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New Sensitive High-Performance Liquid Chromatography–Tandem Mass Spectrometry Method for the Detection of Horse and Pork in Halal Beef

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Supporting Information

ABSTRACT: The accidental or fraudulent blending of meat from different species is a highly relevant aspect for food product quality control, especially for consumers with ethical concerns against species, such as horse or pork. In this study, we present a sensitive mass spectrometrical approach for the detection of trace contaminations of horse meat and pork and demonstrate the specificity of the identified biomarker peptides against chicken, lamb, and beef. Biomarker peptides were identified by a shotgun proteomic approach using tryptic digests of protein extracts and were verified by the analysis of 21 different meat samples from the 5 species included in this study. For the most sensitive peptides, a multiple reaction monitoring (MRM) method was developed that allows for the detection of 0.55% horse or pork in a beef matrix. To enhance sensitivity, we applied MRM^3 experiments and were able to detect down to 0.13% pork contamination in beef. To the best of our knowledge, we present here the first rapid and sensitive mass spectrometrical method for the detection of horse and pork by use of MRM and MRM^3.

KEYWORDS: *Halal food, meat speciation, authenticity, mass spectrometry, peptide, pig, cattle, peptide biomarker*

INTRODUCTION

The total consumption of meat is constantly increasing worldwide. Despite considerable regional differences, pork, beef, and chicken meat are economically most important. In 2011, about 62 million tons of beef,[1] 89 million tons of chicken meat,[2] and about 110 million tons of pork[3] were produced. The annual global production of horse meat is considerably lower at an estimated 700 000 tons.[4] Besides microbiological and chemical product safety, species authentication is an important quality control parameter for meat products because the fraudulent substitution with meat from lower priced species drastically increases the profit margin. As an example, prices for beef are about twice as high compared to pork. The undeclared total or partial substitution of beef with pork or other low-priced meat species is therefore highly relevant, especially for minced meat, which is used as a basis for numerous different convenience products.

The practice of adulterating meat was exemplified by recent events in Europe were at least 50 000 tons of beef meat contained horse and 5–7.5% of samples analyzed in the European Union (EU) and national action plans were positive for undeclared horse meat.[5]

Besides the economical aspect, the intended or careless false declaration of meat products is a severe problem for consumers that have ethical or religious concerns about the consumption of pork or horse meat products. More specifically, Islam and Judaism both have dietary restrictions concerning the consumption of food containing porcine meat or other edible parts of pig. Such products are called "non-kosher" in Judaism or "haram" in Islam and are clearly forbidden in both religions. In addition, parts of the muslim community consider the consumption of meat or other products from domesticated horses as "makruh", which means that these products should be avoided if possible. In Judaism, the consumption of horse products, in general, is non-kosher.

An estimated 1.5 billion Muslims and 14 million Jews represent about 23% of the worldwide population; therefore, the undeclared content of above-mentioned products is a consumer problem on a global scale.

Because the tolerance level for porcine and equine contents in other foods is 0% regarding the religious requirement, the limit of detection (LOD) should be as low as possible and steady development of more sensitive methods is necessary. Methods that have been applied to authentication of meat samples include two-dimensional sodium dodecyl sulfate–polyacrylamide electrophoresis (2D SDS–PAGE), polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and mass spectrometry (MS).

SDS–PAGE methods were first developed for meat identification and use the comparative analysis of protein patterns in different species. As a consequence, these methods are not applicable for the detection of lower or even trace amounts of undeclared meat. A vast number of SDS–PAGE methods was available at the end of the 1980s with a LOD of about 5% porcine meat in different matrices.[6]

In addition, numerous ELISA and PCR methods are available for the identification of meat species. For ELISA methods, the LOD is currently about 0.5% for porcine meat in beef matrix.[7] However, ELISAs suffer from the fact that a multiplexed

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analysis of different species in a single run is hard to achieve and, if successful, quite expensive. The most commonly used method for meat speciation is therefore PCR, which has been mostly applied for the recent food fraud issues in Europe.[8] Simultaneous detection of different meat species in one sample is, in general, possible by PCR.[9]

Besides the advantage of multiplexed species detection, PCR methods nevertheless have some disadvantages, even though a recent publication determined a LOD of 0.1% porcine meat in a poultry matrix.[10] Usually, expensive DNA extraction kits are used, which, dependent upon the matrix amount and sample composition, need up to 8 h for the extraction, followed by cleanup and the PCR reaction. This makes PCR methods not necessarily faster than MS. In addition, DNA is prone to degradation especially in processed food,[11] and consequently, the detection of trace amounts of DNA in processed food samples is still problematic.[12]

In contrast, the primary structure of proteins is, in general, quite stable against processing.[13] Furthermore, a limited degree of protein degradation is less critical than (the equivalent amount of) DNA shearing and when (tryptic) peptides are used as biomarkers as fragmentation events within the relatively short peptide sequences is less likely compared to degradation of the longer DNA templates. We therefore decided to apply a shotgun proteomic approach using high-resolution MS to identify novel marker peptides for the mass spectrometrical detection of horse (*Equus caballus*) and pig (*Sus scrofa*). The identified marker peptides were used to establish a method for triple-quadrupole MS systems because these instruments are widely spread in routine laboratories and allow for excellent sensitivity. Specificity of peptides was evaluated against different mammalian species, and beef samples were spiked with pork and horse meat to simulate contamination with non-declared meat.

Because no official regulation is in place concerning the minimal amount of pork and horse in meat products and quantitation is not relevant for halal/haram labeling (see above), we decided not to perform absolute quantification using stable isotope-labeled peptides. To estimate the sensitivity of our method, we spiked beef samples with decreasing amounts of horse and pork.

