzenesulphonic acid as functional groups) solid-phase extraction cartridges were purchased from Varian (Harbor City, CA, USA) and J and W Scientific (Folsom, CA, USA), respectively. Unlabeled clenbuterol was purchased from Sigma and d_9-clenbuterol [1-(4-amino-3,5-dichlorophenyl)-2-*tert*.-butylamino-d_9-ethanol] >99% isotopic purity, >95% chemical purity) was custom synthesized by Toronto Research Chemicals (Ontario, Canada).

2.2. Extraction of clenbuterol from meat products

Calf meat and pork livers of different animals were purchased at local butcheries, whereas calf livers originated from a slaughterhouse in Santiago de Chile. An adequate portion (approximately 0.2–0.5 kg) of the meat or liver was cut into small slices and homogenized in a laboratory mixer (Braun Multipractic Plus UK40, Braun, Zurich, Switzerland) for 1–2 min. Twenty grams of the test portion were removed and spiked with exactly 8 ng (0.4 μg/kg) of d_9-clenbuterol and subsequently extracted with 50 ml of 0.4 mol/l perchloric acid in an ultrasonic bath for 5 min, followed by 10 min under vortexing before centrifugation (2225 *g*) for 15 min at 4°C. The supernatant was filtered over a 5-μm filter (Schleicher and Schuell, Riehen, Switzerland). The extraction step was repeated twice and the 100-ml filtrate was adjusted to pH 3.0 with 32% sodium hydroxide before loading onto a C_{18} Bond Elut cartridge (500 mg), positioned on a Supelco Visiprep vacuum manifold (Supelco, Buchs, Switzerland), pre-conditioned with 10 ml each of methanol and water with vacuum-induced flow at approximately 0.6 ml/min. The column was rinsed again with 10 ml of water. Clenbuterol was eluted with 12 ml of 4% ammonia in ethanol (v/v). After evaporation of the solvent under a stream of nitrogen, the sample was re-suspended in 6 ml of 0.1 mol/l potassium phosphate buffer (pH 6) and charged onto an SCX cartridge (AccuBond, 500 mg), pre-washed consecutively with 2 ml methanol, water and 0.1 mol/l potassium phosphate (pH 6). Clenbuterol was eluted with 12 ml of 1.3% ammonia in ethyl acetate (v/v). The effluent was concentrated to dryness under a stream of nitrogen and re-suspended in 0.2 ml water

and filtered over a 0.45-µm filter (Millipore). A 10-µl aliquot was analysed by LC–ESIMS–MS (triplicate injections).

2.3. Liquid chromatography

LC analysis was done with a Hewlett Packard 1100 HPLC instrument (HP Instruments AG, Urdorf, Switzerland) equipped with a Vydac C_{18} column, 1.0×150 mm, 5 µm (Vydac, Hesperia, CA, USA), at a flow-rate of 460 µl/min. Buffers employed were as follows: (A) 10 mmol/l ammonium acetate containing 5% acetonitrile (v/v), 0.1% acetic acid pH 4.3 (v/v) and (B) 100% acetonitrile. The gradient was 0% B for 5 min at a flow-rate of 460 µl/min, increasing to 100% B in 15 min, then 100% B for 10 min at a flow-rate of 500 µl/min before returning to 0% B within 2 min followed by a re-equilibration time of 15 min. A LC-Packings splitter (Omnilab Biosystems AG, Mettmenstetten, Switzerland), ca. 1:10, was used in a pre-injector position so that all the injected sample was loaded onto the C_{18} column and entered the mass spectrometer at a flow-rate of approximately 50 µl/min. In order to avoid clogging of the MS interface, a Rheodyne valve diverter (Omnilab Biosystems AG, Mettmenstetten, Switzerland) was used at the beginning of the gradient (Fig. 1).

2.4. Mass spectrometry

Mass spectrometry was performed on a Finnigan TSQ-7000 mass spectrometer (Finnigan, San Jose, CA, USA) equipped with the API 2 interface in positive electrospray ionization (ESI). The spray voltage was set at 3.8 kV with a capillary temperature of 240°C and a sheath gas pressure of 345 kPa. Instrument tuning was done by infusion onto a Harvard syringe pump (Harvard Apparatus, South Natick, MA, USA) of a 1 ng/ml clenbuterol solution at a flow-rate of 5 µl/min equipped with a Tee and 40 µl/min of 20% buffer B flushing through the HPLC pump. Full mass spectra were recorded by scanning a m/z range of 130–350 with a scan time of 2 s and the electron multiplier set at 1600 V.

