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Development and validation of a liquid chromatographic–electrospray tandem mass spectrometric multiresidue method for anthelmintics in milk

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Abstract

A liquid chromatographic–tandem mass spectrometric multiresidue method for the simultaneous quantitative determination of the tetrahydroimidazole, levamisole and the benzimidazoles thiabendazole, oxfendazole, oxibendazole, albendazole, fenbendazole, febantel and triclabendazole in milk has been developed and validated. The anthelmintic residues were extracted with ethyl acetate. The liquid chromatographic separation was performed on a reversed-phase C_{18} column with gradient elution. The analytes were detected by tandem quadrupole mass spectrometry after positive electrospray ionisation by multiple reaction monitoring. The confirmatory method is very sensitive and each component can be detected at a residue level lower than 1 μg/l. The method is validated according to the revised European Union requirements and all parameters were found conform the criteria. The evaluated parameters were linearity, specificity, stability, recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (detection limit, decision limit and detection capability). This analytical method is applied in the Belgian monitoring programme for classical anthelmintic veterinary drugs in raw farm cow's milk.
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1. Introduction

Within the group of antiparasitic drugs, anthelmintic products are widely used in veterinary medicine in cattle. A lot of antiparasitic immunity is already achieved through treatment of young cattle. Nevertheless, in wet seasons the medication of dairy cows with endoparacides can also be necessary for protecting or treating the animals mainly against gastrointestinal nematodes, lungworms and liver fluke. Moreover, Gross et al. [1] reported an increased milk production due to an effective anthelmintic treatment. However, if the recommended withdrawal times are not respected or if nonauthorised substances are administered, the residue level in milk can be very high [2]. To protect the consumer, maximum residue limit (MRL) values were laid down by the European Union (EU) [3].

The veterinary drug substances involved in this study are the tetramisole levamisole (LE) and the seven benzimidazoles thiabendazole (TB), oxfendazole (OF), oxibendazole (OB), albendazole (AB), fenbendazole (FB), febantel (FE) and triclabendazole (TC). Several chromatographic methods for anthel-

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mintic residues have been developed and published already. UV or fluorescence were the most commonly used detection techniques [4–8]. However, detection based on the molecular structure of the compound with mass spectrometry is much more powerful for confirmation purposes. Various mass spectrometric detection methods have already been described [9–18]. A lot of these methods used a rather old ionisation mechanism or only single mass spectrometry. To our knowledge, no liquid chromatographic multiresidue method with electrospray tandem quadrupole mass spectrometry detection for the simultaneous determination of benzimidazoles and levamisole in milk was published. The objective of this study was to develop and to validate such an analytical procedure for the simultaneous detection and quantitative determination of residues of the seven above-mentioned benzimidazoles and the tetrahydroimidazole levamisole. Because levamisole and triclabendazole are not authorised for lactating milk cows, a very sensitive method was needed. A maximum permitted limit (MPL) of 1 μg/l was chosen. The MRL values for TB, OF, OB, AB, FB and FE are 100, 10, 50, 100, 10 and 10 μg/kg, respectively. A fast and relatively simple sample preparation was required in order to be able to use the method routinely in a monitoring programme to observe the contamination of raw farm cow's milk by anthelmintic residues.

2. Experimental

2.1. Reagents and chemicals

Analytical standards of LE, TB, OB, AB, FB and the internal standard (I.S.) mebendazole were purchased from Sigma (St. Louis, MO, USA). The standard substances of OF, FE, TC and flubendazole (FLUB) were kindly provided by Merial (Toulouse, France), Bayer (Leverkusen, Germany), Novartis (Munchwilen, Switzerland) and Janssen Animal Health (Beerse, Belgium), respectively. The molecular structures of these tetramisole and benzimidazole compounds are shown in Fig. 1. Dimethyl sulfoxide (HPLC grade), ethyl acetate (HPLC grade) and formic acid (analytical grade) were from Panreac (Barcelona, Spain). Acetonitrile and methanol of HPLC gradient grade and sodium hydroxide of analytical grade were obtained from Merck (Darmstadt, Germany). Water was purified by a Maxima Ultra LC 113 water purification system (Elga, Bucks, UK) to HPLC grade. Blank milk samples were received from the Department of Animal Nutrition and Husbandry (Melle, Belgium).

2.2. Standard solutions

The analytical standards were dissolved in 10 ml of dimethyl sulfoxide. Stock solutions of 0.1 and 0.2 mg/ml were prepared by dilution with methanol and stored refrigerated at 5 °C for up to 2 months. The working standard solutions were prepared immediately before use by dilution with water containing 0.1% formic acid–acetonitrile (50:50, v/v). Tuning the mass spectrometer and acquisition of the analyte identification spectra were performed with standard solutions of 1 μg/ml. During the validation procedure, the I.S. was spiked using a solution of 1 and 5 μg/ml for fortification to a final milk sample concentration of 10 and 100 μg/l, respectively. The anthelmintic analytes were fortified using a standard solution of 0.1, 1 and 5 μg/ml for matrix concentrations up to 2.5 μg/l, between 2.5 and 25 μg/l and above 50 μg/l, respectively.