MATERIALS AND METHODS

Chemicals. Liquid chromatography−mass spectrometry (LC−MS)-grade methanol (MeOH) and acetonitrile (ACN) were purchased from Sigma-Aldrich. Formic acid (FA) was purchased from Grüssing (Filsum, Germany). Water (H_2O) was prepared on a Milli-Q gradient A10 system by Millipore (Schwalbach, Germany) and used for all buffers and other solutions including high-performance liquid chromatography (HPLC) eluents.

Samples. Meat samples were purchased from local supermarkets, butcher shops, and farmer's markets and directly stored at −20 °C in a Corning (Amsterdam, Netherlands) 50 mL plastic centrifugal tube. For all samples, species identity was verified and we did not use minced meat samples to avoid contaminations. For details of included samples, see Supplementary Table 1 of the Supporting Information.

Sample Preparation. Chunks of meat or larger slices were diced to a length of 1 cm and frozen in liquid nitrogen until the liquid nitrogen stops to boil. The frozen dices were ground at 8000 rpm for about 5 min in a Fritsch Pulverisette 14 rotor mill (Idar-Oberstein, Germany) with a 2 mm sieve.

Preparation of Sample Mixtures. Samples containing more than one meat species were either prepared by weighing different amounts of ground pork or horse meat in ground beef followed by extraction of the mixture or mixing desalted peptide solutions of relevant species (see also the Results and Discussion).

In detail, approximately 300 mg of ground beef sample and amounts down to 1.0 mg of ground pork or horse meat were weighed in a 15 mL Corning tube and were processed as described under "Extraction", "Digest", and "Desalting".

For samples with pork or horse contaminations below 0.25%, we prepared desalted beef, pork, or horse peptide solutions as described below and spiked the respective amount of pork or horse extract in beef peptide solution.

Extraction. Approximately 300 mg of ground sample was weighed in a Corning 15 mL plastic centrifugal tube, and 4 mL of extraction buffer (0.3 M KCl, 0.15 M KH_2PO_4, and 0.15 M K_2HPO_4 at pH 6.5, all Sigma-Aldrich, Steinheim, Germany) was added. Samples were vortexed for 30 s and extracted on a GFL 3005 rotary lab shaker (Burgwedel, Germany) at room temperature for 2 h at 400 cycles/min.

Following extraction, samples were centrifuged for 60 min at 4 °C with 12000*g*. A 100 μL aliquot of the supernatant was transferred into a 1.5 mL Eppendorf reaction tube (Hamburg, Germany), and the buffer was removed at 39 °C under continuous nitrogen flow.

Digest. The dried residue was redissolved using 100 μL of 6 M urea solution and subjected to tryptic digest following a standard protocol.[14] Briefly, an aliquot of 100 μL containing about 1.5 mg of protein of each sample was reduced with dithiothreitol, alkylated using iodoacetamide, diluted 1:10 with H_2O, and supplemented with 20 μg of Promega sequencing grade modified trypsin (Promega, Mannheim, Germany). To allow for complete digest, samples were incubated in a thermoshaker at 37 °C under slow shaking overnight.

Desalting. Digested samples were diluted 1:2 with H_2O and desalted using Phenomenex (Aschaffenburg, Germany) Strata-X 33u polymeric reversed-phase (RP) cartridges filled with 30 mg/mL RP material. Cartridges were washed and activated using 1 mL of MeOH followed by equilibration with 1 mL of 1% formic acid (FA) according to the manual. The samples were loaded onto the cartridge and washed with 1 mL of 5% MeOH/1% FA. Finally, the peptides were eluted with 1 mL of ACN/H_2O (90:10; 0.1% FA), and the eluate collected in Eppendorf reaction tubes was prefilled with 5 μL of dimethyl sulfoxide (DMSO) purchased from Roth (Karlsruhe, Germany).

Preparation for HPLC. After desalting, solvent was removed under vacuum and 40 °C temperature using a S-Concentrator BA-VC-300H purchased from Sauer Laborbedarf (Reutlingen, Germany). DMSO is not removed during this procedure, which prevents peptides from sticking to the vessel surface and enhances peptide recovery. The samples were redissolved in ACN/H_2O (3:97; 0.1% FA) and analyzed.

Peptide Identification with Fourier Transform Mass Spectrometry (FTMS). Identification of species-specific biomarker peptides was performed with a shotgun proteomic approach using either a LTQ Orbitrap XL (Thermo Scientific, Bremen, Germany) or an AB SCIEX TripleTOF 5600 system (AB SCIEX, Darmstadt, Germany). The Orbitrap system was coupled to the Accela HPLC system (Thermo Scientific, Dreieich, Germany), and chromatography was performed on a Phenomenex Kinetex, 2.6 μm, C18, 100 Å (100 × 2.10 mm), column. For data evaluation and analysis, the XCalibur 2.07 software and Proteome Discoverer 1.1 were used (both Thermo Scientific). Potential biomarker peptides were searched against the UniProt knowledge base (KB) database, and only specific peptides were used for further method development on the QTRAP instrument.

The AB SCIEX TripleTOF 5600 system was coupled to a Eksigent nanoLC, and separation was performed on a 2 μm C18 (250 mm × 75 μm) column (Thermo Scientific). Data processing was performed with the ProteinPilot software package, version 4.0.8085.

For detailed MS parameters, see Supplementary Table 2 of the Supporting Information.