The ESIMS–MS daughter ions spectra of clenbuterol and d_9-clenbuterol were obtained using argon as collision gas. In-source collision was set at 6 eV whereas the second quadrupole was set with a collision energy of 14 eV and a gas pressure of 293 mPa. Quantitative analysis of clenbuterol was done by monitoring the multiple transition reaction corresponding to clenbuterol and its deuterated cognate at m/z 277→203 and 259, m/z 279→205 and 261 and m/z 286→204 and 268, m/z 288→206 and 270, respectively. The complete LC–MS coupling system was fully automated via the unix station using ICIS 8.3 version software. Peak integration was done using LC Quan software (Finnigan).

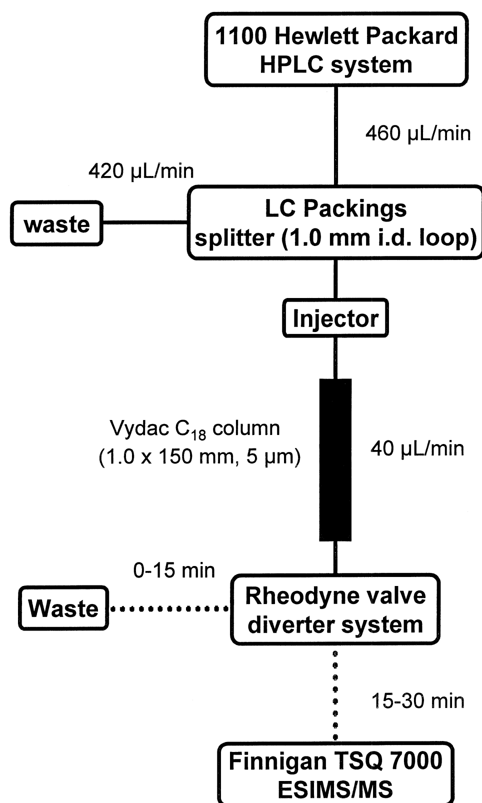

Fig. 1. Scheme of the LC–MS coupling system.

3. Results and discussion

3.1. MS characteristics

Clenbuterol and its internal standard were first analysed by ESIMS to optimize the MS–MS parameters. The full mass spectra of clenbuterol and its

deuterated internal standard display intense ions at *m/z* 277 and 279, and at *m/z* 286 and 288, respectively, which correspond to the characteristic isotopic cluster of the two chlorine atoms (Fig. 2). The isotope ratio of the two chlorine ions (^{35}Cl and ^{37}Cl) showed a relative intensity of 69% (*m/z* 279) and 66% (*m/z* 288), which is in good agreement with the theoretical values calculated at 65.1%. To additionally strengthen our confidence in analyte identity and quantitation, both ions at *m/z* 277 and 279 were used as precursor ions for clenbuterol.

Tandem MS–MS experiments were then conducted using varying collision energies to define the fragmentation pathways and optimize the mass spectral conditions (Fig. 3). Under mild MS–MS conditions, the major fragment ions for clenbuterol and d_9-clenbuterol $(M+H)^+$ were found at *m/z* 259, 203 and *m/z* 268, 204, respectively (Fig. 3a), which represents the loss of a water molecule from the benzylic hydroxyl group and additional loss of the *N*-substituted isobutene moiety. By increasing the collision energy, other ions were observed at *m/z* 168, 132, 57 for clenbuterol, and at *m/z* 169, 133, 66 for the deuterated cognate molecule (Fig. 3b and 3c). An earlier report on the fragmentation of clenbuterol under high in-source collison energy assigned the fragment ion at *m/z* 168 as a result of loss of a chlorine atom, consistent with the elimination of a neutral *tert*.-butylammonium chloride from the protonated molecular ion [21]. Rearrangement of a methyl group hydrogen atom (deuterium) to the protonated secondary amine is clearly illustrated in the fragmentation pattern of the d_9-labeled analogue, i.e. *m/z* 204 vs. 203, *m/z* 169 vs. 168, and *m/z* 132 vs. 133 [22]. Complete loss of the isotopic cluster of chlorine is evident by the fragment observed at *m/z* 132. Moreover, the observation of fragment ions at *m/z* 57 and 66 for clenbuterol and its internal standard, respectively, correspond to the *tert*.-butyl fragment ion (Fig. 4).