2.3. Apparatus

A shaker (Bühler, Hechingen, Germany), a RC-5B Sorvall centrifuge (DuPont Instruments, Wilmington, DE, USA) and a vortex mixer (Scientific Industries, Bohemia, NY, USA) were used during the sample preparation. A high-performance liquid chromatograph combined with a mass spectrometer already applied for previous research studies [15,17,18] was used. The high-performance liquid chromatographic–tandem mass spectrometric system (LC–MS–MS) consisted of a LC system (Kontron, Biotech Instruments, Milan, Italy) with a 325 ternary pump system, a vacuum degasser and a 465 autosampler, coupled with a Quattro LCZ tandem quadrupole mass spectrometer (Micromass, Altrincham, UK) provided with a z-spray electrospray ion interface. The mass spectrometer was fully controlled by the MASSLYNX software version 3.3. A syringe pump (Harvard Apparatus model 11, Holliston, MA, USA) con-

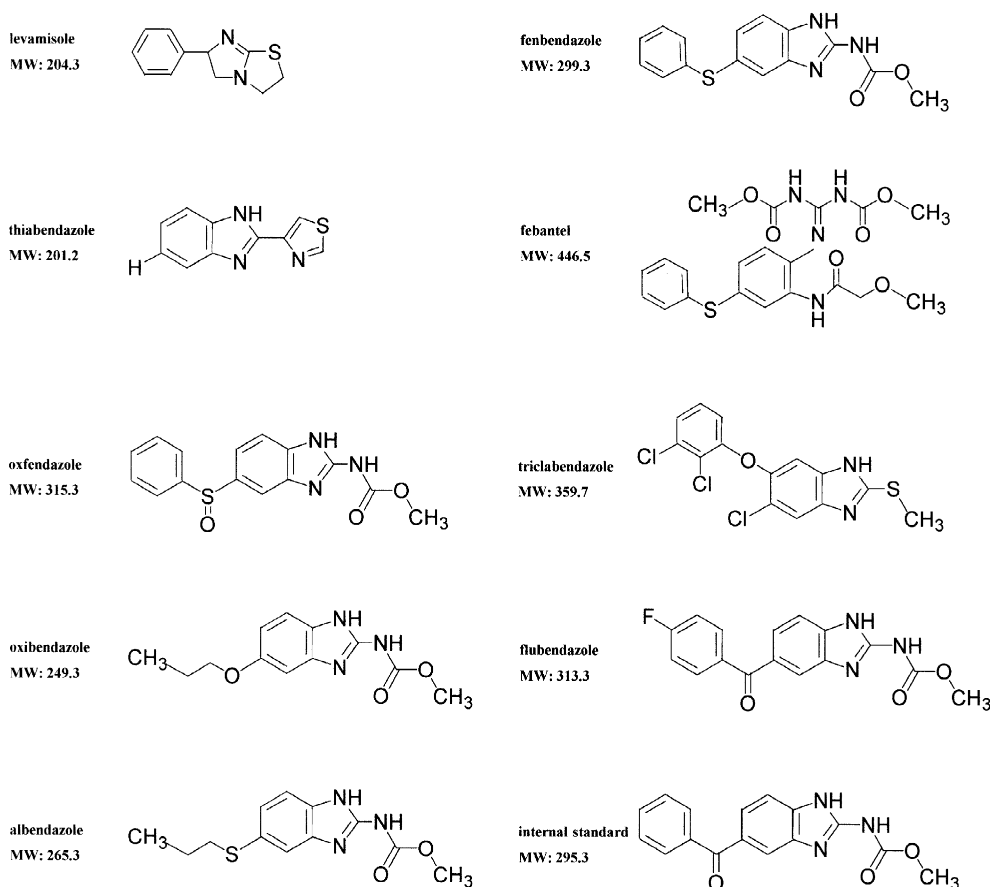

Fig. 1. Chemical structures of the examined anthelmintics (MW = molecular weight).

nected to the interface was very useful for tuning purposes.

2.4. Sample preparation

A 5-ml volume of milk was transferred into a 50-ml centrifuge tube. The milk was spiked with the anthelmintic substances and/or the I.S. After standing for 30 min, the milk sample was made alkaline with 100 μl of a 10 *M* sodium hydroxide solution. The extraction of the analytes was performed with 15 ml of ethyl acetate on a shaker (amplitude: 30 mm, frequency: 90 per min) for 5 min. The extract was centrifuged (5000 rpm, 10 min) and the supernatant was removed using a pipette and transferred into a graduated tube. The organic fraction was evaporated to dryness under a stream of nitrogen in a water bath at 50 °C. The dry residue was redissolved in 600 μl of mobile phase consisting of 0.1% aqueous formic acid–acetonitrile (50:50, v/v) while vortex mixing. The mixture was heated at 50 °C in a waterbath for 5 min. After vortex mixing, the mixture was made up to 1 ml with mobile phase and homogenised by vortex mixing. After cooling to room temperature, the cleaned sample extract was filtered through a 0.2-μm PTFE filter into an autosampler vial.

2.5. Liquid chromatography

The HPLC separation of the anthelmintic analytes was performed on a reversed-phase column and was based on a previously developed procedure [18]. The HPLC runs were carried out at room temperature on

an Alltima C_{18} column (Alltech, Deerfield, Il, USA), 5 μm, 150×2.1 mm I.D. protected by a Alltima C_{18} guard cartridge of 7.5×2.1 mm I.D. The mobile phase consisted of water containing 0.1% formic acid (to increase the ionisation) (A) and acetonitrile (B). Gradient elution was applied: A–B (65:35) (0 min), A–B (65:35) to (50:50) (0–0.1 min), A–B (50:50) to (25:75) (0.1–3 min), A–B (25:75) (3–5 min), A–B (25:75) to (50:50) (5–7 min), A–B (50:50) to (65:35) (7–15 min) and A–B (65:35) (15–25 min). A flow-rate and injection volume of 0.25 ml/min and 20 μl, respectively, were applied.

2.6. Mass spectrometry

The analytes were detected and identified with a tandem quadrupole MS without splitting the HPLC eluent flow. Atmospheric pressure electrospray ionisation in the positive mode (ESI^+) was applied. Nitrogen gas flows of 80 and 600 l/h for nebulising the HPLC eluent and for drying the solvents, respectively, were used. The source block and solvent desolvation temperatures were set at 130 and 250 °C, respectively. The parent molecular ions were fragmented in the collision cell with argon gas.

The analytes were detected by tandem MS using the multiple reaction monitoring (MRM) function of two transitions. The mass spectrometer was tuned optimising the specific cone voltage and collision energy to maximise the ion current of the three induced ions of each drug substance. The ESI^+ mode leads to addition of a H-atom to the ions so that protonated positive ions are formed. The optimisation of the transition of the precursor ion, the molecular parent ion, to the two most abundant daughter ions was performed by infusion of a standard solution of 1 μg/ml using a syringe pump. A summary of the monitored protonated cations and the optimised MS operating parameters obtained for the examined anthelmintics is given in Table 1. Identification and confirmation of a positive sample is proved by detection of these three diagnostic ions above the detection limit.