Tandem Mass Spectrometry (MS/MS). Method development for the QTRAP 5500 LC−MS/MS system (AB SCIEX, Darmstadt, Germany) was performed with a VWR Hitachi HPLC (pump L-2160U, autosampler L-2200U, and column oven L-2300) using a Phenomenex Kinetex, 2.6 μm, C18, 100 Å (100 × 2.10 mm), column

Table 1. Characteristic Marker Peptides for Various Meat Species Identified in This Study[a]

marker	species	protein	UniProt accession	biomarker peptide sequence	AA position
1	pig/horse	troponin T/unknown	Q7SNG7/F6X010	YDIINLR	239−245/185−191
2	pig	myosin-4	Q9TV62	TLAFLFAER	619−627
3	horse	myosin-2	Q8MJV1	EFEIGNLQSK	1086−1095
4	pig	myosin-1 and myosin-4	Q9TV61/Q9TV62	SALAHAVQSSR	1331−1341/1329−1339
5	horse	myoglobin	P68082	YLEFISDAIIHVLHSK	104−119
6	horse	myosin-1 and myosin-2	Q8MJV0/Q8MJV1	VVETMQTMLDAEIR	1596−1609/1595−1608
7	horse	pyruvate kinase	F6W3M5	IYVDDGLISLQVK	184−196
8	horse	hemoglobin	P01958	FLSSVSTVLTSK	129−140
9	horse	myoglobin	P68082	HGTVVLTALGGILK	65−78
10	horse	myoglobin	P68082	VEADIAGHGQEVLIR	18−32
11	horse	myosin-1	Q8MJV0	LVNDLTGQR	1272−1280
12	cattle	myosin-1	Q9BE40	TLALLFSGPASGEAEGGPK	619−637

[a]Markers 1−3 are discussed in more detail in the text. AA position = position of the biomarker peptide in the sequence of the identified protein.

(Phenomenex, Aschaffenburg, Germany). A Turbo V electrospray ionization (ESI) source was used. For details on the HPLC gradient, source, and MS parameters, see Supplementary Table 3 of the Supporting Information.

Data were evaluated using Analyst software, version 1.5.2. The multiple reaction monitoring (MRM) method was developed using the iterative workflow of the AB SCIEX MRMPilot software, version 2.1. On the basis of the predicted MS/MS spectra of target peptides, possible MRM transitions were identified and introduced in the initial method. Optimization of collision energy (CE) and determination of the retention time of target peptides was performed with purified peptide extracts from respective species or with synthesized peptides. In addition, most intense transitions were identified and subjected to a second round of optimization. To further enhance sensitivity, we changed the HPLC in the final method to a Eksigent ekspert microLC 200 system (AB SCIEX, Darmstadt, Germany) using either the Agilent ZORBAX XDB C18, 3.5 μm, 0.5 × 150 mm, column (Böblingen, Germany) or the Eksigent HALO C18, 2.7 μm, 90 Å, 0.5 × 100 mm, column. Although both columns performed well and are suitable for the analysis of complex peptide samples, the Zorbax column delivers lower backpressure and slightly better retention of some of the relevant peptides. It was therefore preferably used. To survey retention time stability and relative intensity of MRM transitions, we added positive control samples from respective species to sample batches. For micro-LC gradient, source, and MS parameters, see Supplementary Table 4 of the Supporting Information.

RESULTS AND DISCUSSION

For the identification of species-specific biomarker peptides, we focused mainly on the myofibrillar and sarcoplasmic proteins because these fractions contain highly abundant proteins necessary to obtain the highest sensitivity and are completely soluble in buffer solutions, which is optimal for downstream applications. Different extraction buffers and conditions have been evaluated by SDS−PAGE and the Bradford assay to optimize extraction (data not shown).

A further advantage of the chosen buffer system is the suitability for direct tryptic digest without the necessity of buffer change after sample extraction.

Identification of Specific Peptides Using FTMS. DNA polymorphisms used for species differentiation by PCR are not directly transferable to protein-based methods because extractability and copy numbers of target proteins have to be taken into account as well as the compatibility of the respective peptides with mass spectrometrical detection. In addition, proteome-wide database searches to identify species-specific differences are not feasible. It was therefore necessary to first identify species-specific polymorphisms, ideally on a (sub)-proteome wide scale, which are suitable for sensitive MS detection. We therefore extracted the myofibrillar and sarcoplasmic fraction from commercially available meat samples derived from cattle, pig, wild boar, horse, chicken, and lamb. Beef, lamb, and chicken meat were included as negative controls because of their high economic impact and the fact that meat from these species is considered "halal". The extracts were subjected to tryptic digest, and resulting peptide mixtures were analyzed using the AB SCIEX TripleTOF 5600 system (Darmstadt, Germany) and the Orbitrap XL (Thermo Scientific, Bremen, Germany). The number of identified peptides was about 20% higher for the TripleTOF 5600 system compared to the Orbitrap, probably because of the use of the nano-LC system (Eksigent 400) for the TOF instrument compared to conventional high flow on the Orbitrap. More specifically, about 4000−4500 peptides from about 400 proteins were identified on the Orbitrap, while about 5500 peptides from ∼500 proteins were identified for identical samples on the TOF instrument. Details of the proteomic data will be published elsewhere. Briefly, MS and MS/MS data of tryptic peptides were searched against FASTA protein sequence databases of the respective species to allow for unambiguous sequence identification of peptides and, where possible, assignment to a protein. Species specificity of potential marker peptides was evaluated by a database search against FASTA files from all mammalians and comparative data analysis of pork, horse, or beef data sets against all other data sets. In addition, potential marker peptide candidates were searched against the UniProt KB database to exclude interferences with other plant or animal species that can be expected in food.

To ensure that the occurrence of biomarker peptides is independent from breed or muscle tissue, 21 commercially available samples from different muscles and different breeds of the above-mentioned species were analyzed (see Supplementary Table 1 of the Supporting Information for an overview).

Most abundant biomarker peptides for horse, beef, and pork identified by the non-target proteomic approach were used for method development of the MRM method (overview given in Table 1). In addition, a marker peptide specific for beef was used as a positive control in spiking experiments, where horse or pork was spiked into beef matrix. To ensure unambiguous identification, at least three MRM transitions with matching signal ratios and retention time match were considered as specific. Optimization of MS parameters and MRM development was performed using peptide extracts or synthetic peptides. Optimized MS conditions are given in Supplementary

Tables 2−4 of the Supporting Information, and the complete list of MRM transitions is given in Supplementary Table 5 of the Supporting Information.