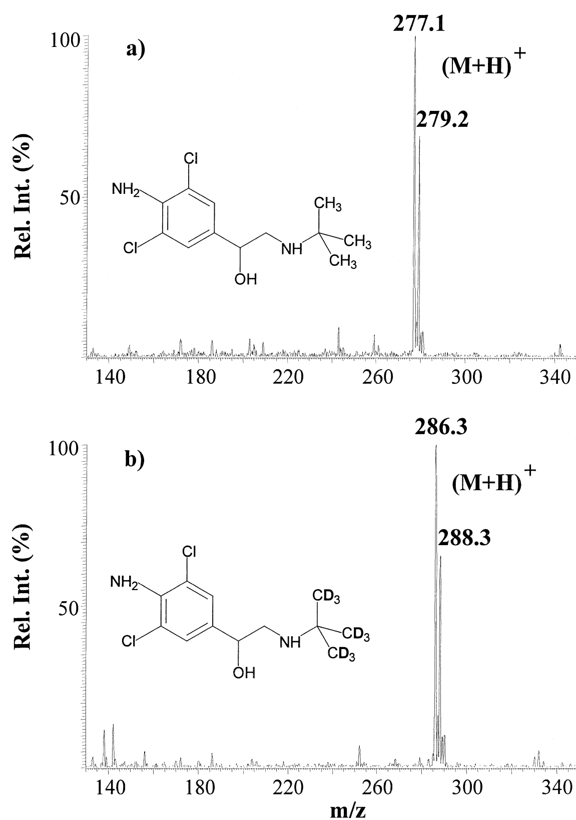

Fig. 2. Full scan electrospray mass spectra of (2a) clenbuterol and (2b) d_9-clenbuterol, depicting also their respective chemical structures.

3.2. Calibration graphs

A twelve-point calibration graph was established in water, and for each curve the area ratios of the transition reactions *m/z* 277→259 and 203 versus *m/z* 286→268 and 204 and *m/z* 279→261 and 205 versus *m/z* 288→270 and 206 plotted against their respective amount ratios. The calibration curve in water showed a linear response from 10 to 5000 pg with a correlation coefficient $r^2 = 0.9992$ ($y = 1.2634x - 0.0124$) and $r^2 = 0.9979$ ($y = 1.3171x - 0.0245$) for the ^{35}Cl and ^{37}Cl isotope transitions, respectively.

Calf meat, pork and calf livers were fortified with clenbuterol at three different concentration levels (0.2, 0.5 and 1.0 μg/kg) with a fixed amount of d_9-clenbuterol (0.4 μg/kg, 0.8 ng injected) in order to determine the impact of the food matrix on the calibration graph. Similarly, the matrix-based calibration curve also depicted linearity over the given range, giving for a non-contaminated calf liver a correlation coefficient $r^2 = 0.9999$ ($y = 1.3861x + 0.0097$) and $r^2 = 0.9999$ ($y = 1.3609x + 0.005$) for ^{35}Cl and ^{37}Cl, respectively. A slight increase in slope was observed in the matrix-matched solutions for

