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Quantification of anthelmintic drug residues in milk and muscle tissues by liquid chromatography coupled to Orbitrap and liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

A simple method for the determination of some anthelmintic drugs and phenylbutazone residues in milk and muscle was developed. Following a fast and easy extraction and evaporation procedure, the extract was injected into an ultra performance liquid chromatography system coupled to a single stage Orbitrap detector. The high mass resolution of 50,000 full width at half maximum and corresponding narrow mass windows permitted a very selective and sensitive detection of analytes without requiring fragmentation of the observed $[M+H]^+$ or $[M+Na]^+$ ions. This eliminated some difficulties which have plagued the analysis of compounds belonging to the group of avermectins. The analytical method was validated according to the EU commission decision for Orbitrap based, but also for more traditional tandem mass spectrometry based detection and quantification. Equal repeatability but significantly higher sensitivity for critical compounds (avermectins) was obtained for the Orbitrap based detection. A result of this study was the conclusion that analytes with poor fragmentation properties (e.g. sodium-cationized molecules) can be more easily quantified by single stage high resolution mass spectrometry than by tandem mass spectrometry.

1. Introduction

Anthelmintic drugs can be used to treat animals against parasites like nematodes and arthropods. The term anthelmintic refers to the spectrum of pharmaceutical activity and not to a common chemical sub-structure of these drugs. Drugs belonging to the chemical classes of macrocyclic lactones (avermectins), benzimidazoles, flukicides and individual compounds like levamisole or coumaphos are considered to be anthelmintics. These drugs show significantly higher toxicity against parasites than mammals. Still, food (e.g. meat and milk) produced from treated animals should not contain residues of such drugs to ensure food safety. Therefore, governmental bodies like the EU require the monitoring of a number of these drugs in their control programs.

Residues of anthelmintic drugs in milk or meat were initially determined by liquid chromatography coupled to a variety of photometrical detection modes. The drug class of benzimidazoles can be monitored by utilizing ultra violet [1] or fluorescence [2] based detection. The currently preferred mode of detection and quantification appears to be liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) [3,4]. There is an excellent review paper covering the topic of benzimidazole residue analysis in biological matrices [5].

Detection is much less straightforward for avermectins which do not contain chromophoric moieties. Until recently, the preferred mode of detection was based on a pre-column derivatisation step which permits the chromatographic separation and the sufficiently sensitive fluorescence based detection [4,6]. However, the strict requirements for confirmation of suspected positive findings, require the use of mass spectrometry based detection. Quantification by fluorescence and confirmation by tandem mass spectrometry represents the currently most often practiced workflow among laboratories involved in analysis of avermectin residues [4,7]. This contrasts with residue monitoring strategies utilized for more commonly analyzed drugs like tetracyclines, quinolones and sulfonamides. These drugs are generally detected, quantified and confirmed by a single step tandem mass spectrometry (LC–MS/MS) based procedure. The analysis of avermectins by LC–MS/MS has not yet reached the same level of maturity as achieved for most other veterinary drugs. This is probably to be explained by their specific physical and chemical properties. Avermectins are rather heavy compounds with a molecular weight ranging between 600 and 900 Da. Despite of their relatively high masses, most avermectins, contain only one nitrogen atom within their chemical structure. Some drugs like ivermectin, doramectin

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and abamectin do not contain any heteroatoms besides C, H and O in their elemental composition. This has negative effects on the efficacy of ionization. Furthermore, avermectins which are devoid of nitrogen atoms produce significantly higher abundances for $[M+Na]^+$ than $[M+H]^+$ ions [8,9] under a positive electrospray (ESI) environment. This is even occurring in the absence of a sodium ion containing mobile phase. Traces of sodium, originating from sample processing, the mobile phase, glassware, etc. are sufficient to shift the $[M+Na]^+/[M+H]^+$ ratio [9]. This can result in non-linear calibration curves [9–11]. An additional difficulty posed by avermectins is the poor fragmentation properties of sodium adducts in the collision chamber of the MS/MS instrumentation [4,12]. Some compounds like abamectin were found to produce no significant product ions when attempting to fragment the $[M+Na]^+$ precursor ion [12]. This is the reason why several authors investigated alternative forms of ionizations. Negative ESI was reported to circumvent many of these problems. However, this comes at a price of lower sensitivity [4,9,12]. These problems could partially be mitigated by employing high pH mobile phase e.g. trimethlyamine in acetonitrile [9,12]. However, such an environment is not ideal for benzimidazoles, which produce best results when utilizing conditions favouring positive ionization (low pH values). Another strategy was the use of atmospheric pressure chemical ionization [10,13] instead of ESI. The currently most popular approach relies on the use of an ammonium salt containing mobile phase and positive ESI based detection [14]. It was the strategy to detect those avermectins containing no nitrogen atoms as $[M+NH_4]^+$ ions. Such adducts produce significantly more intensive product ions than the $[M+Na]^+$ precursor ions. Unfortunately, the required high ionic strength mobile phases suppress the signal intensity of nitrogen containing drugs (avermectins and other drugs like benzimidazoles). In addition, nitrogen containing avermectins were observed to form $[M+NH_4]^+$ ions besides the more desired $[M+H]^+$ ions [4,12]. Several authors were carefully optimizing pH and ionic strength of their mobile phase to create favourable ionization conditions for the different compounds covered by their analytical method [4,7,11,12,14]. However, the suggested mobile phase composition differs significantly in ion strength and pH among the various published papers [4,7,11,12,14]. Compared to other drugs, avermectins show relatively poor sensitivity. This makes it difficult to reach the required maximum residue levels (MRL). Therefore, authors developing multiresidue methods were compelled to assign relatively long MS/MS dwell times for these compounds [4,11]. An excellent review regarding the topic of avermectin residue analysis covering many of the discussed limitations has been recently published [14].

Phenylbutazone is not an anthelmintic but a non-steroidal anti-inflammatory drug. It has frequently been given to animals to treat musculoskeletal disorders or entritis. The lack of an MRL value for this drug mandates a sensitive detection and unequivocal confirmation to prove a possible illicit use. Therefore, LC–MS/MS has become the method of choice [15,16]. A problem related to the analysis of phenylbutazone is the instability of the compound. Irreproducible degradation reactions during extraction, clean-up and storage in the injection vials have been reported [17,18].

Analyzing a number of different compounds with a single clean-up, separation and detection method (multi residue methods) always requires compromises. Therefore, even the careful optimization of relevant conditions will only produce acceptable results for the limited set of analytes and matrices on which the optimization process focused on. Such settings may become sub-optimal when changing the underlying set-up (e.g. enlarging the number of compounds to be analyzed or shifting to other matrices to be analyzed). This is most relevant for the inclusion of avermectins into multi-residue methods. Current technologies permit the sufficiently sensitive detection of avermectins, if a method can focus on these compounds only. However, the selected conditions (mobile phase, ionization mode or interface) required for analyzing these compounds create difficulties when expanding the method to cover additional compounds not belonging to this chemical family (multi-residue methods). Furthermore, not discussed in all these cited avermectin papers was the concern that the carefully optimized elution conditions (e.g. pH and ion strength of ammonium containing mobile phase) might be far from ideal when utilizing an ESI interface from another manufacturer. Hence, it was the aim of this paper to present an alternative strategy which does not impose such limitations. Therefore, the proposed separation is based on a classical mobile phase containing only formic acid, water and acetonitrile. The resulting sodium adducts created in the ESI were not fragmented, but detected with a high resolution mass spectrometer (HRMS) operated at 50,000 full width at half maximum (FWHM) and a corresponding narrow mass window width, to provide an equal level of selectivity as MS/MS. Such high resolutions which have become available with the latest HRMS instrumentation provide significant advantages over previously reported methods [19,20] since the high selectivity improves signal to noise of analytes peaks in complex sample matrices. The quantitative performance of this approach has been critically compared to MS/MS.