Determination of Peptide Specificity in the MRM Method. Peptides identified as specific using database searches and analysis of shotgun proteomic data were in addition verified by measuring the different matrices (beef, pork, wild boar, horse, lamb, and chicken, respectively) using the MRM method. This approach is necessary for several reasons. First, proteomic or genomic data stored in the respective repositories are, in general, not complete, which might lead to false identification of unique biomarker peptides. Second, peptide cross-talk can occur especially in non-high-resolution MS methods. For the latter, the matrix can contain peptides with nearly identical precursor masses compared to the biomarker peptide. In addition, these matrix peptides can show partial coverage of MRM transitions, which consequently might lead to false-positive results, when not excluded experimentally.

To this end, samples given in Supplementary Table 1 of the Supporting Information were again analyzed using the optimized MRM method to substantiate species specificity under non-high-resolution MS conditions and to verify that the chosen analytes are not breed- or tissue-dependent. These measurements were performed in triplicate for all samples.

Figure 1 gives an overview of the results and displays the signals of the three most intense MRM transitions from every biomarker peptide in representative samples from different species. To reduce complexity, the most intense transition of every peptide is given in red, while the second and third most intense signals are shown in blue and green, respectively. Peptide signals considered as specific are numbered according to the nomenclature for biomarker peptides given in Table 1. Notably, marker peptide 1 YDIINLR is found in porcine troponin T as well as in horse (uncharacterized protein F6X010) according to the UniProt KB database. As expected, we detected this peptide in our set of samples in pork, wild boar, and horse but not in other species. The peptide YDIINLR can therefore be used as a combined marker for both "haram" or "non-kosher" species. Within the 21 samples analyzed, we did not observe false-positive signals and were always able to detect all biomarker peptides for a given species in the corresponding samples. Taken together, species specificity was confirmed for all chosen peptides using the newly developed MRM method. The complexity of the analyzed samples is indicated by a number of unspecific MRM signals (marked with stars), which are seen in beef, lamb, and especially chicken. In most cases, only single MRM transitions were observed that, in addition, showed shifts in the retention time compared to the respective biomarker peptides. Signals consisting of more than one MRM transition always showed clear retention time shifts and non-matching fragment ion ratios and were thus all identified as unspecific.

The results shown in Figure 1 have been generated on the micro-LC system and can be easily transferred to conventional HPLC (for details, see Supplementary Table 3 of the Supporting Information). However, it has to be kept in mind that the micro-LC system is less time- and solvent-consuming and shows enhanced sensitivity. The estimated LOD for porcine meat in beef matrix when detecting marker 1 is about 2% when using HPLC (data not shown) and could be reduced by a factor of 3−4 using micro-LC (see below).

Detection of Pork and Horse Meat Contaminations in Beef Matrix. For the detection of trace contaminations of pork

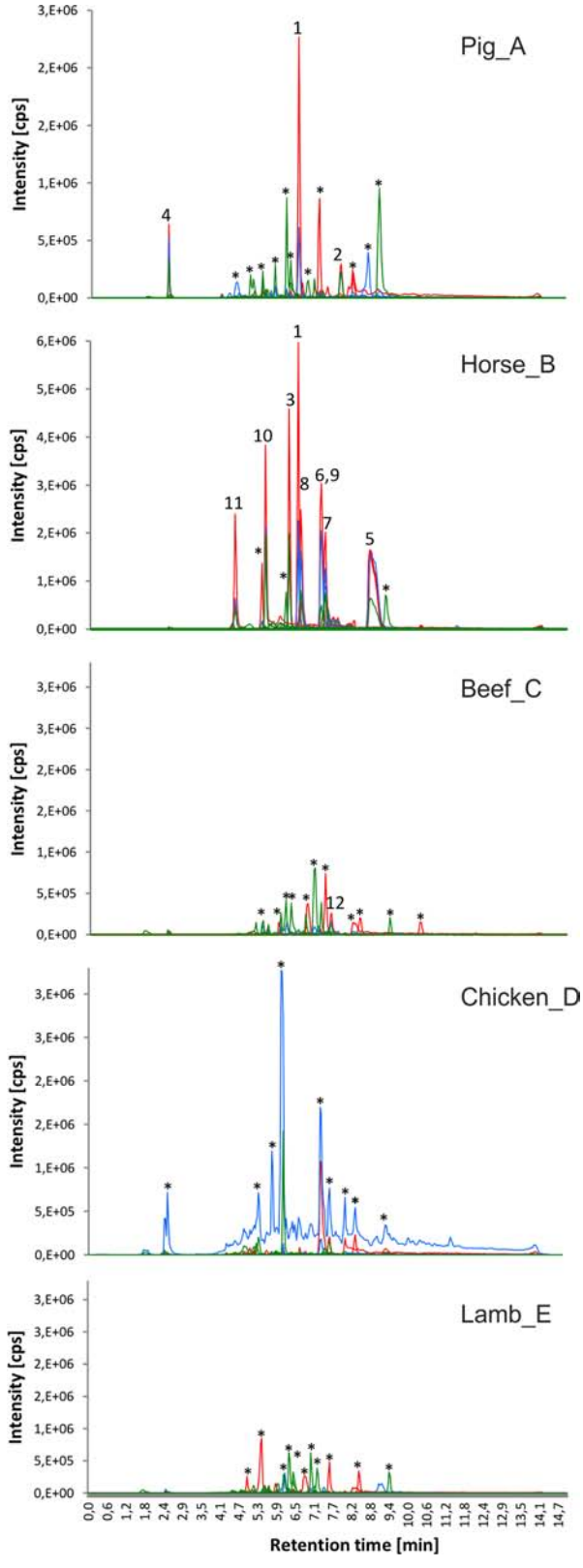

Figure 1. Micro-LC−MS chromatograms of five different meat species detecting the characteristic biomarker peptides by MRM. All identified marker peptides are numbered according to Table 1. To reduce complexity, only the three most intense MRM traces are shown for every peptide. The most intense transition is given in red; the second most intense is given in blue; and the third most intense is given in green. Unspecific signals are marked with an asterisk.