Quantification was obtained by internal calibration. The generated data of the samples by MRM of the transition from the parent ion into the most abundant daughter ions were evaluated by an internal standard procedure based on matrix calibration curves. The quantitative results were calculated automatically by the MASSLYNX software version 3.3 of the mass spectrometer. The calibration curves were always calculated using the best fit of two replicated determinations per concentration level. The calibration curves were obtained using at least five concentration points, including the zero level. The response factors (y values) (response factor= peak area ratio of sample and I.S. multiplied by the concentration level of the I.S.) were plotted against the concentration levels (x values).

2.7. Validation procedure

The proposed LC–MS–MS method was validated by determining the various parameters which are stipulated as the revised EU requirements for detecting residues of veterinary drug substances in animal

Table 1
Summary of the protonated diagnostic ions and the MS operating parameters

Analyte	Parent ion (m/z)	Daughter ions (m/z)	Cone voltage (V)	Collision energy (eV)
Levamisole	205.0^+	91.1^{+a}, 123.1^+	40	29
Thiabendazole	202.0^+	175.2^{+a}, 131.1^+	45	28
Oxfendazole	316.2^+	191.2^{+a}, 159.1^+	40	22
Oxibendazole	250.1^+	176.2^{+a}, 218.1^+	35	23
Albendazole	266.2^+	191.2^{+a}, 234.3^+	35	27
Fenbendazole	300.1^+	268.2^{+a}, 159.0^+	40	28
Febantel	447.4^+	383.4^{+a}, 415.3^+	25	15
Triclabendazole	359.0^+	274.1^{+a}, 344.2^+	40	30
Flubendazole	314.2^+	282.2^{+a}, 123.0^+	29	40
Internal standard	296.1^+	264.2^{+a}, 104.9^+	35	25

[a] Most abundant daughter ion.

products [19]. Some supplementary validation parameters were also evaluated.

An evaluation of the mass spectrometry identification criteria was performed during the whole validation procedure. Concerning the chromatographic separation, the difference between the relative retention time (RRT) of the analytes in the validation sample, and those of the matrix calibration standards was measured. Concerning the ion recognition, each diagnostic ion was measured when it had a signal-to-noise ratio of at least 3:1. Furthermore the difference of the relative intensities of the detected ions (RI), expressed as a percentage of the peak area of the most intense ion, in the validation samples and those of the matrix calibration standards was evaluated.

The linearity of the LC–MS–MS response was checked by repeated LC–MS–MS analysis of standard solutions of a mixture of the anthelmintics at concentration levels of 0, 1, 5, 25, 100 and 500 μg/l.

The examined validation parameters for the complete analytical procedure were the linearity, the specificity, the stability, the recovery, the precision (repeatability and within-laboratory reproducibility) and the analytical limits (limit of detection, decision limit and detection capability). During this whole validation procedure, blank milk samples were fortified with a standard solution of a mixture of the anthelmintic compounds, unless otherwise stipulated. The I.S. was added with a separate standard solution. The analytical method was validated at two concentration levels. To evaluate the usefulness of the quantitative determination method for the non-authorised substances levamisole and triclabendazole, at residue concentration values as low as possible, the validation was performed at MPL (1 μg/l) level. Moreover, the method can also be used in depletion studies to quantify low residue levels. On the other hand the method is also validated on MRL level which is very important to make the right decision for the licensed substances for statutory testing purposes.

To evaluate the specificity, possible interferences were checked by analysing 20 blank milk samples. Possible hindered identification and quantification was detected by addition of flubendazole, a benzimidazole substance very similar to the target compounds. Hindered identification was checked by analysing blank milk samples separately spiked at MRL level with the authorised substances and at 10 μg/l for LE, TC and FLUB. Interferences on the quantification were evaluated by comparing the peak areas of blank samples spiked at 100 μg/l, respectively with a mixture of ME+FLUB (ME=LE+TB+OF+OB+AB+FB+FE+TC) and ME.

The linearity of the method was demonstrated using blank milk samples spiked with the analytes at concentration levels of 0, 1, 5, 25, 100 and 500 μg/l.

The stability of fortified milk samples was tested by repeated determination of a spiked milk sample for 3 months at 2-week time intervals. The samples were fortified at the MRL level for the authorised compounds and at 10 μg/l for levamisole and triclabendazole and stored at −18 °C until determination. The regression data and measured residue values were compared with those obtained for a freshly fortified milk sample. Furthermore, the stability of a frozen (−18 °C) incurred milk sample positive for LE was followed for 4.5 months.

Because no certified reference material of the anthelmintic substances exists, the analyte extraction recovery instead of the trueness was determined. The recovery as well as the repeatability and the within-laboratory reproducibility were evaluated at two concentration levels. In a first series of analyses, blank milk samples spiked at 1 MPL, 1.5 MPL and 2 MPL what corresponds to 1.0, 1.5 and 2.0 μg/l, were analysed. These validation parameters were also calculated at MRL level with blank milk samples spiked only with the authorised anthelmintic compounds at their respective MRL level. To obtain the recovery efficiency, the I.S. was added after the clean-up procedure and six replicates at each level were analysed.

To evaluate the precision of the method, the repeatability and the within-laboratory reproducibility were determined. The repeatability was calculated by analysing six replicates on 3 different days. To determine the within-laboratory reproducibility the analyses were performed on 5 different days, using other standard solutions and by two different operators. To obtain six values, one value obtained for the repeatability experiments data was also taken into account.

The limits of detection (LODs) were calculated as the apparent residue content corresponding to the value of the mean plus three times the standard

deviation (SD) obtained for at least 20 representative blank sample determinations. If only noise was obtained, technical LODs were calculated as those concentrations which yield a signal-to-noise ratio (S/N) of 3:1.