The current paper focused on the capabilities and limitations of the latest HRMS technology in the field of quantitative multi-residue methods. The selection of compounds to be quantified was intended to cover a wide range of physical and chemical properties. Hence, the list of anthelmintic drugs is not exhaustive, since it does neither cover some less relevant avermectins nor the benzimidazole metabolites. The described analytical method is considered to be a building block, capable to accept further compounds belonging to the group of anthelmintics or other drug classes.

2. Materials and methods

2.1. Standards and stock solutions

The veterinary drug reference substances albendazole, febantel, fenbendazole, fubendazole, mebendazole, oxfendazole, oxibendazole, parbendazole, thiabendazole, triclabendazole, phenylbutazone, ivermectin and abamectin (avermectin B1a und B1b) were of the highest available purity and were bought from Sigma–Aldrich (Buchs, Switzerland).

Individual stock solutions (1000 mg/l)*:* They were prepared by accurately weighing 50 mg of reference substance into a 50 ml volumetric flask. Depending on the specific solubility properties, the compounds were dissolved and diluted to volume with acetonitrile, dimethlysulfoxide or methanol.
Mixed stock solution (10 mg/l): The solution was prepared by transferring 1 ml of the individual stock solutions (1000 mg/l) into a 100 ml volumetric flask and diluted to volume with dilution solution.
Mixed spike solution for A and B spikes (1000 μg/l): 2 ml of mixed stock solution (10 mg/l) was transferred into a 20 ml volumetric flask and diluted to volume with dilution solution.
Reference solution (100 μg/l): 1 ml of mixed spike solution (1000 μg/l) was transferred into a 10 ml volumetric flask and diluted to volume with dilution solution.
Reference solution (50 μg/l): 1 ml of mixed spike solution (1000 μg/l) was transferred into a 20 ml volumetric flask and diluted to volume with dilution solution.
Reference solution (10 μg/l): 2 ml of reference solution (100 μg/l) was transferred into a 20 ml volumetric flask and diluted to volume with dilution solution.

Reference solution (5 μg/l): 1 ml of reference solution (100 μg/l) was transferred into a 20 ml volumetric flask and diluted to volume with dilution solution.

2.2. Reagents and solvents

Acetonitrile, LC–MS grade was obtained from Carl Roth (Karlsruhe, Germany), methanol, HPLC grade from J.T. Baker (Deventer, The Netherlands), dimethylsulfoxide (DMSO), ammonium sulphate, formic acid (98%), ethylenediamino-tetraacetic acid (EDTA) and ascorbic acid were of analytical grade and from VWR (Darmstadt, Germany).

Mobile phase A: 50 ml of acetonitrile and 3 ml of formic acid were added into a 1 l graduated flask and made up with water to the mark.
Mobile phase B: 50 ml of water and 3 ml of formic acid were added into a 1 l graduated flask and made up with acetontrile to the mark.
Antioxidant solution: 0.07 g EDTA and 9 g of ascorbic acid was dissolved in a 250 ml graduated flak and made up with water to the mark.
Acetonitrile (40%): 200 ml of acetonitrile and 300 ml of water were added into a beaker and mixed.
Dilution solution: Mix 25 ml of antioxidant solution with 475 ml of acetonitrile 40% in a beaker.

2.3. Extraction and sample processing instrumentation

Homogenization was done by a Polytron PT300, Kinematica (Littau, Switzerland). Extracts were centrifuged by a Centrifuge Sorval RC6C plus Thermo (Zürich, Switzerland) and evaporated by a stream of nitrogen, utilizing the equipment: Liebisch; Type: 51006101 (Zürich, Switzerland).

2.4. Sample preparation

Some 10 g of milk or muscle tissue was homogenized with 10 ml of acetonitrile, 5 g of ammonium sulphate and 1 ml of antioxidant solution. In the case of a test for recovery, 200 μl of mixed spike solution (1000 μg/l) was added. The resulting solution was centrifuged (5 min at 3500 rpm = 8000 × *g*). 4 ml of the supernatant organic layer was transferred into a pre-weighted test tube. This was followed by the addition of 100 μl of antioxidant solution. Evaporation proceeded under a stream of nitrogen at 50 °C. A small volume remains (approximately 0.2 ml). The pre-weighed test tube was put onto a pair of scales and dilution solution was added until a net weight of 2 g resulted. After mixing the contents, the extract was micro-filtered. 0.63 ml of the extract was diluted with 0.07 ml dilution solution. Another 0.63 ml of the extract was spiked with 0.07 ml spike solution (1000 μg/l) to produce a B spike solution. The unspiked and the spiked extracts were transferred into two different HPLC vials for injection. Typical chromatograms showing mass traces of a spiked liver sample, showing some selected analyte traces are shown in Figs. 1 and 2.

2.5. UHPLC separation

The equipment consisted of an Acquity system from Waters (Millford MA) and a Kinetex Core-Shell, C18 150 mm × 2.1 mm 2.6 μm column with installed pre-filter, both from Phenomenex (Torrance, CA, USA), were used. The column was maintained at 25 °C and the injection volume was 10 μl. The following linear gradient was used: 0–1 min 20% B and 0.4 ml/min, 1–2 min 20–30% B and 0.4 ml/min, 2–10.5 min 30–100% B and 0.4 ml/min, 10.5–13 min 100% B and 0.4 ml/min, 13–13.1 min 100–20% B and 0.4 ml/min, and 13.1–14.5 min 20% B and 0.4 ml/min.

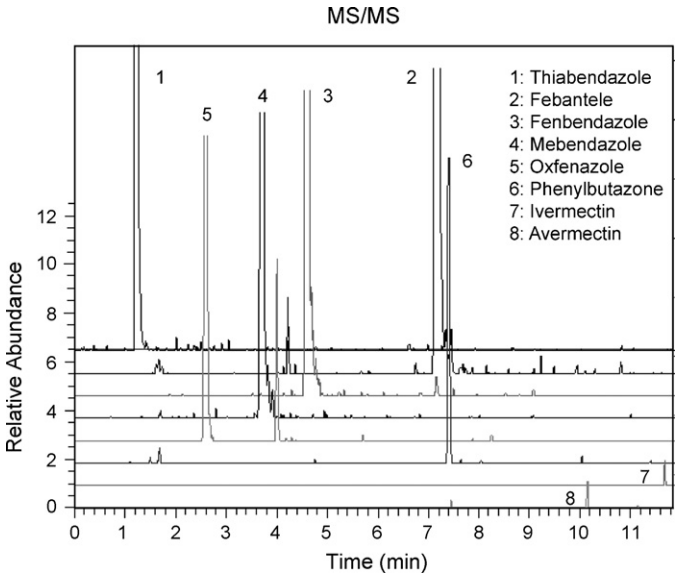

Fig. 1. Milk sample spiked at 5 μg/l. Measurement technique: MS/MS.

2.6. Orbitrap parameters

The utilized instrument was a single stage Orbitrap instrument; Exactive HCD (Thermo Fischer Scientific, Bremen, Germany) operated under Exactive Tune 1.1 and XCalibur 2.1 software.