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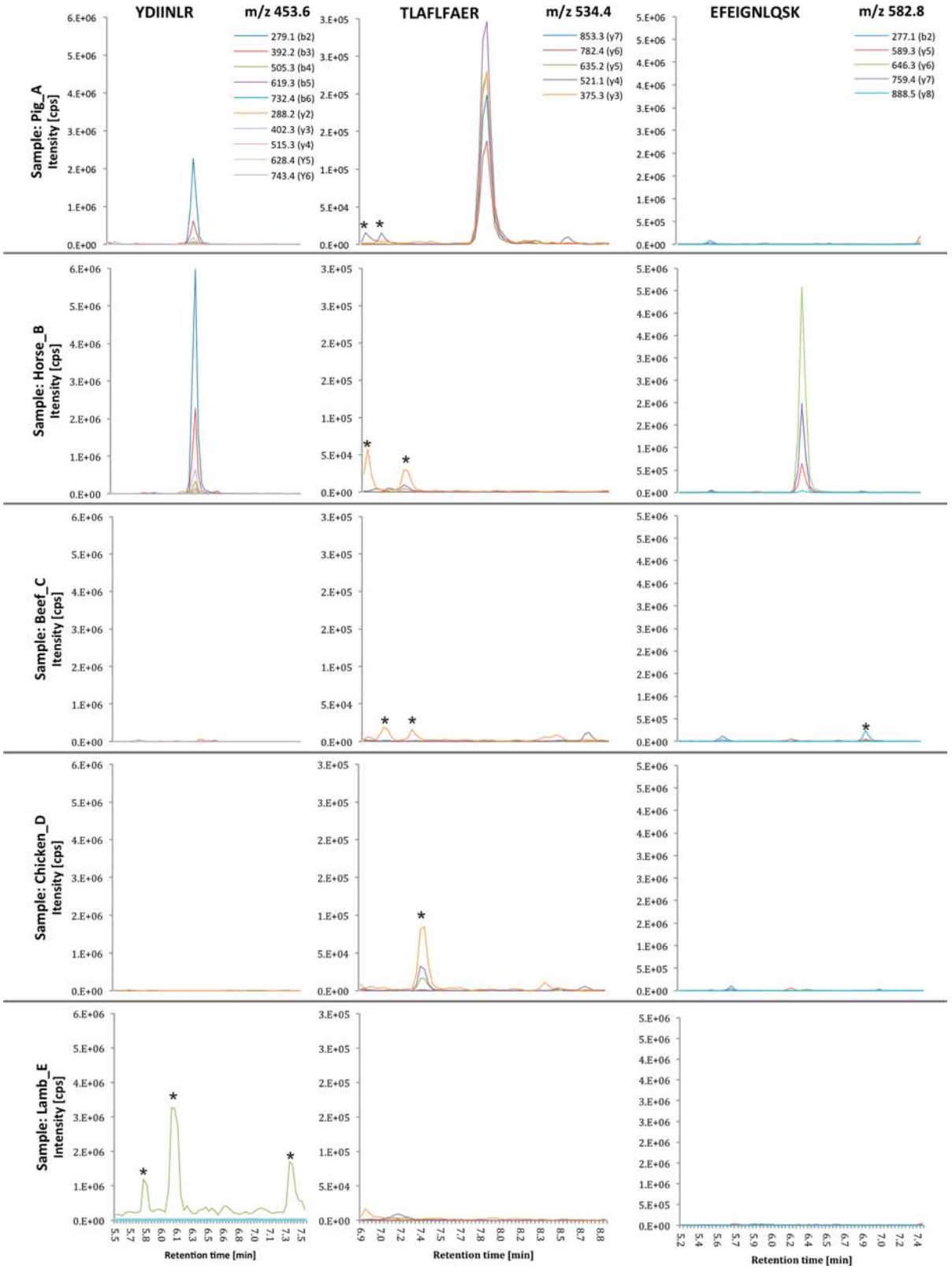

Figure 2. Micro-LC−MS chromatograms (2 min window) of the meat samples from five different species with the characteristic MRM transitions of the three most intense marker peptides. Unspecific signals are marked with an asterisk.

or horse in beef, we focused on the three most intense peptides (markers 1−3) and exclusively used the micro-LC system (detailed method given in Supplementary Table 6 of the Supporting Information). Figure 2 shows extracted ion chromatograms of MRM transitions of markers 1−3 in a more detailed 2 min window of the 14 min chromatographic run. As expected, marker 1 YDIINLR was found in pork and horse and was not detectable in the other species. We observed