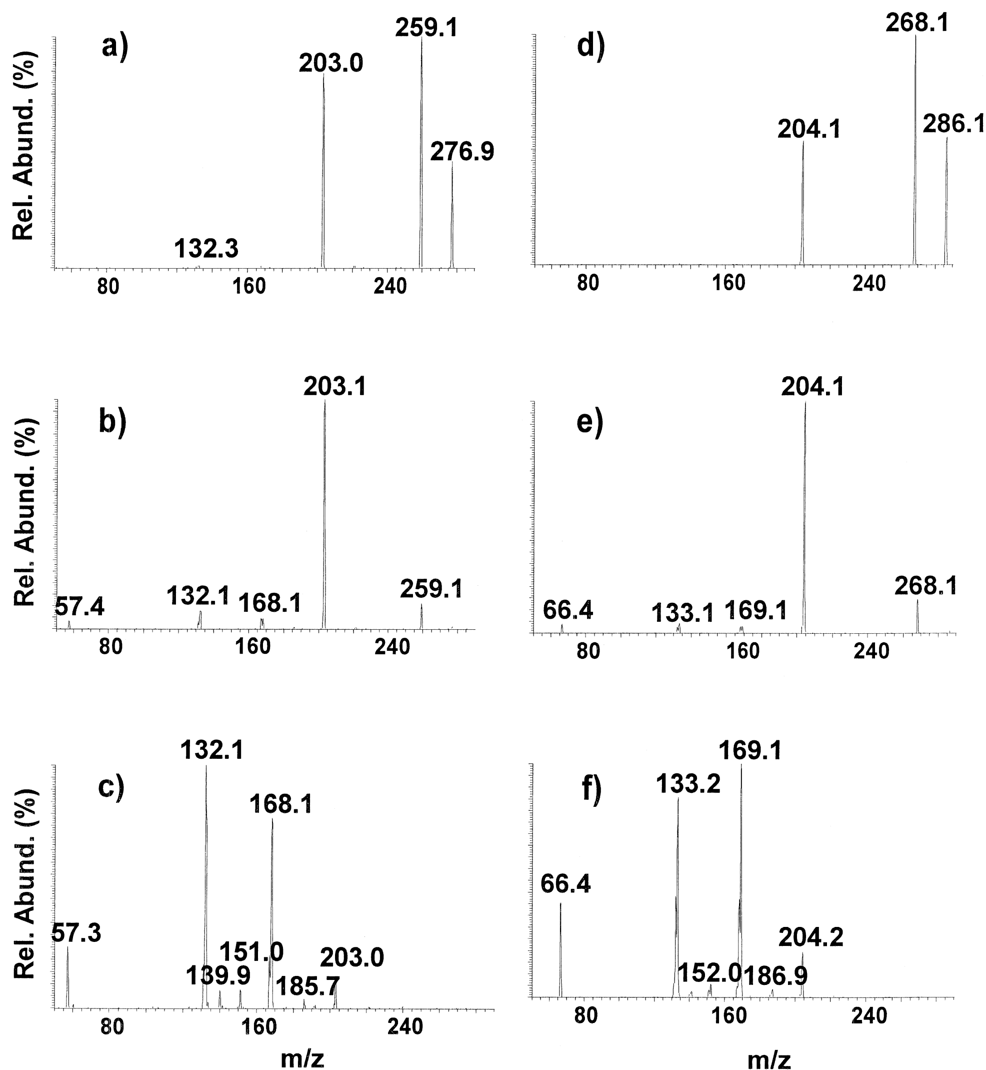

Fig. 3. ESIMS–MS daughter ion mode spectra of (3a), (3b) and (3c) clenbuterol [$(M+H)^+$ 277] and (3d), (3e) and (3f) d_9-clenbuterol [$(M+H)^+$ 286] with a collision energy set at (3a) and (3d) 14 eV, (3b) and (3e) 20 eV and (3c) and (3f) 33 eV. Spectra were recorded by scanning a mass range from m/z 50–290 at a scan time of 2 s. Argon was used as the collision gas with a pressure set at 2.2 mTorr.

both isotope transitions, which indicates that the analyte may be overestimated if extrapolated from a purely water-based calibration graph.

3.3. Performance of the method

The major quality parameters of the method are summarized in Table 1. The limit of detection (LOD), estimated from the value of the mean of multiple injections ($n=10$) of a blank liver plus three-fold standard deviation was at 10 ng/kg in liver. The limit of quantitation (LOQ), calculated similarly plus ten-fold standard deviation of the blank, was found at 15 ng/kg in liver. The recovery of clenbuterol was determined by fortifying a series ($n=3$) of blank samples at 0.4 μg/kg of the analyte prior to acid extraction and solid-phase clean-up. The mean recovery of clenbuterol in the food matrices