The capillary of the ESI interface (HESI-II) was set to +3600 V. The heater temperature was adjusted to 350 °C and the capillary temperature to 200 °C. Sheath gas and auxiliary gas were set to 50, respectively 12 units. The capillary voltage was 37.5 V and the tube lens voltage 125 V. The scan range covered (*m*/*z*: 190–1000). Resolution was set to 50,000 full width at half maximum (FWHM) which provides two full scans (data points) per second. The target capacity of the C-trap was always defined at 3,000,000 charges and the maximum injection time was limited to 50 ms. All extracted mass traces were based on a 10 ppm mass window. The exact masses of the quantification and confirmation traces are given in Table 1.

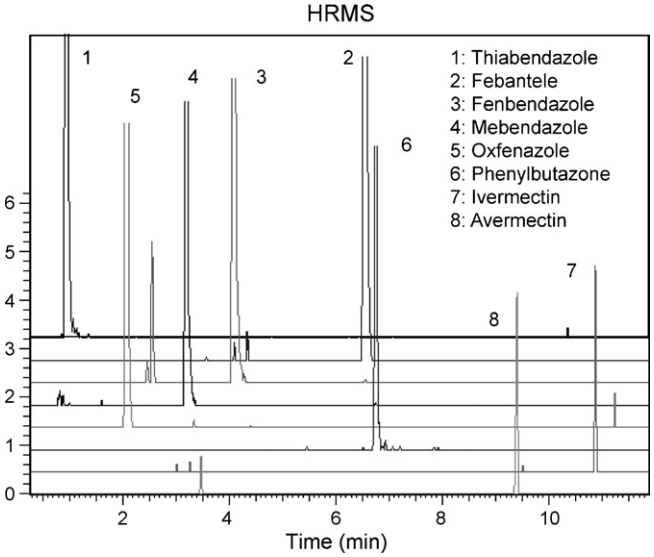

Fig. 2. Milk sample spiked at 5 μg/l. Measurement technique: HRMS.

Table 1
Exact mass information for analytes quantified by HRMS. "Elemental composition" refers to the unionized analyte. "ion" refers to the elemental composition of the detected ion possessing an accurate mass of "*m*/*z*". "Relative intensity" denotes the intensity of the confirmation peak area a compared to the quantification peak area.

Analyt	Elemental composition	Quantification ion		Confirmation ion		
		Ion	*m*/*z*	Ion	*m*/*z*	Relative intensity (%)
Albendazole	$C_{12}H_{15}N_3O_2S$	$[M+H]^+$	266.09576	$[C_{11}H_{11}N_3OS]^+$	234.06955	20
Avermectin	$C_{48}H_{72}O_{14}$	$[M+Na]^+$	895.48143	$[C_{48}H_{72}O_{14}+NH_4]^+$	890.52603	40
Febantel	$C_{20}H_{22}N_4O_6S$	$[M+H]^+$	447.13324	$[C_{19}H_{19}N_4O_5S]^+$	415.10707	60
Fenbendazole	$C_{15}H_{13}N_3O_2S$	$[M+H]^+$	300.08011	$[C_{14}H_{10}N_3OS]^+$	268.05391	15
Flubendazole	$C_{16}H_{12}FN_3O_3$	$[M+H]^+$	314.09352	$[C_{15}H_9FN_3O_2]^+$	282.06733	10
Ivermectin	$C_{48}H_{74}O_{14}$	$[M+Na]^+$	897.49708	?	551.33655	15
Mebendazole	$C_{16}H_{13}N_3O_3$	$[M+H]^+$	296.10295	$[C_{15}H_{10}N_3O_2]^+$	264.07614	10
Oxfendazole	$C_{15}H_{13}N_3O_3S$	$[M+H]^+$	316.07502	$[C_{14}H_{10}N_3O_2S]^+$	284.04882	5
Oxibendazole	$C_{12}H_{15}N_3O_3$	$[M+H]^+$	250.1186	$[C_{11}H_{12}N_3O_2]^+$	218.0924	20
Parbendazole	$C_{13}H_{17}N_3O_2$	$[M+H]^+$	248.13934	$[C_{12}H_{14}N_3O_1]^+$	216.11314	20
Phenylbutazone	$C_{19}H_{20}N_2O_2$	$[M+H]^+$	309.15975	$[C_{13}H_{11}N_2O]^+$	211.08659	2
Thiabendazole	$C_{10}H_7N_3S$	$[M+H]^+$	202.04334	–	–	–
Triclabendazole	$C_{14}H_9Cl_3N_2OS$	$[M+H]^+$	358.95739	$[C_{14}H_8Cl_3N_2OS]^+$	360.95444	95

2.7. Tandem mass spectrometer parameters

The utilized instrument was a tandem quadrupole instrument; TSQ Quantum Access Max (Thermo Fischer Scientific, Bremen, Germany) operated under XCalibur 2.1 software.

The same type of interface was used as for the Orbitrap based detection. Furthermore, identical operating conditions (e.g. gas flow and voltages were selected). Tube lens and collision energy were individually optimized for every compound. The quantification and confirmation transitions are given in Table 2. The dwell times were defined to produce a cycle time of 0.5 s, which corresponds to the setting utilized for the Orbitrap instrument.

2.8. Validation concept and system suitability

The validation procedure as described in the EU Commission Decision 2002/657/EEC (CD) was used as a guideline. Minor deviations were required since some quantified compounds belong to the group of regulated compounds which have a defined maximum residue levels (MRL), while other drugs are not authorized. Instead of using two independent validations, as proposed by the CD, a large calibrated range described by the spiking levels of: 1, 5, 10, 40, and 200 μg/kg was chosen. This range is wide enough to cover the relevant quantification levels for the different compounds in the two tested matrices (milk and muscle). Each validation series consisted of seven unspiked matrix samples and four repetitions of each of the five spiking levels. This resulted in $7+5\times4=27$ individual extractions and injections. Three independent milk validation series were performed on three different days by different operators to determine the within-day and between-day reproducibility. This included 21 different blank milk samples. In addition, injection ready extracts obtained from processing blank samples were spiked (called B spikes) to quantify for possible signal suppression effects (response obtained from blank extract, spiked prior to injection into the instrument, versus equal analyte concentration in pure standard solution). These calculated, matrix dependent signal suppression effects were used to correct measured analyte concentrations. Calibration was based on an external calibration curve based on five concentration levels. A quadratic calibration fitting function with a $1/\times$ weighting was used. The observed calibration data was linear for most compounds over the tested two orders of magnitude, but of heteroscedatic nature (higher absolute standard deviations at high analyte concentrations). Using un-weighted, linear calibration curves, extending over a wide dynamic range, would make the calculation of low concentration measurements unreliable. The strong influence of some high level data points on the linear regression is expected to induce high intercepts at the zero level and produce unrealistic high CCα and CCβ values for banned or unlisted compounds (based on a decision level of 1 μg/kg).

Validation was performed for the following matrices: milk and bovine muscle. Care was taken to select samples which reflect the variety as observed within a given matrix. The bovine samples originated from local slaughtering houses and reflected different origins, growing regimes and age of animals. Milk samples containing different amount of fat and shoving various histories of heat treatment (pasteurization) were selected.

System suitability focused on monitoring critical detection parameters of ivermectin and phenylbutazone. These compounds were chosen because of their low MRM levels, the possible oxidative degradation of phenylbutazone and the occurrence of peak broadening of late eluting ivermectin. Peak areas and peak widths of these compounds obtained from injected neat standard solution and fortified final extracts were monitored. Drifts exceeding more than two standard deviations from the average were generally found to be related to a contamination of the electrospray interface (peak areas) or separation column aging (peak width of ivermectin).