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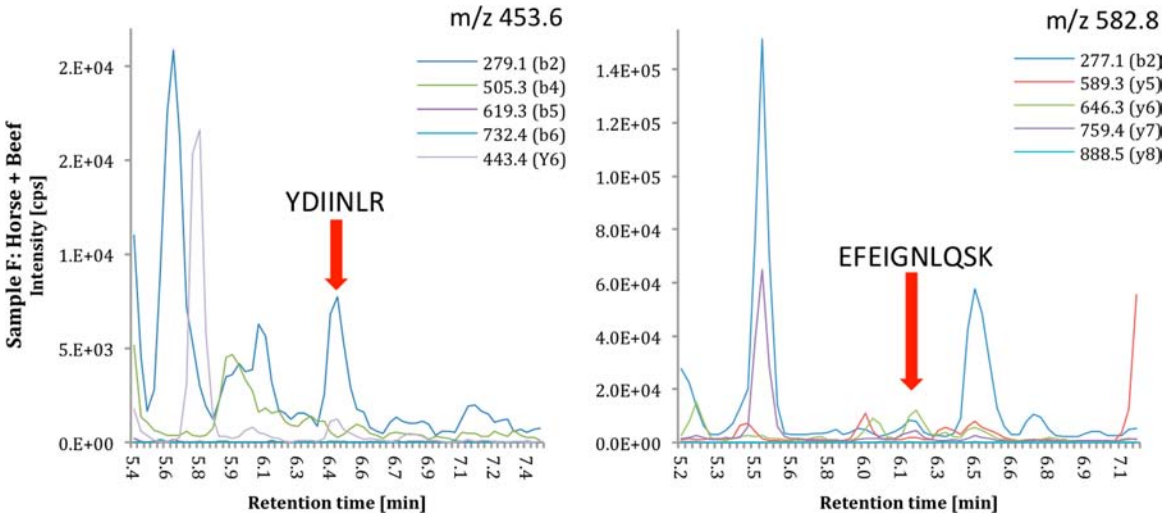

Figure 3. Micro-LC−MS chromatograms of beef containing 0.55% horse meat. MRM transitions for marker peptides 1 (left) and 3 (right) are shown. For marker 1, only one MRM was detectable at this concentration. For marker 3, three transitions are just above the background level.

an interference with the b4 fragment (m/z 505.3) of YDIINLR in lamb. Because none of the further MRM transitions of YDIINLR was found in the lamb matrix, this signal is clearly unspecific (Figure 2). Similarly, three MRM transitions of the second porcine marker TLAFLFAER were detected in chicken. However, neither the retention time nor the fragment ion ratio matched in comparison to the positive controls. Therefore, this signal is also unspecific for chicken and can be clearly distinguished from the characteristic signals observed in the pork samples. For marker 3 EFEIGNLQSK, no relevant interferences were observed.

To simulate a contamination of halal meat, we spiked different amounts of pork or horse meat in beef and measured the characteristic marker peptides. Figure 3 gives an example of 0.55% horse meat spiked into beef. At this concentration, the detection limit for peptide YDIINLR is reached and the three MRM transitions of marker EFEIGNLQSK are hardly distinguishable from background noise. To achieve higher sensitivity and enhance the signal-to-noise ratio, we then took advantage of the MRM^3 mode of the QTRAP system. This approach uses the linear ion trap at Q3 to generate either a full spectrum from a certain fragment ion (e.g., m/z 453.6 → m/z 743.4 → "spectrum") or a second-generation MRM (e.g., m/z 453.6 → m/z 743.4 → m/z 628.6) to enhance specificity and reduce background. Because of prolonged filling times of the linear ion trap, signal intensities were generally enhanced in MRM^3.

Figure 4 displays the improved signal-to-noise ratio when MRM^3 is used, and Supplementary Table 7 of the Supporting Information gives the improved MS conditions. The shift in retention time compared to Figures 1−3 is due to the HALO column used in this set of experiments. For the 0.24% pork contamination in beef, only the most abundant MRM transition of the b2 fragment of YDIINLR can be observed (upper left panel of Figure 4). In contrast, the MRM^3 signal of the y6 fragment (m/z 743.4) shows enhanced signal intensities and huge improvement of the signal-to-noise ratio (upper right panel of Figure 4). It has to be noted that, for the MRM^3, not the most abundant b2 ion (YD) has been used because the respective MRM^3 would generate a single amino acid fragment from the dipeptide precursor, which is likely to result in a MRM^3 with low specificity. Despite the low signal intensities of the y6 precursor, a specific MRM^3 with excellent signal-to-noise ratio has been triggered. Similar results can be observed when horse is spiked into beef. Again, only the b2 fragment is clearly visible, but the y6 precursor also triggers a specific MRM^3 signal (data not shown). To confirm that the MRM^3 is specific only for horse or pork, we analyzed all "halal" species included in this study with the MRM^3 method. No MRM^3 was triggered in beef and lamb samples, while in chicken, we observed a single MRM^3 signal with a clear shift in retention time (see Supplementary Figure 1 of the Supporting Information). Using this approach, both "haram" species pig and horse can therefore be detected down to concentrations of at least 0.25%, but it is not possible to differentiate both species at this low concentration. We therefore developed a second MRM^3 for the horse-specific peptide EFEIGNLQSK (marker 3) to allow for discrimination of both species. Figure 4 (lower right) shows the MRM^3 of marker 3 at a concentration of 0.55% (m/z 582.8 → m/z 646.4 → m/z 345.4; see Supplementary Table 7 of the Supporting Information for details), which also shows very good specificity and no interference with the other species included in this study (see Supplementary Figure 2 of the Supporting Information).

To further enhance the sensitivity, we then used a QTRAP 6500 system equipped with micro-LC and analyzed a sample containing 0.13% pork in beef. Because homogeneous spiking of beef with pork is hard to achieve with these low amounts, we mixed respective aliquots of tryptic-digested protein extracts from both species (see "Preparation of Sample Mixtures" for details). The MRM and MRM^3 data of peptide YDIINLR of this sample is given in Figure 5. Again, only the most sensitive MRM transition was detectable, but we observed a strong and specific MRM^3 signal. To enhance specificity, we additionally performed "MRM^3 spectrum" experiments (m/z 453 → m/z 732 → "spectrum") and identified the respective y5, y5*, y4, y3, y3*, and y2 fragments (see Supplementary Figure 3 of the Supporting Information). This further demonstrates the specific identification of peptide YDIINLR at concentrations of 0.13% for pork.