Besides the more commonly used LOD, the new concept of decision limit ($CC\alpha$) was also studied. $CC\alpha$ is the concentration, above which it can be decided with a statistical certainty of $1-\alpha$ that the identified content is truly above the MRL/MPL. The $CC\alpha$ values of the proposed determination method are defined as the mean values of the obtained concentration levels by determining blank milk samples spiked at MRL and MPL (=1 μg/l) level plus 1.64 times the corresponding standard deviations. This validation parameter was calculated for the various anthelmintic substances with already obtained values of the calibrations curves calculated for the evaluation of the validation parameters repeatability, recovery, within-laboratory reproducibility and stability.

The detection capability ($CC\beta$) is the concentration at which the method is able to detect the MRL/MPL residue level with a statistical certainty of $1-\beta$. The $CC\beta$ values were calculated as the sum of the respective $CC\alpha$ value plus 1.64 times the SD obtained on the measured concentration values by determining 20 blank milk samples spiked at the respective decision limits obtained at MRL and MPL concentration level.

3. Results and discussion

3.1. Liquid chromatography

A relatively simple, very fast, sensitive and reliable multiresidue method for the determination and the quantification of residues of LE, TB, OF, OB, AB, FB, FE and TC at concentration levels lower than 1 μg/l was developed. A previously published method for the determination of mebendazole and metabolites, substances of the same anthelmintic group, in sheep liver [18] was optimised for determination of the studied anthelmintic substances in milk. Optimisation of the sample extraction and the clean-up indicated that defatting of the sample extract with *n*-hexane could be omitted. A slightly higher sensitivity for some of the substances was obtained without this supplementary step. At the beginning of the method development, netobimin and albendazolsulfoxide, two other authorised benzimidazole anthelmintics [3] were also involved in this study. However, the proposed determination procedure which is very useful for the described analytes was not successful for these substances. A new separate method has to be developed. Probably, the sample extraction and/or the MS ionisation mode would have to be changed. Compared with our previously described HPLC method [2], this LC–MS–MS method is much faster, the scoop of substances is broader and the confirmation power of MS is much higher than those of diode array UV detection. Because of the high selectivity of MS, the sample clean-up could be kept very simple.

The mobile phase and gradient programme of our previously developed LC–MS–MS method [18] was successful for the LC separation of the eight anthelmintics and the I.S. To increase the sensitivity, 20 μl, instead of 10 μl of cleaned sample extract, was injected into the LC–MS–MS apparatus. To shorten the running time, the recommended solvent flow-rate of 0.20 ml/min for the column with an I.D. of 2.1 mm was increased to 0.25 ml/min. An example of a chromatogram of a blank milk sample fortified at 1 μg/l with a mixture of the anthelmintic analytes and at 10 μg/l for the I.S. mebendazole is shown in Fig. 2. All the compounds were eluted within 11 min. The initial mobile phase composition was already obtained after a run time of 15 min. The column had to be equilibrated for a further 10 min. The total run time was 25 min which means that 38 cleaned samples can be determined per night. The sample preparation was more time consuming and thus the time-limiting step. Twenty-four samples could be prepared during a 7-h working day. Because of the stability of spiked samples, the prepared samples were collected and stored refrigerated or frozen until determination during the validation study.

3.2. Mass spectrometry

Mebendazole was used as I.S. because deuterated analytical standards for the anthelmintics involved in this study are not available. Mebendazole is authorised for use in food animals, but not in lactating dairy cows. The proposed method is only useful for