3. Results and discussion

3.1. Extraction and clean-up

Benzimidazoles and their metabolites have been extracted by relatively polar, aqueous extraction solutions [1], but also by liquid–liquid extraction, involving an aqueous buffer and an organic solvent like ethylacetate [2,3]. Some benzimidazoles, respectively metabolites of benzimidazoles were reported to be covalently bound to matrix components present in urine and liver [5]. This caused some authors to include chemical [5] or enzymatic hydrolysis [1,5] steps into their methods, to liberate the covalently bound parent drugs. The need for such a pre-treatment has been controversially discussed [5]. The benefits of enzymatic hydrolysation of benzimidazoles in liver was investigated [1]. The authors finally selected an extraction technique which avoided the use of such a time consuming enzymatic pre-treatment [1]. Based on such information, the need of hydrolyzing benzimidazoles in simpler matrices like milk or muscle was considered to be of even less importance. Hence, this step was not included in the proposed method. Recent papers focusing on benzimidazoles [5] use simplified extraction procedures based on anhydrous acetonitrile. A benefit related to the use of this solvent is the extensive precipitation of possible interfering proteins. Time consuming liquid–liquid

Table 2
MS/MS transition information. Compound specific tube lens voltages and collision energies are given for each analyte.

Analyt	Elemental compositon	Quantification transition				Confirmation transition		
		Precursor ion	Transition	Collision energy [eV]	Tube lens [V]	Transition	Collision energy [eV]	Tube lens [V]
Albendazole	$C_{12}H_{15}N_3O_2S$	$[M+H]^+$	266.0 > 234.0	20	100	266.0 > 191.0	30	100
Avermectin	$C_{48}H_{72}O_{14}$	$[M+Na]^+$	895.5 > 751.3	20	100	895.5 > 681.0	50	100
Febantel	$C_{20}H_{22}N_4O_6S$	$[M+H]^+$	447.0 > 383.0	20	90	447.0 > 415.0	20	90
Fenbendazole	$C_{15}H_{13}N_3O_2S$	$[M+H]^+$	300.0 > 268.0	20	100	300.0 > 159.0	40	100
Flubendazole	$C_{16}H_{12}FN_3O_3$	$[M+H]^+$	314.0 > 282.0	20	110	314.0 > 123.0	40	110
Ivermectin	$C_{48}H_{74}O_{14}$	$[M+Na]^+$	897.5 > 753.3	40	150	897.5 > 609.0	50	150
Mebendazole	$C_{16}H_{13}N_3O_3$	$[M+H]^+$	296.0 > 264.0	20	110	296.0 > 105.0	40	100
Oxfendazole	$C_{15}H_{13}N_3O_3S$	$[M+H]^+$	316.0 > 284.0	20	110	316.0 > 159.0	30	110
Oxibendazole	$C_{12}H_{15}N_3O_3$	$[M+H]^+$	250.0 > 176.0	30	100	250.0 > 148.0	40	100
Parbendazole	$C_{13}H_{17}N_3O_2$	$[M+H]^+$	248.0 > 216.0	20	100	248.0 > 173.0	30	100
Phenylbutazone	$C_{19}H_{20}N_2O_2$	$[M+H]^+$	309.0 > 120.0	20	95	309.0 > 211.0	16	90
Thiabendazole	$C_{10}H_7N_3S$	$[M+H]^+$	202.0 > 175.0	30	90	202.0 > 131.0	40	90
Triclabendazole	$C_{14}H_9Cl_3N_2OS$	$[M+H]^+$	359.0 > 344.0	30	105	359.0 > 274.0	40	105

or solid phase extraction procedures are less commonly used, since nowadays modern analytical methods rely on the selectivity provided by LC–MS/MS detection.

Avermectins are apolar drugs which can easily be extracted by solvents of relatively low polarity. The presence of metabolites seems to be of low relevance [14], which facilitates extraction and clean-up procedures. Most reported methods [8–14] depend on acetonitrile as extraction solvent. Acetonitrile is preferred by most authors because of its capability to deproteinate extracts.

Older methods use a clean-up with alumina based [6,11] solid phase extraction (SPE). There has been a shift to C-18 or polymer sorbent based reversed phase SPE materials [8–10,12,13]. Most recent developments include an approach which is closely related to the pesticide residue extraction and clean-up technique called QuEChERS [4,7]. This methodology relies on acetonitrile based extraction which is aided by a high amount of solid anhydrous sodium sulphate, sodium chloride and anhydrous magnesium sulphate. A fast clean-up is performed by dispersive SPE utilizing a C-18 material. This technique was reported to be capable in extracting benzimidazoles, their metabolites, avermectins and flukicides [7].

Phenylbutazone has been analyzed together with other non-steroidal anti-inflammatory agents [15], but often alone respectively together with another closely related drug [16,17]. Due to the low polarity of the analyte, acetonitrile was a preferred extraction solvent. The analysis of phenylbutazone appears to be hampered by irreproducible degradation of the analyte. As a matter of fact, the proposed method was in the final stage of validation when these problems were observed in our laboratory. Some spiking experiments showed very poor recoveries. Initially no explanation could be found for these irreproducible events. Screening the published literature yielded the information that phenylbutazone can undergo irreproducible degradation and oxidation reactions [15–17]. The authors of these papers suggested the combined use of a reducing and complex binding agent. We did not observe these degradation problems anymore after including these additives in the modified method. It appears that most authors of published methods focusing on phenylbutazone seem not to be aware of this potential problem.

Based on these data we concluded that these three classes of drugs (benzimidazoles, avermectins and phenylbutazone) can be easily extracted by one extraction step. Furthermore experiments showed that good quantitative results can be obtained even without a clean-up step (e.g. C-18 in the form of classical SPE or dispersive SPE). Furthermore, the QuEChERS approach which relies on a mixture of different inorganic salts was simplified by the use of anhydrous sodium sulphate only. The quantitative performance of the method was not significantly affected by the presence or absence of sodium chloride or magnesium sulphate in the extraction vessel.

Initial attempts to directly inject the diluted acetonitrile extracts failed because the avermectins could not be detected at the required levels. Hence, an evaporation followed by a solvent exchange step was included. The apolar character of the analytes requires the dissolution of the evaporated extracts with a solvent mixture containing a relatively high organic solvent (e.g. acetonitrile) content. The chosen acetonitrile concentration was sufficiently strong to ensure a quantitative recovery (dissolution) of analytes from the evaporated extracts, but low enough to prevent a deformation of early eluting chromatographic peaks. Dimethylsulfoxide was initially used as a keeper during evaporation, however, the very intense dimers produced in electrospray tend to fill the C-trap of the Orbitrap, reducing the dynamic range and hence sensitivity. This is an Orbitrap specific limitation and not relevant for MS/MS based methodologies.

The current method does not yet include metabolites like sulphoxides and sulphones of certain benzimidazoles. It should be possible to integrate such metabolites into the proposed method, since the utilized extraction procedure is a simplification (avoidance of dispersive C-18 treatment) of an extraction and clean-up technique which was reported to cover such metabolites [4,7].