In the current study, we present a MS-based approach for the detection of trace amounts of horse or pork in beef samples. MS has been used previously to analyze, for example, milk quality, animal health,[15] and meat authentication.[16] However,

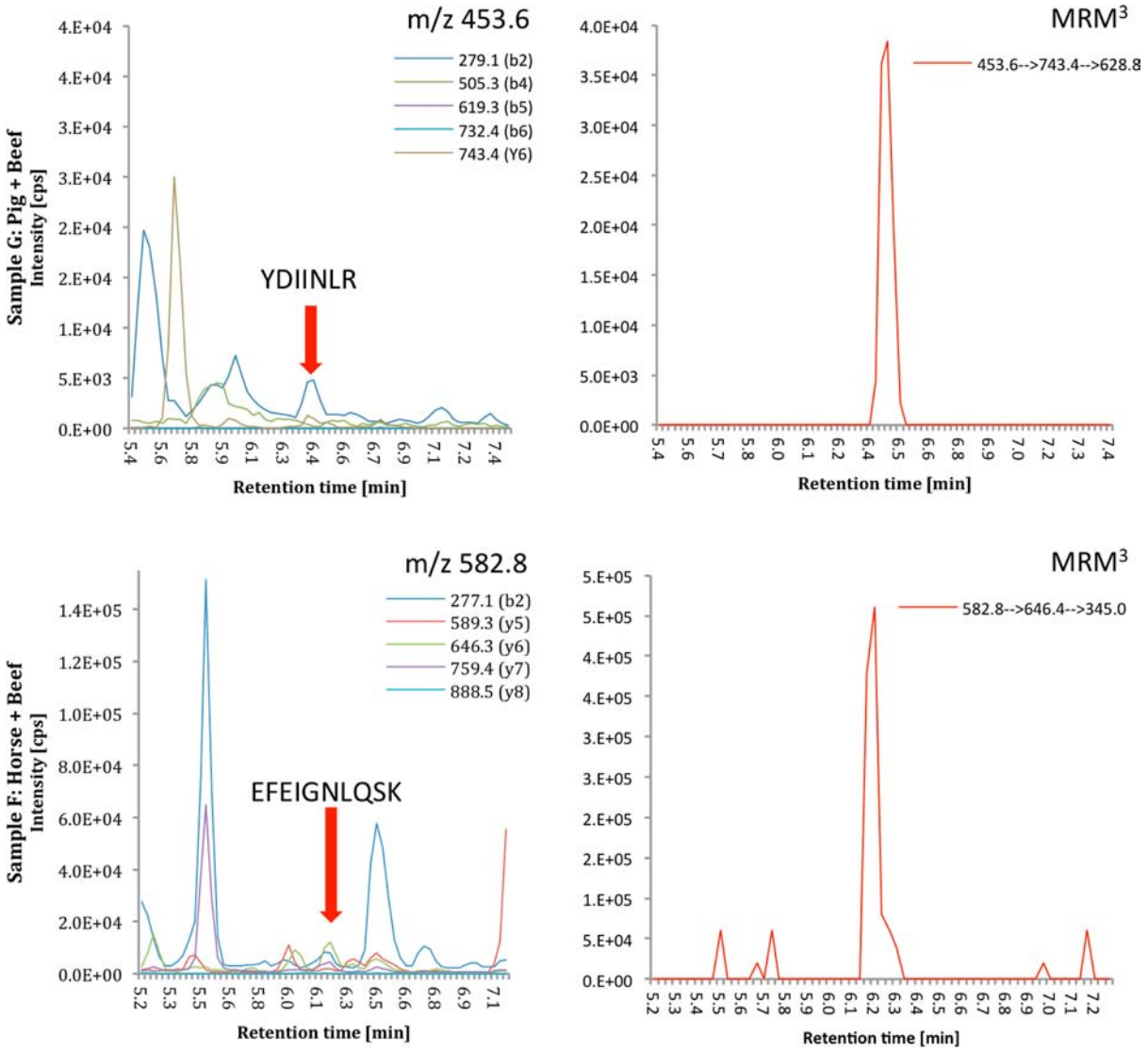

Figure 4. Detection of marker 1 (upper lane) and marker 3 (lower lane) using MRM^3. (Upper panels) Sample G contains 0.24% pork in beef. Only the MRM transition m/z 453.6 → m/z 279.1 is observable (left). The MRM^3 experiment m/z 453.6 → m/z 743.4 → m/z 628.8 (right) gives an additional specific signal with a highly improved signal-to-noise ratio. (Lower panels) Sample F contains 0.55% horse meat in beef, and the MRM method (left) is at the detection limit. Using the MRM^3 experiment m/z 582.8 → m/z 646.4 → m/z 345.0 (right), sensitivity is enhanced by a significantly improved signal-to-noise ratio.

attempts to analyze the muscle proteome and distinguish species mainly rely on laborious and expensive separation techniques, such as 2D gel electrophoresis, to separate the proteome of meat samples, followed by matrix-assisted laser desorption−ionization time-of-flight mass spectrometry (MALDI−TOF−MS) of species-specific protein spots.[17,18] An alternative approach to reduce sample complexity and enrich target proteins is the fractionation of proteins in solution by their isoelectric point (known as OFFGEL fractionation). Using this technique, Sentandreu and colleagues were able to detect 0.5% chicken in a pork matrix.[19] However, OFFGEL fractionation is also time-consuming, and in quality-control laboratories, this technique is often not available.

In this study, we identified 12 tryptic biomarker peptides specific for pork and/or horse meat. To the best of our knowledge, we present the first MRM and MRM^3 methods for the rapid and sensitive detection of both species using routinely available MS techniques. Importantly, the MRM and MRM^3 methods show sensitivities that are comparable to the most sensitive PCR[10] and ELISA[7] methods available, demonstrating that MS has enormous potential as an alternative analytical technique for meat speciation and authentication.