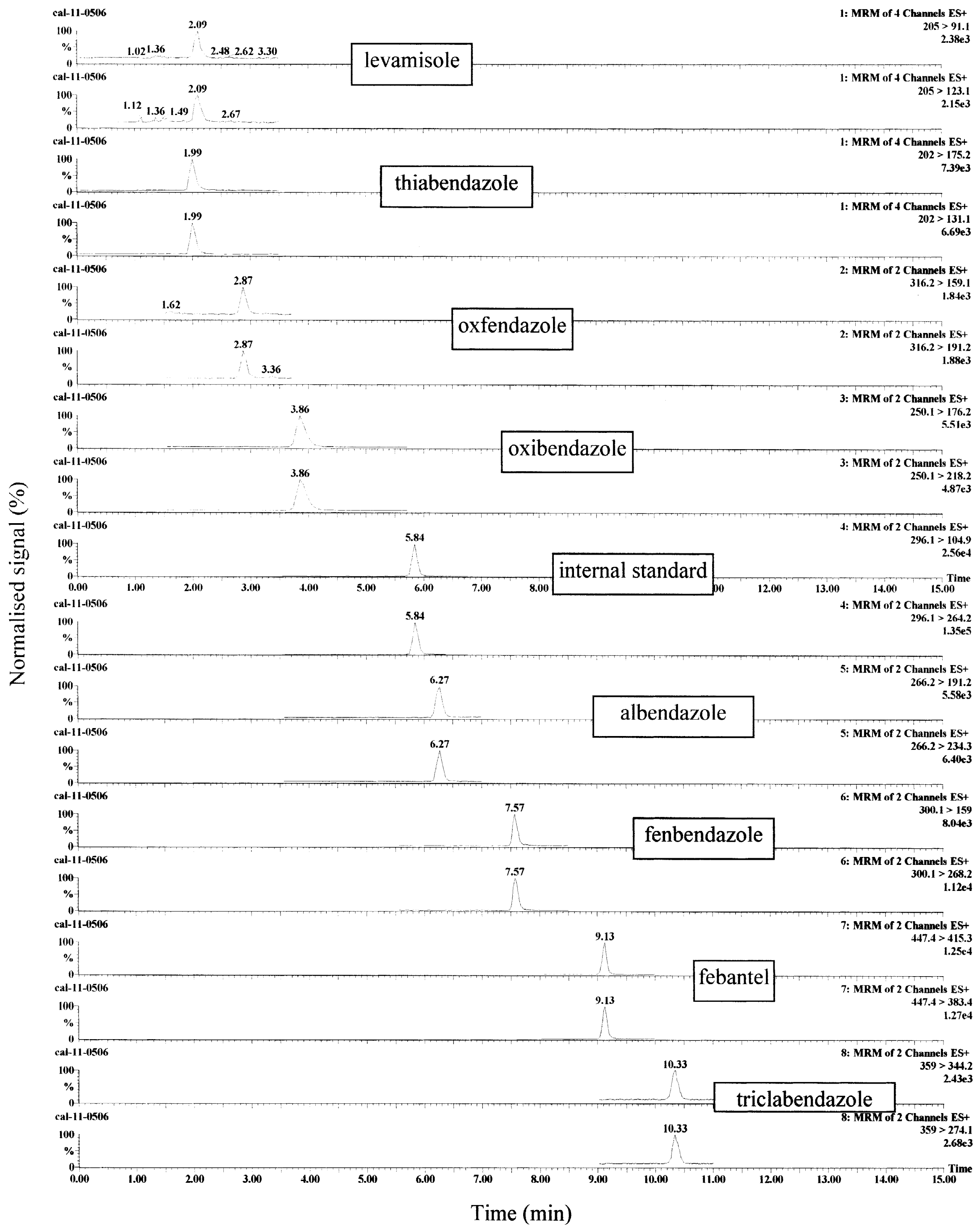

Fig. 2. Chromatogram of a blank milk sample spiked with a mixture of the anthelmintics at 1 μg/l and with the internal standard mebendazole at 10 μg/l.

milk matrices where it is practically impossible to find a mebendazole contamination. Furthermore, the tandem MS detection technique has a very high sensitivity and specificity. Any mebendazole contamination of unknown samples will immediately be detected and can be compensated for after double analysis.

Identification and quantitative determination of the eight anthelmintics involved in this study, FLUB and the I.S. were carried out by MRM where two transitions from parent ion to daughter ion are measured. An explanation of the fragmentation route for the various compound molecules into the daughter diagnostic ions, as summarised in Table 1, are given below. The most abundant protonated daughter ion for LE with *m/z* 91.1 is induced by fragmentation of the protonated product ion to C_6H_5CH–. The fragment ion with *m/z* 123.1 can be explained by splitting off the fragment –$NCSCH_2CH_2N$– from the LE molecule. The consecutive loss of –CN and –CS from the molecular structure of TB give rise to the fragments with *m/z* 175.2 and *m/z* 131.1. The two daughter fragments with *m/z* 175.2 and *m/z* 159.1 of OF originates from consecutive losses of –SOC_6H_5 and –OCH_3. The loss of –OCH_3 from the chemical structures of OB, AB, FB, FE, FLUB and the I.S. results in the fragments with *m/z* 218.1, 234.3, 268.2, 415.3, 282.2 and 264.2, respectively. The daughter fragment with *m/z* 104.9 for I.S. originates from fragmentation into –COC_6H_5. The remaining fragment with *m/z* 123.0 for FLUB originates from fragmentation into –COC_6H_4F. The loss of –$CH_2CH_2CH_3$ from the fragment with *m/z* 218.1 of OB results in the second daughter fragment with *m/z* 176.2. The second fragment of AB with *m/z* 191.2 is obtained by loss of –CONH from the main daughter ion with *m/z* 234.3. A second loss of –OCH_3 from the fragment ion of FE with *m/z* 415.3 results in the remaining daughter ion with *m/z* 383. The daughter fragment with *m/z* 159.0 for FB originates from the loss of –SC_6H_5 from the fragment with *m/z* 268.2. The fragment ions of TC with *m/z* 344.2 and *m/z* 274.1 can be explained by the consecutive loss of –CH_3 and 2 Cl atoms, respectively.

For the confirmation of the drug compounds involved in this study and belonging to the drug substances of group B, veterinary drugs and contaminants, of Annex I of Council Directive 96/23/EC, at least three identification points have to be obtained with MS detection [19]. The detection of the analytes in standard solutions and spiked milk samples during this study was performed by the transitions of the parent ion $(M+H)^+$ to the two most abundant daughter ions. The two MRM transitions studied are equal to four identification points.

As can be seen in the chromatogram in Fig. 2, levamisole and thiabendazole were not chromatographically separated. However, LC separation was not needed because of the powerful separation capacity of the mass spectrometer in the MRM mode.

3.3. Validation study

3.3.1. Mass spectrometric detection

The specific criteria on relative retention time (RRT) and the ion ratio (RI) were examined for all the samples used for the validation study. The values for RRT and for RI were in agreement with the European requirements [19] for all of the samples used to calculate these parameters. As an example, the values of a sample spiked at 1 μg/l and of a matrix calibration sample spiked at 1 μg/l to evaluate the repeatability, are given. The values for the RRT in the spiked sample were 0.31, 0.31, 0.49, 0.51, 1.00, 1.27, 1.69 and 1.86 for LE, TB, OF, OB, AB, FB, FE and TC, respectively. These values are within the tolerance ranges of ±2.5% obtained with the figures of the calibration samples. The values for the RI in the spiked sample were 31, 96, 81, 83, 80, 77, 84, 94 and 22% for LE, TB, OF, OB, AB, FB, FE, TC and the I.S., respectively. These figures are within the allowed ranges obtained for the calibration sample. These ranges are 27±25, 99±20, 92±20, 87±20, 81±20, 79±20, 81±20, 90±20 and 22±25% for LE, TB, OF, OB, AB, FB, FE, TC and the I.S., respectively.

3.3.2. Linearity

The linear instrumental response was proven for the eight analytes by six points standard calibration at concentration levels of 0, 1, 5, 25, 100 and 500 μg/l. The slope and the intercept values for the linear regression standard lines of LE, TB, OF, OB, AB, FB, FE, TC were 0.33 and −0.002, 1.44 and 20.71, 0.21 and 0.002, 0.66 and −0.0002, 0.46 and 0.35, 0.82 and 0.20, 0.11 and 0.08, 1.21 and 0.03,

respectively. Because of the rather broad concentration range a better fit for the low concentration levels was obtained with a weighting factor of $1/y^2$.