3.2. Optimization of LC–MS parameters

The majority of LC–MS methods based on positive ESI utilize a mobile phase which contains formic or acetic acid. Alternative ionic additives like buffers e.g. (ammonium formate or ammonium acetate) respectively volatile ion pair agents like perfluorinated acids are only used if poor chromatographic or ionization properties require such additives. The price to be paid for employing such buffers or ion pair agents is an intensified analyte signal suppression. As mentioned in Section 1, a careful optimization of additives and gradient timing can minimize such negative suppression effects. However, it is very likely that even carefully optimized conditions produce disappointing results when applied to an interface from another instrumental vendor. Hence, it was one of the aims of this work to utilize classical mobile phases (based on formic acid) to ensure the use of this method on different instruments. Furthermore, the proposed method is understood to be a building block which can accept other analytes belonging the other drug groups. Selecting a formic acid mobile phase increases the likelihood that such additional compounds can be separated with good peak shape and detected at sufficient sensitivities. As discussed earlier, this choice forces the analyst to deal with sodium-cationized molecules.

For the majority of analytes the $[M+H]^+$ ion was sufficiently intensive that it could be selected as precursor ion for MS/MS. This was not the case for avermectin and ivermectin which showed a much higher ion abundance for sodiated than protonated molecules. As discussed, fragmentation of these ions produces a relatively low abundance of product ions.

3.3. Selection of HRMS parameters

Only limited efforts were invested into optimizing HRMS conditions. This included a common capillary voltage for all compounds. Such a common value reflects a compromise, because some analytes would produce higher ion abundances at higher voltages, while others would undergo already extensive interface induced fragmentation. Again, the $[M+H]^+$ ions were selected for the majority of analytes, only avermectin and ivermectin were detected in the form of $[M+Na]^+$ adducts. The Orbitrap was operated at a resolution of 50,000 FWHM which provides two data points per second. This is acceptable, considering the average peak width ranging from 4 to 5 s. The chosen resolution, in combination with a 10 ppm mass extraction windows provides a selectivity which corresponds to the traditional MS/MS performance [22]. The capability of the Orbitrap to produce such high resolutions provides significant advantages over older TOF instruments operating at 10,000–15,000 FWHM. This refers to the sensitivity and the dynamic range. Our old TOF based multiresidue method [26] did not include avermectins, because the required MRL could not be met. Other authors [20] reported LOQ for ivermectin in milk of 25 μg/l. This resulted not only in critical sensitivities but also poor RSD of 122% at the required detection levels [20]. The high resolution of the utilized Orbitrap instrumentation permits the definition of very narrow mass extraction windows, providing higher selectivity and sensitivity in complex matrix samples by improving the signal to noise ratio. Lower resolving HRMS instrumentation also permit the definition of narrow mass windows, however, this can lead to false negative findings because of possible isobaric interferences with endogenous matrix compounds [21]. The mass window utilized for this study was 10 ppm. This value is appropriate for a resolution of 50,000 FWHM. A value of 50 ppm would be permitted for a 10,000 FWHM resolving instrument. The implication of this setting was tested by measuring a low level spiked muscle extract with the Orbitrap at 50,000 FWHM and processing the acquired data by two different mass windows (10 and 50 ppm, respectively). Extracted mass traces of some analytes are shown in Figs. 4 and 5. Visible is the appearance of additional chromatographic peaks when selecting the 50 ppm setting (e.g. parabendazole and febantel). Furthermore the appearance of spikes (e.g. ivermectin and phenylbutazone) at 50 ppm setting, reduces signal to noise, as caused by isobaric interferences. Such interferences become more relevant when analyzing more complex matrices like kidney or liver or expanding the number of analytes to be covered by the method. Hence, high resolution is an important prerequisite when building multi matrix, multiresidue methods.

The "high dynamic range" trap setting was selected, to accumulate some 3,000,000 charges in the C-trap. This setting provided a high dynamic range and therefore a high sensitivity. The applicability of this value was carefully evaluated. We know from previous method developments, that the presence of co-extracted proteins can cause significant signal suppression in the C-trap [23]. This phenomena, which we termed "post interface signal suppression", was not observable when injecting the extracts produced by the proposed method into the Orbitrap. We attribute this to the relatively low concentration of co-extracted proteins (anhydrous acetonitrile extraction). This permitted the utilization of the high dynamic range setting. The high mass stability of the instrument did not require the use of a lock mass. Weekly external mass calibrations provided to be sufficient.

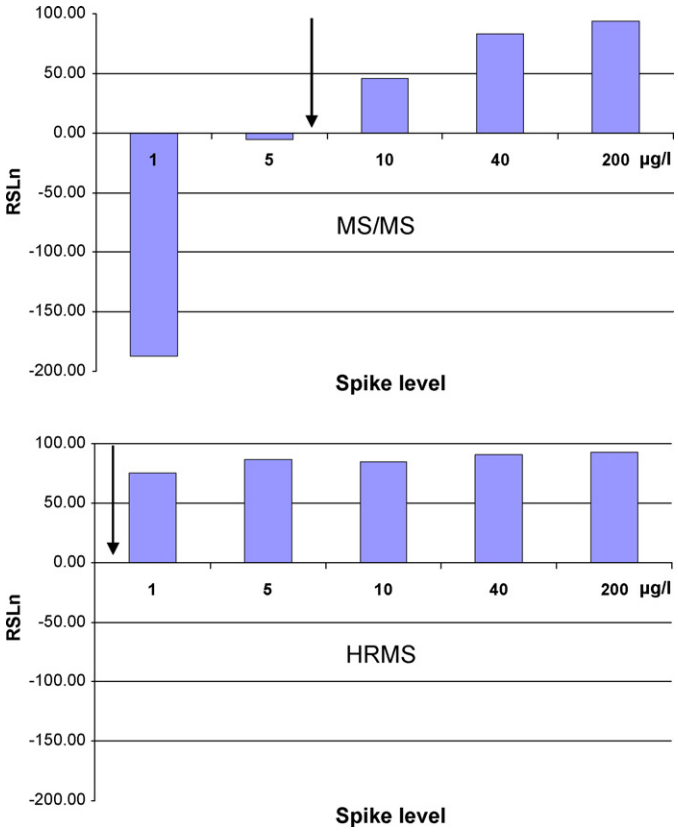

Fig. 3. Comparison of the limit of determination (ivermectin) for the both detection techniques. The depicted relative significance of the level (RLSn) is calculated according to the following formula: RSLn = 100 × [average peak area − 2.33 RSD × (average peak are) − noise (blank)]/(average peak area). The flipping of the bars (indicated by a arrow) show the estimated limits of quantification.

The fact that the monitored compounds belong to three different compound groups would require a number of different internal standards in order to combat analyte recovery related losses and possible signal suppression. Such compounds, preferably stable isotope labelled analogues could not be found, hence quantification relied on external standards and matrix matched calibrations techniques.

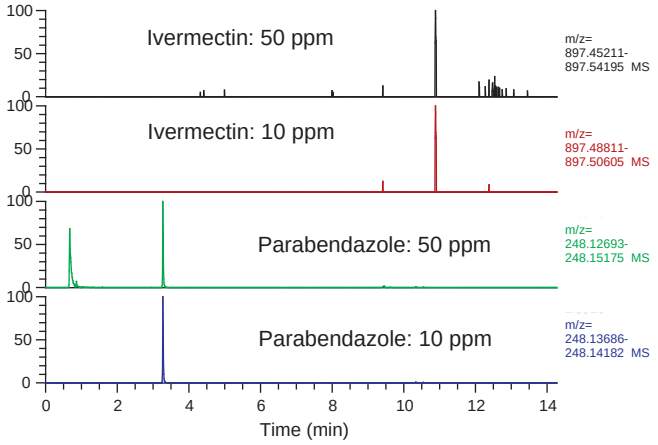

Fig. 4. Effect of different mass extraction windows on the detection selectivity of ivermectin and parabendazole at low levels in muscle matrix extracts.