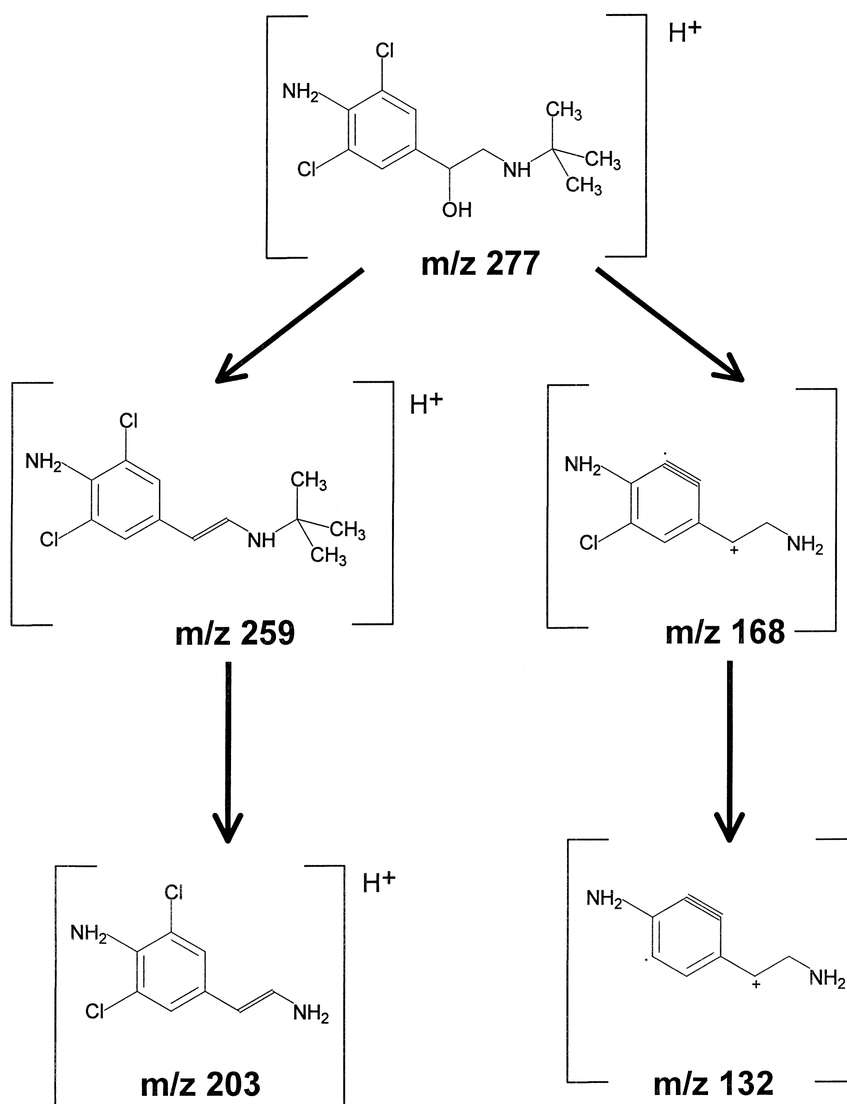

Fig. 4. Fragmentation pathway of clenbuterol.

Table 1
Method performance characteristics

LOD (μg/kg)	0.01
LOQ (μg/kg)	0.015
Recovery±RSD (%)[a]	63±7%
Intra-assay coefficient of variation[b]	
df=8	3.8%
Inter-assay coefficient of variation[b]	
df=10	5.8%

[a] Blank samples spiked at 0.4 μg/kg, $n = 3$.
[b] Calculated using robust statistics.

was calculated at 63% (C.V. 7%) using the ^{35}Cl isotope transition. Coefficients of variation (C.V.) within and between series were calculated from sets of replicate analyses using robust statistics [23] and are shown in Table 1.

3.4. Analysis of meat and liver samples

The total ion currents of a blank pork liver and naturally contaminated calf liver are depicted in Figs.