The linearity of the LC–MS–MS confirmation method was demonstrated for the eight anthelmintics in a residue concentration range of 0–500 μg/l. A six-point matrix calibration analysis at concentration levels of 0, 1, 5, 25, 100 and 500 μg/l was analysed. The obtained linear regression parameters slope and intercept for LE, TB, OF, OB, AB, FB, FE, TC were 0.19 and −0.0001, 0.66 and 0.017, 0.14 and 0.00005, 0.83 and 0.014, 0.43 and 0.0069, 0.67 and 0.0043, 0.82 and 0.0041, 0.24 and 0.0026, respectively. Again, to increase the fit for the low residue levels, weighted least square regression was applied with a weighting factor of $1/y^2$.

3.3.3. Specificity

No interferences of endogenous material above a *S/N* ratio of 3 for all of the eight anthelmintics could be detected by analysing twenty blank milk samples. False positive or false negative results might be generated due to hindered identification. The absence of possible interference of other substances belonging to the same class of compounds as the analyte was demonstrated. No false positive or false negative results were obtained by analysing blank milk samples, separately spiked at MRL level for the licensed compounds and at 10 μg/l for LE, TC and FLUB. The quantification was not influenced by the presence of flubendazole in the samples. The differences on the peak areas between the samples with and without flubendazole were very small and within the normal ranges of repeatability. Clearly, the tandem MS system is a very powerful analytical tool with a high separation capacity and a high specificity. The identification and confirmation properties of the LC–MS–MS technique are superior in comparison with the more traditional LC–diode array detection (DAD) technique using UV detection applied in our previous determination methods [2].

3.3.4. Stability

The stability of fortified milk samples stored at −18 °C was tested by repeated determination of samples at 2-week intervals. The regression data and calculated concentration values were comparable with those obtained for freshly spiked milk samples during an observation period of 3 months. For practical reasons, this observed stability for fortified samples is very important. The numerous samples to be analysed can be collected, stored and analysed in the most efficient way.

The demonstrated instability of an incurred milk sample indicated that laboratories have to be careful with the conservation of real samples for testing purposes of anthelmintic residues for a long time period. A decrease of 30% of the residue value of levamisole was observed in a positive milk sample of the Belgian monitoring programme after 3 months storage at −18 °C. The levamisole residue level decreased from 183.8 to 128.8 μg/l. This level stabilised the next 1.5 month of measurement during subsequent storage under frozen conditions.

3.3.5. Recovery

The trueness was determined by recovery experiments because of the lack of certified reference material. The results of the sample extraction efficiency analyses are given in Table 2. The overall recovery values ranged from 89.6 to 102.0%. These values are in agreement with the EU requirements [19] which are correlated with the concentration level. The variabilities on the recoveries were acceptable and the RSD values varied from 5.4 to 11.6. The obtained variabilities are in agreement with the criteria of the Horwitz equation [19] which depend on the spiked concentration. The obtained extraction recoveries are higher than those of our previous LC–DAD method [2]. The supplementary clean-up during the sample preparation probably influenced the analyte recovery unfavourably. In spite of the very simple sample preparation, Facino et al. [10] reported somewhat lower recoveries, probably due to the not fully optimised extraction procedure or to the choice of extraction solvents. Chapell et al. [11] used a more intensive solid-phase extraction (SPE) sample clean-up for the determination of LE in milk. Their recovery was acceptable and reached 82.6%.

3.3.6. Precision

The precision was proven by means of the repeatability and the within-laboratory reproducibility.

The repeatability was checked at MPL and MRL

Table 2
Analyte recovery values for the determination of anthelmintics in fortified milk samples ($n=6$ for each concentration level)

Spiked level (µg/l)	LE		TB		OF		OB		AB		FB		FE		TC	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
1.0	102.9	6.1	94.8	5.3	102.1	13.1	104.7	7.9	88.8	8.0	85.1	7.4	96.6	6.4	96.8	14.6
1.5	100.0	7.2	98.8	5.1	96.6	8.9	109.3	2.2	99.4	3.1	95.2	4.7	101.7	1.0	91.6	4.6
2.0	89.6	8.3	93.9	4.0	82.7	6.5	101.7	1.3	90.3	3.6	82.7	8.4	98.4	2.6	87.0	9.1
5	–	–	–	–	108.7	9.5	–	–	–	–	103.6	1.9	79.9	8.2	–	–
10	–	–	–	–	100.5	2.2	–	–	–	–	102.2	2.8	79.9	5.3	–	–
15	–	–	–	–	104.3	3.3	–	–	–	–	106.0	1.8	81.3	5.4	–	–
25	–	–	–	–	–	–	99.0	2.1	–	–	–	–	–	–	–	–
50	–	–	85.6	15.8	–	–	96.1	2.1	95.1	4.4	–	–	–	–	–	–
75	–	–	–	–	–	–	101.1	1.4	–	–	–	–	–	–	–	–
100	–	–	90.5	3.9	–	–	–	–	96.2	3.7	–	–	–	–	–	–
150	–	–	98.4	1.8	–	–	–	–	100.5	0.4	–	–	–	–	–	–
Overall recovery (%)	97.3	9.2	93.7	8.2	99.1	11.4	102.0	5.4	95.0	6.1	95.8	10.5	89.6	11.6	91.8	10.8

levels. The results of repeated analysis of spiked samples on different days at MPL and MRL levels are summarised in Tables 3 and 4, respectively. The precision data are shown with mean values of measured residue concentrations together with the variability on these found levels expressed as RSD. Almost all of these mean values fell within the ranges stipulated by the EU criteria [19], except for a few very small deviations. Following the validation criteria, the within-day precision expressed as RSD values for analyses carried out under repeatability conditions would have to be between one half and

Table 3
Repeatability results for the determination of anthelmintic substances in milk samples spiked at 1, 1.5 and 2 MPL (=1.0, 1.5 and 2.0 µg/l) ($n=6$ for each concentration level)