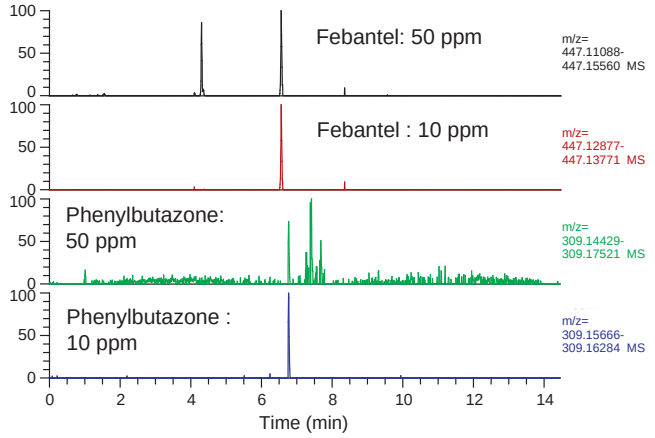

Fig. 5. Effect of different mass extraction windows on the detection selectivity of febantel and phenylbutazone at low levels in muscle matrix extracts.

3.4. Validation

The proposed method is intended to quantify drugs with defined maximum residue levels (MRL). On the other hand, it is also intended to detect drugs for which no such values exist. Compounds lacking an MRL are either explicitly banned, or no value has been defined yet for whatever reason. The European commission decision proposes two different ways in validating such compounds to ensure the quality of decisions regarding the question if a compound exceeds the defined maximum level or if the presence of a banned or unregulated compound can be proven. The problem is complicated by the fact that some compounds might have an MRL in milk but not in muscle. Hence depending on the matrix a compound might be considered regulated (having an MRL) or banned, respectively not regulated. As a consequence validation of the two matrices would require two different set-ups. A further complication is observed when regulations change. For example, the relatively recent detection of ivermectin in Brasilian beef caused the EU to define a MRL for this compound in beef products. Strictly applied, this would require that an existing and validated method for ivermectin had to be revalidated in order to take the new MRL value into account. A possible solution for this problem was the validation of a large dynamic range [24]. The tested levels should be high enough to include the compound with the highest MRL but should go down to the detection level in order to meet the requirements for banned or unregulated compounds. Another, more time consuming approach is the repetition of the whole validation process utilizing dedicated settings as the "dual validation" proposed in a recent paper [25].

Validation data are given in Tables 3–6. Comparing the average coefficients of determination (r^2) for all compounds in milk shows that HRMS (0.9958) performs equally well as MS/MS (0.9962). For muscle, a slightly poorer (not statistically significant) performance is observed for HRMS (0.9934) versus MS/MS (0.9975). This difference is to be explained by the relatively low r^2 for ivermectin. That r^2 value was caused by a single deviating measurement of a 200 μg/kg spiking level. Clearly evident is the higher sensitivity of HRMS based measurements for avermectins. A fair comparison of MS/MS and HRMS sensitivity is not easily done. HRMS signals based on narrow mass windows are often devoid of any noise [24]. Hence, a calculation of signal to noise delivers arbitrary results. More realistic values for the limit of determination are obtained when calculations are based on the relative standard deviation observed among the different utilized spiking levels. The basic idea behind this approach is the fact that a compound becomes undetectable when the absolute standard deviation of repeated measurements

Table 3
HRMS validation data for milk. Relative standard deviation within and between a day. The values in brackets refer to the deviations between a day. Average recoveries (corrected for signal suppression) are given for each spiking level. CCα and CCβ values are calculated for the appropriate concentration levels. These levels are given in brackets. A "*" indicates that no MRL has been defined for this compound, hence an arbitrary level of 1 μg/kg, μg/l respectively was defined. Limit of determination was estimated according to the relative standard deviations at the different spiking levels. Coefficient of determination refers to the whole dynamic range. Signal suppression refers to the peak area ratio of a given analyte concentration present in a matrix extract and in a pure standard solution, respectively.

Analyte	RSD [%] within a day (between a day)					Recovery [%]					CCα (MRL) [μg/l]	CCβ (MRL) [μg/l]	Limit of determination [μg/l]	Coefficient of determination [r^2]	Signal suppression
	Level A (1 μg/l)	Level B (5 μg/l)	Level C (10 μg/l)	Level D (40 μg/l)	Level E (200 μg/l)	Level A (1 μg/l)	Level B (5 μg/l)	Level C (10 μg/l)	Level D (40 μg/l)	Level E (200 μg/l)					
Milk (HRMS)															
Albendazole	7.6 (35.1)	11.9 (18.5)	7.7 (9.8)	11.5 (3.1)	8.4 (21)	73.6	86.8	86.7	81.7	72.9	112.1 (100)	147.9 (100)	0.5	0.9915	1.18
Fenbendazole	4.1 (38.8)	2.2 (22)	3.1 (8.3)	2.3 (6.7)	5.2 (18.9)	85.1	82.2	83.1	75.4	77.9	10.4 (10)	11.5 (10)	0.5	0.9974	1.24
Flubendazole	4.5 (12)	3.6 (11.1)	2.5 (14.2)	1.4 (8.5)	3 (9.3)	82.3	84.9	89.2	85.0	79.7	1.07 (1*)	1.24 (1*)	0.5	0.9987	0.99
Mebendazole	16.5 (133)	11.2 (50.8)	7.3 (31.7)	4.6 (4.4)	8.9 (14.3)	61.5	78.0	86.0	88.4	81.2	1.16 (1*)	1.65 (1*)	0.5	0.9934	1.02
Oxfendazole	3.3 (16.9)	3.3 (9.8)	2.9 (8.8)	3 (5.8)	7.4 (12)	84.8	88.8	91.8	89.6	84.4	10.5 (10)	11.7 (10)	0.5	0.9953	0.99
Oxibendazole	3.1 (16.2)	2.9 (1.2)	2.1 (3)	2.9 (6.6)	8.6 (13.4)	88.7	88.6	93.3	87.4	88.3	1.04 (1*)	1.14 (1*)	0.5	0.9935	1.06
Parbendazole	3.5 (8.5)	6.2 (2.8)	3.6 (10.7)	3.7 (8.7)	3.5 (17)	87.0	83.9	87.0	82.2	83.3	1.06 (1*)	1.19 (1*)	0.5	0.9986	0.99
Phenylbutazone	5.3 (40.7)	4.6 (17.5)	7.3 (4.1)	4.1 (7.2)	4 (29.3)	73.2	79.6	83.6	81.2	78.3	1.07 (1*)	1.25 (1*)	0.5	0.9979	0.85
Thiabendazole	6.2 (40.9)	7.3 (26.8)	7.8 (31.1)	4 (13.9)	8 (13.9)	82.1	87.4	91.1	88.2	81.5	105.5 (100)	117.4 (100)	0.5	0.9932	0.95
Triclabendazole	6.1 (21.1)	18.2 (6.9)	3.7 (20.9)	4.9 (15.1)	2.4 (43.6)	70.1	67.9	69.4	67.9	73.9	1.06 (1*)	1.20 (1*)	0.5	0.9976	0.82
Febantel	4.7 (22)	4.8 (25.4)	4.1 (31.2)	3.8 (27.7)	5.3 (18)	83.4	82.9	86.0	81.5	85.9	10.7 (10)	12.1 (10)	0.5	0.9973	1.04
Avermectin + Na	18.9 (117)	10.4 (12.2)	11.6 (17.7)	6.4 (13.6)	4.8 (30.2)	49.1	86.6	87.6	86.3	72.9	1.18 (1*)	1.81 (1*)	0.5	0.9944	0.9
Ivermectin + Na	6.5 (346)	27.3 (107)	25.4 (36.5)	7.4 (11)	4.1 (26.9)	28.5	56.6	74.4	85.2	76.9	1.01 (1*)	1.06 (1*)	0.5	0.9971	1.05

Table 4
MS/MS validation data for milk.