Outlook and Concluding Remarks. Following the identification of specific biomarker peptides, we have developed a sensitive and rapid MRM-based method that can be applied routinely in quality-control laboratories that are equipped with triple-quadrupole instruments. Using MRM^3 transitions in a QTRAP instrument, we were able to detect contaminations of pork with the most sensitive biomarker peptide down to 0.13% in beef matrix without pre-enrichment of analytes, while the LOD for the applied MRM method is reached at about 0.55%. However, it has to be noted that we used unprocessed meat and that the sensitivity in processed samples or low-quality meat is likely to be reduced. One future direction is therefore the analysis of processed samples, including optimization of extraction and sample preparation.

Using micro-LC systems, the total chromatographic run time was only 17 min and, in general, it is possible to integrate

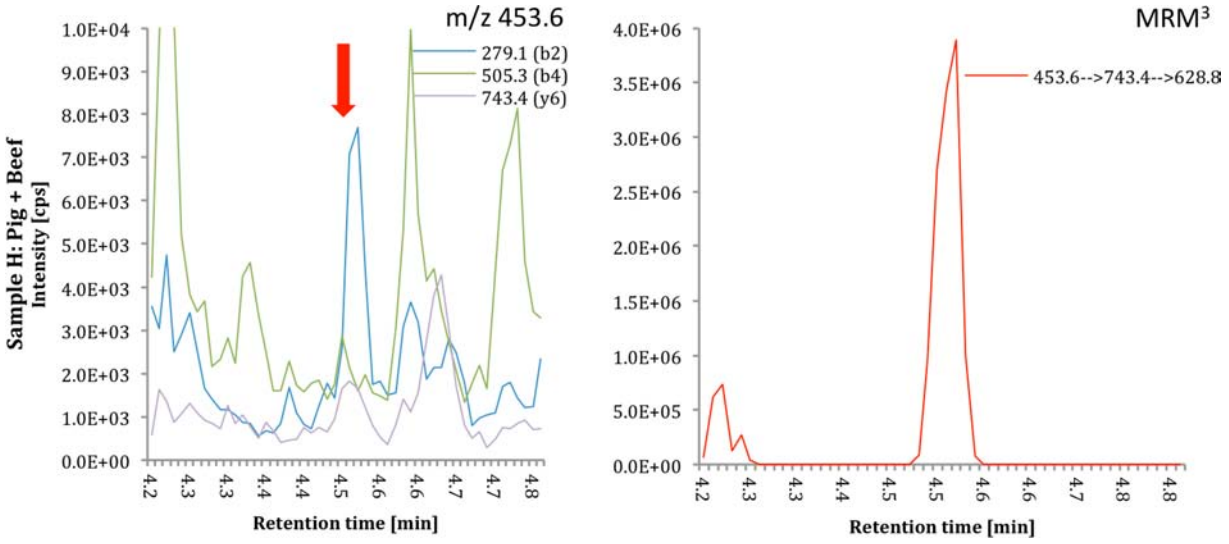

Figure 5. Detection of marker 1 (YDIINLR) at a concentration of 0.13% pork spiked in beef using the QTRAP 6500 MS by MRM and MRM^3. Again, only the most sensitive transition m/z 453.6 → m/z 279.1 is observable in MRM mode (left), while the MRM^3 experiment m/z 453.6 → m/z 743.4 → m/z 628.8 (right) gives an additional specific signal with a highly improved signal-to-noise ratio.

additional species in a multiplexed meat authentication method. As a first step, we already integrated the bovine marker peptide as a positive control for our spiking experiments. In addition, the generally time-consuming tryptic digest could be shortened to a 2 h incubation step. We included a method published by Carrera et al.[20] in our sample preparation, and initial experiments showed promising results after 2 h of incubation time (data not shown). Together, we present a promising approach to establish MS for the sensitive and rapid targeted detection of different meat contaminations in food as an alternative for PCR and ELISA methods.

■ ASSOCIATED CONTENT

Supporting Information

Samples analyzed in this study (Supplementary Table 1), samples used for screening shown in Figure 1 (Supplementary Table 1a), mixed samples as shown in Figures 3−5 (Supplementary Table 1b), FTMS parameters and HPLC parameters for identification of biomarker peptides (Supplementary Table 2), QTRAP 5500 and HPLC parameters for method development and sample screening with conventional HPLC (Supplementary Table 3), QTRAP 5500 parameters and Eksigent micro-LC parameters for MRM method and MRM^3 method (Supplementary Table 4), MRM conditions used for peptide identification (Supplementary Table 5), optimized MRM method for best LOD (Supplementary Table 6), parameters for the MRM^3 experiments (Supplementary Table 7), retention time (min) and specificity of marker peptides as shown in Figure 1 (Supplementary Table 8), LC−MS chromatograms showing MRM and MRM^3 (m/z 453.6 → m/z 743.4 → m/z 628.6) of peptide YDIINLR in the negative controls beef, chicken, and lamb (Supplementary Figure 1), LC−MS chromatograms showing MRM and MRM^3 (m/z 582.8 → m/z 646.4 → m/z 345.4) of the horse-specific peptide EFEIGNLQSK in the negative controls pork, beef, chicken, and lamb (Supplementary Figure 2), and MRM^3 spectrum of a sample containing 0.13% pork (QTRAP 6500 system) (Supplementary Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Co-authors Dietmar Waidelich and Jörg Dojahn are employees of AB Sciex, a vendor of MS instrumentation. They supported the measurements on QTRAP 6500 and TripleTOF 5600 instruments in the AB Sciex Application Laboratory in Darmstadt, Germany, but had no influence on the study design, sample analysis, or content of the manuscript.

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