Spiked level (µg/l)	LE		TB		OF		OB		AB		FB		FE		TC	
	Mean (µg/l)	RSD (%)	Mean (µg/l)	RSD (%)	Mean (µg/l)	RSD (%)	Mean (µg/l)	RSD (%)	Mean (µg/l)	RSD (%)	Mean (µg/l)	RSD (%)	Mean (µg/l)	RSD (%)	Mean (µg/l)	RSD (%)
Within day																
Day 1																
1.0	1.1	17.1	1.1	16.4	0.9	11.9	0.9	10.8	0.9	6.2	0.8	12.6	1.0	15.9	0.9	9.8
1.5	1.5	8.6	1.4	7.9	1.5	12.1	1.4	3.5	1.3	10.3	1.3	10.2	1.4	16.3	1.2	14.4
2.0	2.1	2.4	2.1	7.0	1.9	9.0	1.9	5.6	1.9	3.5	1.7	5.9	1.6	6.3	1.4	8.9
Day 2																
1.0	0.9	19.0	0.8	24.7	1.0	9.6	0.9	14.2	0.8	7.5	1.0	5.8	1.1	7.9	1.0	5.3
1.5	1.6	10.1	1.4	12.4	1.5	10.1	1.4	2.8	1.2	8.8	1.4	11.0	1.5	9.9	1.3	6.0
2.0	2.1	10.6	2.0	9.8	1.9	14.7	2.0	6.3	1.8	5.4	1.7	7.4	1.7	13.2	1.4	11.2
Day 3																
1.0	1.0	13.6	0.8	18.5	0.8	28.5	0.9	11.7	1.0	10.7	1.0	12.5	1.1	8.4	1.0	10.0
1.5	1.6	8.7	1.3	11.4	1.1	8.7	1.3	9.3	1.4	5.5	1.4	6.3	1.5	11.8	1.5	10.0
2.0	1.8	6.8	1.6	3.9	1.6	13.8	1.7	4.0	1.9	8.4	1.8	4.7	1.9	3.1	1.8	10.9
Between days overall ($n=18$)																
1.0	1.0	18.2	0.9	21.7	0.9	18.9	0.9	11.9	0.9	11.5	1.0	15.3	1.0	11.0	0.9	9.4
1.5	1.5	9.1	1.4	10.8	1.4	16.0	1.4	6.1	1.3	9.0	1.3	10.3	1.5	12.2	1.4	13.8
2.0	2.0	10.2	1.9	13.9	1.8	14.5	1.9	8.8	1.9	6.1	1.7	7.0	1.7	12.2	1.6	16.0

Table 4
Repeatability results for the determination of anthelmintic substances in milk samples spiked at 0.5, 1.0 and 1.5 MRL level ($n=6$ for each concentration level)

Analyte	Spiked level (μg/l)	Within day						Between days overall ($n=18$)	
		Day 1		Day 2		Day 3			
		Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)
TB	50	41.8	6.9	46.4	9.8	48.9	2.8	45.7	9.4
	100	83.6	3.0	91.8	12.3	97.3	1.0	90.9	9.4
	150	133.4	6.0	145.0	5.0	157.4	6.2	145.3	8.8
OF	5	5.4	7.1	4.6	7.8	4.9	4.0	5.0	9.1
	10	9.0	9.6	9.7	8.5	10.1	7.7	9.6	9.5
	15	15.1	3.5	15.3	8.5	15.2	5.3	15.2	5.8
OB	25	22.0	3.5	24.7	1.6	25.1	2.7	24.0	6.3
	50	42.8	3.1	49.0	5.9	49.9	2.9	47.2	8.0
	75	66.9	3.9	77.2	4.5	78.9	3.8	74.3	8.3
AB	50	44.3	7.1	48.3	4.9	52.0	3.0	48.2	8.2
	100	93.9	2.2	96.4	6.4	102.0	1.3	97.4	5.2
	150	143.8	2.8	151.3	4.4	161.8	4.5	152.3	6.3
FB	5	5.5	5.1	5.4	8.1	5.8	5.0	5.5	6.8
	10	10.7	4.1	10.3	8.2	11.14	2.4	10.7	6.0
	15	16.1	5.1	16.6	4.8	16.8	4.1	16.5	4.7
FE	5	4.8	5.6	4.5	8.0	5.0	5.6	4.8	7.5
	10	9.8	5.9	8.9	10.5	9.9	3.1	9.5	8.3
	15	15.0	5.1	14.7	8.0	16.5	6.9	15.4	8.3

two thirds of these values according to the Horwitz equation. The repeatability of the proposed confirmatory method is good. All of the calculated RSD values were within the allowed range. Moreover, the overall precision values were lower than half of the Horwitz values. The overall precision ranged from 5.2% for the determination of albendazole at 100 μg/l to 21.7% for the determination of thiabendazole at 1 μg/l.

A summary of the within-laboratory reproducibility results of the determinations at MPL and MRL levels are given in Tables 5 and 6, respectively. These analyses were performed on 5 different days, with different standard solutions and by two analysts. A sixth value for the various concentration levels was obtained from the analysis series performed to study the repeatability of the analytical method. The method studied showed a good within-laboratory reproducibility. The allowed ranges for the measured mean values of this validation parameter are equal to those of the repeatability [19]. This criterium was fulfilled for all the calculated mean values at the

Table 5
Within-laboratory reproducibility results for the determination of anthelmintics in milk samples spiked at 1, 1.5 and 2 MPL (=1.0, 1.5 and 2.0 μg/l) $n=6$ for each concentration level

Spiked level (μg/l)	LE		TB		OF		OB		AB		FB		FE		TC	
	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)	Mean (μg/l)	RSD (%)
1.0	1.0	11.4	0.9	14.7	0.9	15.2	1.0	10.2	1.0	1.0	1.1	13.0	1.1	19.2	1.1	13.8
1.5	1.5	6.5	1.4	9.0	1.5	21.8	1.5	8.8	1.5	6.6	1.5	10.0	1.6	5.5	1.6	7.1
2.0	2.0	4.6	1.9	12.6	1.7	7.0	2.0	8.8	2.0	5.6	2.0	14.8	1.9	27.2	2.0	25.4