Analyt	RSD [%] within a day (between a day)					Recovery [%]					CCα (MRL) [μg/l]	CCβ (MRL) [μg/l]	Limit of determination [μg/l]	Coefficient of determination [r^2]	Signal suppression
	Level A (1 μg/l)	Level B (5 μg/l)	Level C (10 μg/l)	Level D (40 μg/l)	Level E (200 μg/l)	Level A (1 μg/l)	Level B (5 μg/l)	Level C (10 μg/l)	Level D (40 μg/l)	Level E (200 μg/l)					
Milk (MS/MS)															
Albendazole	4.7 (1.9)	4.5 (7)	2.6 (8)	3 (14.8)	1.7 (14.4)	81.7	80.2	82.0	79.3	80.9	103.6 (100)	111.3 (100)	0.5	0.9996	0.96
Fenbendazole	4 (11.9)	2.7 (12.6)	2.8 (10.2)	2.4 (14.6)	1.8 (15.6)	80.4	78.9	81.7	79.2	80.4	10.4 (10)	11.3 (10)	0.5	0.9997	1.01
Flubendazole	4.5 (7.8)	4.3 (7.9)	2.5 (5)	2.1 (6.4)	1.9 (9.9)	85.3	85.3	88.1	87.4	89.4	1.06 (1*)	1.21 (1*)	0.5	0.9996	1.05
Mebendazole	3.3 (10.2)	4.4 (12.4)	1.6 (6.5)	2.2 (7.5)	1.8 (8.9)	87.3	82.3	85.9	85.6	88.1	1.05 (1*)	1.16 (1*)	0.5	0.9996	1.03
Oxfendazole	7.7 (18.4)	10.1 (8.5)	5.1 (16.3)	5.6 (28.7)	3.3 (10.8)	84.8	80.2	81.6	83.6	92.0	10.7 (10)	12.3 (10)	0.5	0.9985	0.96
Oxibendazole	4.5 (4)	9.5 (16.6)	3.8 (23.7)	5.4 (42.9)	4.3 (29.6)	93.6	83.0	82.6	81.0	88.3	1.07 (1*)	1.24 (1*)	0.5	0.9978	0.93
Parbendazole	4.1 (7.4)	4.7 (10.9)	1.5 (10.5)	2.1 (21.1)	1.5 (22.5)	89.9	83.7	84.9	79.4	80.6	1.07 (1*)	1.23 (1*)	0.5	0.9997	0.99
Phenylbutazone	17.2 (23.4)	8 (27.1)	4.9 (16.9)	4.5 (6.8)	3 (5.4)	63.5	77.8	78.0	77.6	80.2	1.21 (1*)	1.84 (1*)	1	0.9991	1.05
Thiabendazole	5.2 (10.8)	14.1 (24.5)	9.1 (38.3)	8.6 (43.3)	10.9 (31.7)	87.3	80.4	80.2	88.2	111.8	112.7 (100)	147.2 (100)	0.5	0.988	0.5
Triclabendazole	8.4 (13.7)	5.4 (13)	3.5 (10.5)	3.8 (13.9)	2.7 (12.1)	69.3	69.2	72.5	71.2	71.5	1.12 (1*)	1.44 (1*)	0.5	0.9993	1.02
Febantel	3.2 (8.9)	3.3 (7.9)	1.5 (2.8)	2.3 (12)	2.4 (9.6)	89.6	86.5	88.8	83.5	82.7	10.3 (10)	10.8 (10)	0.5	0.9994	1.1
Avermectin + Na	88.6 (191)	67.9 (141)	30.3 (119)	10.9 (22.5)	3.6 (26.9)	36.1	144.3	129.0	107.0	84.9	1.38 (1*)	2.1 (1*)	10	0.9898	1.8
Ivermectin + Na	96.4 (114)	90.4 (150)	34.7 (135)	9.2 (36.5)	9.1 (17.8)	32.6	82.9	115.6	95.6	72.5	1.64 (1*)	3.04 (1*)	10	0.9802	1.21

Table 5
HRMS validation data for muscle.

Analyt	RSD [%] within a day					Recovery [%]					CCα (MRL) [μg/kg]	CCβ (MRL) [μg/kg]	Limit of determination [μg/kg]	Coefficient of determination [r^2]	Signal suppression
	Level A (1 μg/kg)	Level B (5 μg/kg)	Level C (10 μg/kg)	Level D (40 μg/kg)	Level E (200 μg/kg)	Level A (1 μg/kg)	Level B (5 μg/kg)	Level C (10 μg/kg)	Level D (40 μg/kg)	Level E (200 μg/kg)					
Muscle (HRMS)															
Albendazole	7.3	16.4	10.6	4.1	12.9	78.3	80.3	80.4	81.8	77.0	110.2 (100)	134.1 (100)	1	0.9951	0.92
Fenbendazole	12.1	15	6	4.4	3.5	75.8	79.7	79.3	80.9	77.1	52.1 (50)	56.5 (50)	1	0.9995	0.9
Flubendazole	14.3	12.2	6.7	2.1	3.1	83.1	92.9	94.0	91.1	86.9	51.1 (50)	53.5 (50)	0.5	0.9996	0.92
Mebendazole	10.3	19	6.8	3.5	4.8	84.2	89.9	90.3	88.7	85.0	62.3 (60)	67.3 (60)	1	0.9992	0.94
Oxfendazole	20.5	21.2	9.9	5	12.9	87.7	89.1	84.0	86.7	88.7	53.3 (50)	60.4 (50)	0.5	0.9952	0.95
Oxibendazole	7.9	14.7	10.4	4.5	24.4	95.2	89.1	84.0	86.2	81.5	118.4 (100)	167.8 (100)	1	0.9832	1.1
Parbendazole	9.4	19.6	10.4	6.9	4.5	77.5	83.3	84.0	80.2	68.7	1.1 (1*)	1.3 (1*)	0.5	0.9982	0.97
Phenylbutazone	22.7	20.9	11.8	7.9	6.2	60.4	80.8	82.1	80.4	70.1	1.2 (1*)	1.7 (1*)	1	0.9979	0.9
Thiabendazole	19.8	14.7	49.1	10.9	5.7	61.3	69.7	95.0	93.2	88.4	106.8 (100)	121.7 (100)	1	0.998	0.69
Triclabendazole	21.2	15.9	12.9	7.3	10.7	56.5	69.0	74.5	78.1	74.9	245.3 (225)	292 (225)	1	0.9966	0.82
Febantel	10.7	9.6	17.3	7.3	8.8	83.6	91.5	105.6	106.6	93.9	54 (50)	62.9 (50)	1	0.997	1.17
Avermectin + Na	44.7	19.1	37.1	6.2	22.9	33.4	78.3	66.2	66.9	67.1	23.1 (20)	31 (20)	1	0.9852	2.41
Ivermectin + Na	0	44.9	37.2	9.9	32.6	0.0	69.5	83.0	79.1	76.0	23.9 (20)	34.3 (20)	2	0.9704	1.02

Table 6
MS/MS validation data for muscle.