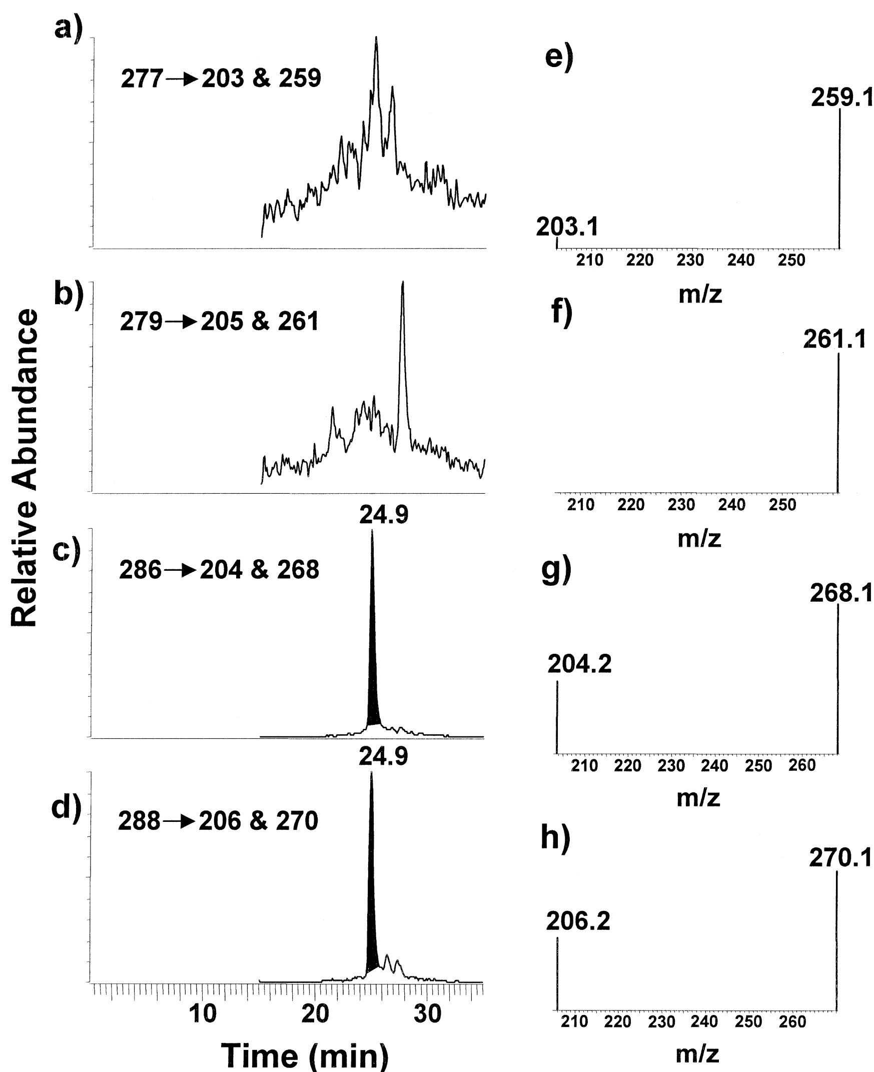

Fig. 5. HPLC–ESIMS–MS of an uncontaminated pork liver with (5a–d) total ion current and (5e–h) their respective mass spectra transition ions monitored.

5 and 6, respectively. The MRM total ion currents of the uncontaminated pork liver lacks a response in both channels (^{35}Cl and ^{37}Cl) at the retention time of clenbuterol. In contrast, clenbuterol is clearly detected in both the ^{35}Cl and ^{37}Cl isotope transitions at the correct retention time of clenbuterol in the contaminated calf liver sample (Fig. 6). Moreover, the peak height ratios of *m/z* 279→261 and 205 versus *m/z* 277→259 and 203 gives similar results of 65% and 67%, respectively, in agreement with the theoretically calculated ratio of ^{35}Cl and ^{37}Cl.

The importance of quantifying clenbuterol based on MRM transitions of both chloride isotope ions is evident in a second calf liver sample that was analysed (Fig. 7). In this case, a peak was detected in the chromatogram at retention time 25.3 min for the transition reaction *m/z* 279→261 and 205 (Fig. 7b), which is close to the retention time of the internal standard (25.1 min). However, the MRM total ion current does not show traces of the transition ions *m/z* 277→203 and 259 for the ^{35}Cl isotope, indicating that this chemical is not clenbuterol. Further-