Table 6
Within-laboratory reproducibility results for determination of anthelmintics in milk samples spiked at 0.5, 1.0 and 1.5 MRL level $n=6$ for each concentration level

Analyte	Spiked level (μg/l)	Mean (μg/l)	RSD (%)
Thiabendazole	50	49.2	6.5
	100	99.7	7.4
	150	159.3	9.9
Oxfendazole	5	4.8	7.8
	10	10.1	4.5
	15	14.5	18.9
Oxibendazole	25	25.0	5.3
	50	49.9	8.5
	75	77.8	12.7
Albendazole	50	51.3	3.4
	100	101.0	1.9
	150	151.8	3.7
Fenbendazole	5	5.5	12.1
	10	10.8	10.6
	15	16.4	7.0
Febantel	5	5.2	9.0
	10	10.7	11.3
	15	16.3	3.5

found concentration levels. The variability for the within-laboratory reproducibility is allowed to be higher [19] than for the repeatability and the RSD values should be lower than the values of the Horwitz equation correlated with the concentration level. The values shown in Tables 5 and 6 are much lower than these limits. The within-laboratory reproducibility expressed as RSD of the mean determined residue levels ranged from 1.0% for the determination of albendazole at 1.0 μg/l to 27.2% for the determination of febantel at 2.0 μg/kg. As expected, this range was somewhat higher than this for the repeatability.

3.3.7. Analytical limits

The development and the full optimisation of this LC–MS–MS method to reach a high sensitivity was one of the objectives of this study. A MPL level of 1 μg/l was chosen to detect low anthelmintic residue contamination levels in milk during the monitoring programme of raw farm milk, especially because two banned substances namely levamisole and triclabendazole are involved. As already expected from the study of the specificity, no interference could be measured for any compound to calculate the LOD values while analysing 20 blank milk samples. The technical LOD values were obtained as those concentrations which yielded a S/N ratio of 3:1 by replicated analysis of blank milk samples spiked at concentration levels of 1.0, 1.5, 3.0 and 5.0 μg/l. The sensitivity of the multiresidue confirmation method was very high and all the anthelmintics could be detected at a contamination level lower than 1 μg/l. The measured LOD values (MRL values within brackets expressed in μg/kg) for LE, TB, OF, OB, AB, FB, FE and TC were 0.5 (−), 0.1 (100), 0.6 (10), 0.1(50), 0.3(100), 0.1(10), 0.1(10) and 0.6 (−) μg/l, respectively. As expected, these LOD values are much lower than those obtained in our previous HPLC methods [2]. The performance of the proposed method is also better than that described in the literature by Macri et al. [8], Facino et al. [10] and Branchflower et al. [12]. Chapell et al. [11] demonstrated a comparable detection limit for LE. They used a multidimensional procedure with online liquid chromatography–gas chromatography including an ion trap multi-mass spectrometry detector but their method can only determine levamisole.

The decision limit values were calculated at MRL levels for the registered anthelmintics as well as at MPL level (1.0 μg/l) for all the substances involved in this study. The $CC\alpha$ values at MPL level were calculated with concentration values already obtained for blank milk samples spiked at 1.0 μg/l for calculating the calibrations curves for the determination of the recovery, the repeatability and the within-laboratory reproducibility. The $CC\alpha$ values at MRL levels were calculated with figures already obtained for the determination of the recovery, the repeatability, the within-laboratory reproducibility and the stability. The calculated mean values plus 1.64 times the corresponding SD equals the decision limit. These $CC\alpha$ values are summarised in Table 7. Obviously the highest percentile deviation on the target concentration was obtained for the analyses at MPL level. The highest value of 1.3 μg/l should be taken into account for decisions concerning the contamination of febantel and triclabendazole. The highest percentile deviation (12%) at MRL level was also observed for the determination of febantel. The obtained $CC\alpha$ was 11.2 μg/l. The decision limit

Table 7
Decision limits ($CC\alpha$) and detection capabilities ($CC\beta$) (in μg/l) for the determination of anthelmintics in milk at MPL and MRL levels[a]

Contamination level	Levamisole		Thiabendazole		Oxfendazole		Oxibendazole		Albendazole		Fenbendazole		Febantel		Triclabendazole	
	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$	$CC\alpha$	$CC\beta$
MPL (=1.0 μg/l)	1.2	1.4	1.1	1.2	1.1	1.3	1.1	1.2	1.1	1.2	1.1	1.3	1.3	1.3	1.3	1.4
MRL	–		105.8	118.2	10.5	12.1	52.6	58.9	104.6	110.5	10.9	12.1	11.2	12.9	–	

[a] $CC\alpha$, mean value of spiked milk samples + 1.64SD (standard deviation) ($n=9$ for MPL level and $n=12$ for MRL level); $CC\beta = CC\alpha +$ 1.64SD ($n=20$).

should be handled as a very useful tool for the laboratory in order to make the right decision concerning a positive sample.

The detection capabilities were obtained by analysing 20 blank milk samples spiked at MPL/MRL level. The respective decision limit values plus 1.64 times the corresponding standard deviations equals the detection capabilities. The $CC\beta$ values are also given in Table 7.

4. Conclusions

A simple, very fast and specific LC–MS–MS confirmatory multiresidue method has been developed for the simultaneous, quantitative determination of levamisole, thiabendazole, oxfendazole, oxibendazole, albendazole, fenbendazole, febantel and triclabendazole in milk at residue levels at 1 μg/l. The proposed method is useful to quantify residues of the anthelmintics at MRL level, providing the laboratory a powerful tool to make decisions on violations for statutory testing purposes. The reliability of the LC–MS procedure was proven by fulfilling all the validation criteria of the final version of European Commission Decision concerning analytical methods for detecting substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC [19].

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