Analyt	RSD [%] within a day					Recovery [%]					CCα (MRL) [μg/kg]	CCβ (MRL) [μg/kg]	Limit of determination [μg/kg]	Coefficient of determination [r^2]	Signal suppression
	Level A (1 μg/kg)	Level B (5 μg/kg)	Level C (10 μg/kg)	Level D (40 μg/kg)	Level E (200 μg/kg)	Level A (1 μg/kg)	Level B (5 μg/kg)	Level C (10 μg/kg)	Level D (40 μg/kg)	Level E (200 μg/kg)					
Muscle (MS/MS)															
Albendazole	4.6	13.9	12.5	6.4	5.6	73.0	74.1	77.1	81.1	77.3	105.6 (100)	117.7 (100)	1	0.9989	0.95
Fenbendazole	4.7	15.4	8.1	6.2	3.1	74.6	72.1	73.8	74.6	73.2	52.6 (50)	58.2 (50)	1	0.9997	1.03
Flubendazole	6.5	11.5	7.5	4	3.1	90.2	84.7	89.5	88.4	86.1	51.8 (50)	55.7 (50)	1	0.9997	0.98
Mebendazole	5.9	13.2	3.6	5.1	3.7	88.5	84.4	85.9	87.9	85.4	62.6 (60)	68.2 (60)	1	0.9995	1.04
Oxfendazole	12	14.6	2.8	5.9	2.8	81.4	85.5	88.8	85.9	83.3	52.5 (50)	57.9 (50)	1	0.9997	1
Oxibendazole	5.6	12.4	3.7	2.5	3.5	87.1	80.5	81.7	81.2	79.1	103.1 (100)	109.6 (100)	1	0.9996	1.1
Parbendazole	1.6	12.8	7.7	6.8	3.4	82.1	76.2	76.4	76.9	74.3	1 (1*)	1.1 (1*)	1	0.9996	1.02
Phenylbutazone	45.6	22.6	6.3	8.9	8.2	54.1	67.6	68.6	72.5	71.6	1.3 (1*)	2.5 (1*)	1	0.998	0.9
Thiabendazole	13.9	8.7	7.8	4.7	7.8	83.1	77.8	83.4	90.7	87.2	106.7 (100)	121.5 (100)	1	0.9982	0.72
Triclabendazole	9.2	16.9	14.6	6.9	6.3	63.2	66.4	67.9	71.4	69.5	236.9 (225)	262.6 (225)	1	0.9988	0.74
Febantel	5.7	6.8	2.6	7.1	7	97.9	90.5	93.7	99.7	96.5	53.4 (50)	61.1 (50)	1	0.9985	0.93
Avermectin + Na	275.7	40.6	106.1	12.7	2	6.3	40.0	57.1	91.7	70.4	27.5 (20)	55.7 (20)	10	0.9949	0.93
Ivermectin + Na	330.3	263.1	65.6	9.5	6.3	42.1	30.8	83.9	104.9	65.5	27.2 (20)	53.4 (20)	10	0.9833	0.75

reaches the amplitude of the measured signal. This idea embedded into a statistical concept [24] was used to estimate the limit of determination. As visible in the validation tables, MS/MS based signals for ivermectin become very unreliable at the lowest three spiking levels. The limit of determination is reached when the statistically corrected absolute standard deviation subtracted from the average analyte signal produces no net signal. Fig. 3 shows this relationship in the form of a graph. The depicted "relative significance of the level" (RLSn) is calculated according to the following formula: RSLn = 100 × [average peak area − 2.33 RSD × (average peak are) − noise (blank)]/(average peak area). The limit of determination is reached at the concentrations where the bars flip from positive to negative. This happens at 10 μg/kg (MS/MS) and at 1 μg/kg (HRMS). See Fig. 3. This difference is also visible when comparing CCα and CCβ values as shown in Tables 3–6.

4. Conclusion

Our initial work with the single stage Orbitrap instrument revealed certain limitations when injecting some concentrated, relatively dirty tissue extracts [23]. Extensive signal suppression effects were observed in the regions of the chromatogram where multiple charged proteins eluted. This signal suppression was termed "post interface signal suppression" since it most likely occurred in the C-trap of the instrument, well downstream from the ESI interface. Hence, the chromatograms produced by the method described in this paper were carefully investigated. No indications of post interface signal suppression could be detected. This even permitted the selection of the high dynamic range mode of the Exactive instrument which provides a larger dynamic range and therefore higher sensitivity. Comparing this anthelmintic method to the mentioned multi-residue method [23,26], where post interface signal suppression was observed, revealed some important differences. The use of a very polar extraction solvent (aqueous organic solvent), in combination with a difficult matrix (e.g. liver and kidney), limited clean-up and a final concentration step produces extracts with contain a very high load of endogenous proteins. The method reported here is based on an extraction based on anhydrous acetonitrile. Limited amounts of proteins will be co-extracted in such an environment. Hence, even after a concentration of the extract, the load of proteins is not high enough to cause the discussed detrimental effect in the C-trap. This is an important conclusion when it comes to access the capability of the single stage Orbitrap technology to deal with complex real live matrices.

HRMS methods, regardless if single stage as provided by TOF, or MS/MS based like Q-TOF have a history of poor sensitivity, generally being significantly below the performance of tandem quadrupole. The Orbitrap instrument used for this study showed a significantly higher sensitivity than the TOF instrument we have used for a previously developed multi-residue method [24]. This is also indicated by the provided validation data, where limits of determination below 1 μg/kg could be achieved. An important aspect of this study is the experimental proof that HRMS sensitivity can be clearly superior over MS/MS for very stable ions (resistant towards fragmentation). This has been shown for the two tested avermectins. This observation is more than just of academical importance. These compounds show a poor ionization efficacy. On the other hand, they have relatively low MRL levels. In other words, there is a need for sensitive detection. The validation data (HRMS and MS/MS) obtained for these compounds still show below average coefficients of determination. This is most likely to be explained by non-linearity as caused by the ions present in the sample solution. A changing concentration ratio of analyte versus cations capable in forming adducts, will affect the $[M+Na]^+$ versus $[M+H]^+$ respectively $[M+X]^+$ ratio.

As stated above, the pressure to utilize multi-residue methods calls for generic separation and detection techniques. Regarding separations, this favours a simple mobile phase (e.g. formic acid in water and acetonitrile). Such a mobile phase provides best peak shapes and optimal signal intensity. Mobile phases with additives like inorganic buffers or volatile ion pair agents might provide better results for a particular group of compounds, when based on a particular brand of instrument & interface. However, including other analytes or moving to another instrument will produce a different performance.

The capability of HRMS to quantify residues with high selectivity and sensitivity, without having to rely on MS/MS fragmentation reactions, provides important benefits. Collision energies which are utilized for fragmentation reactions are highly compound dependent. They are to be individually evaluated for each analyte. Furthermore, compound specific settings complicate building multi-residue methods, consisting of several hundred compounds. This is probably the reason why the majority of Q-TOF based papers report the use of their instrument in the single stage MS mode (collision chamber is switched off, respectively being operated without collision gas under RF mode conditions).

We believe that MS/MS will lose its dominance as quantification technique in the field of multi-residue methods because of the discussed limitations. Having access to rugged and sensitive HRMS techniques and ample software support, will likely cause a shift of MS/MS toward the domain of final confirmation of findings which were produced by HRMS technology.

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