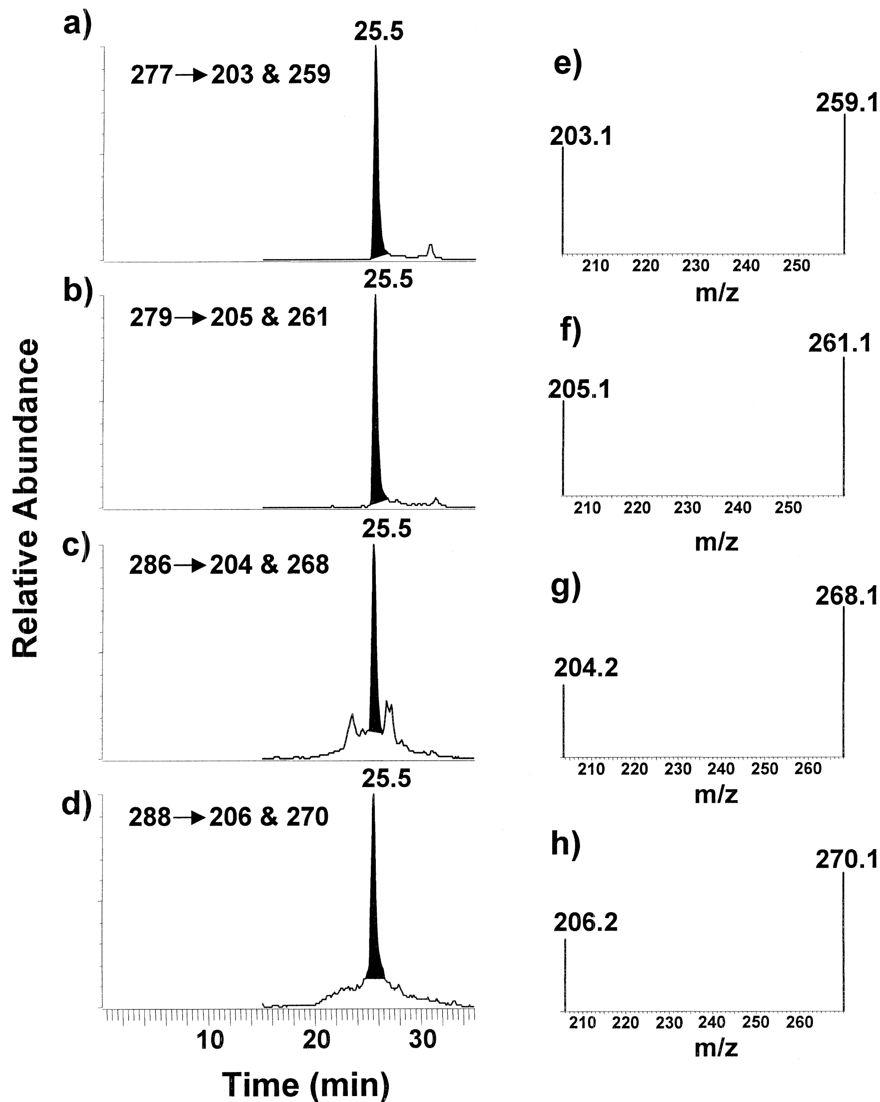

Fig. 6. HPLC–ESIMS–MS of a contaminated calf liver with (6a–d) total ion current and (6e–h) their respective mass spectra transition ions monitored.

more, the transition ion ratio observed for *m/z* 259 and 203 is significantly different from that of the analyte (Fig. 7e), and the absence of the product ion *m/z* 205 in the MRM transition *m/z* 279→261 and 205 corroborates that this response is due to an interfering compound (Fig. 7f).

The amount of clenbuterol found in the respective meat/liver samples that were analysed in this study is summarized in Table 2. Quantitation is based upon MRM transition reactions of both the ^{35}Cl and ^{37}Cl ions of clenbuterol and extrapolation using the calibration graphs established in water and in a fortified matrix. As depicted, the individual values obtained from either of the two calibration curves compare rather well, with slightly lower values estimated from the matrix-matched curve.

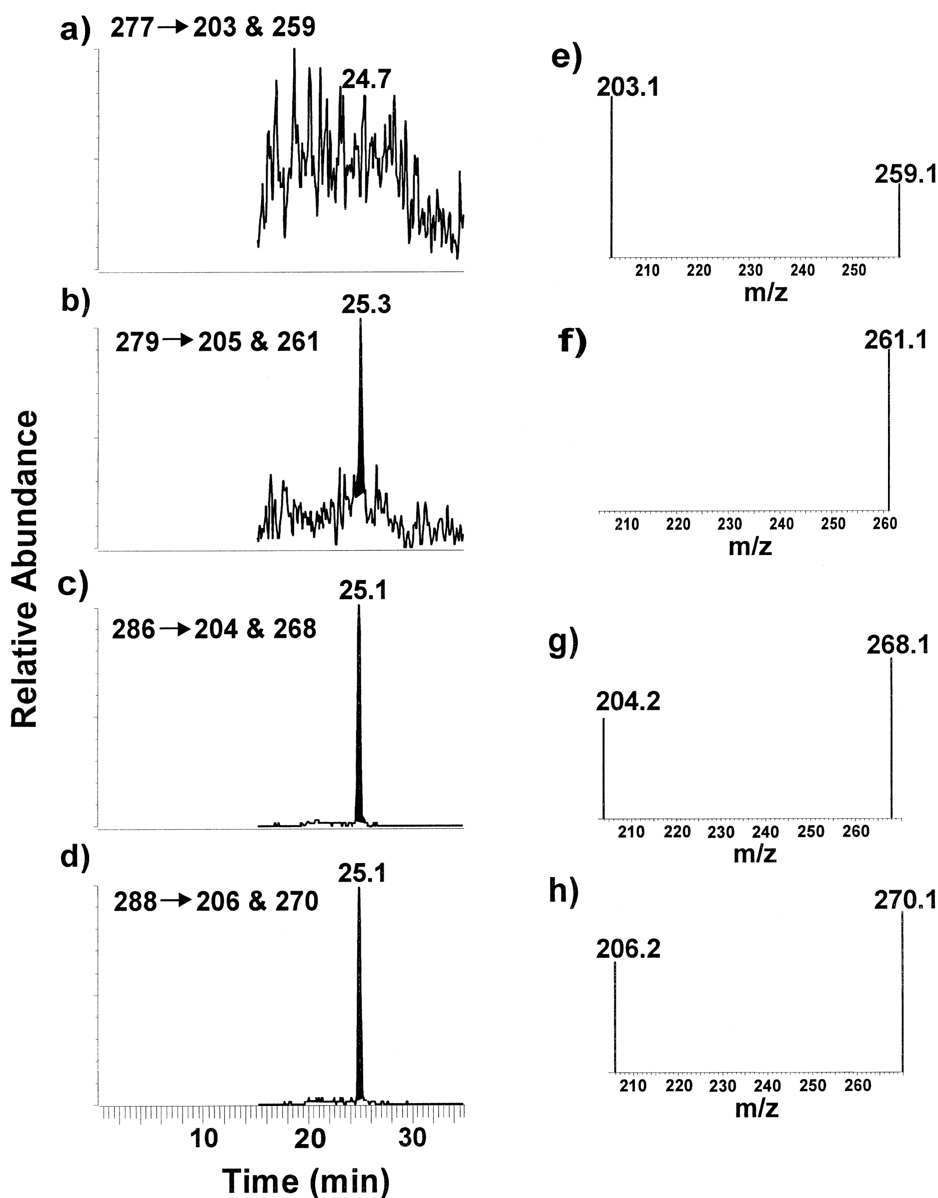

Fig. 7. HPLC–ESIMS–MS of an uncontaminated calf liver with (7a–d) total ion current and (7e–h) their respective mass spectra transition ions monitored.

4. Conclusions

A quantitative and confirmatory method for determining trace levels of clenbuterol in meat and liver products has been developed. Tandem solid-phase clean-up on disposable C_{18} and cation-exchange resin (SCX) cartridges obviates the use of costly immunoaffinity columns. Clenbuterol is determined by isotope dilution and LC–ESIMS–MS using the MRM reactions of the ^{35}Cl and ^{37}Cl

Table 2
Results of analysis of clenbuterol in various meat and liver samples

Sample	Ion	Amount[a] (μg/kg)	C.V. (%)	Amount[b] (μg/kg)	C.V. (%)
Calf meat No. 1	277	0.04±0.00	4.84		
	279	0.05±0.00	4.96		
Calf meat No. 2	277	0.72±0.01	0.75	0.65±0.01	0.78
	279	0.71±0.01	0.74	0.61±0.00	0.78
Pork liver No. 1	277	Nd[c]	–	Nd[c]	–
	279	Nd[c]	–	Nd[c]	–
Pork liver No. 2	277	0.03±0.00	7.30	0.02±0.00	9.29
	279	0.02±0.00	4.62	0.01±0.00	12.13
Calf liver No. 1	277	Nd[c]	–	Nd[c]	–
	279	Nd[c]	–	Nd[c]	–
Calf liver No. 2	277	42±0.11	0.26	38.79±0.10	0.26
	279	40.53±0.01	0.03	39.14±0.01	0.03

[a] Calculated from the standard calibration curve.
[b] Calculated from the matrix effect.
[c] Nondetectable.

isotope precursor ions, which gives additional specificity to the method and diminishes the risk of false positive results. Analysis of various liver and meat products demonstrate that clenbuterol levels may range widely from 20 to 40 000 ng/kg, and emphasizes that more stringent and systematic quality control screens are required to detect the illegal usage of β-agonists in foods of animal origin.

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