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Analysing polypeptide antibiotics residues in animal muscle tissues: The crucial role of HRMS

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ABSTRACT

A confirmatory method for the determination of polypeptide antibiotics (bacitracin, colistin, and polymyxin B) in muscle samples has been developed. Extraction is performed with acidified methanol, and a clean-up step by solid-phase extraction with polymeric cartridges is applied. Separation by ultra-high performance liquid chromatography (UHPLC) is carried out using a solid core C18 column and gradient elution with water/acetonitrile containing 0.2% formic acid. High-resolution mass spectrometry (HRMS) (Q-Orbitrap) detection using different working modes has proved to be highly advantageous in eliminating interfering signals from endogenous matrix components. The analytical method has been successfully validated according to Commission Regulation 2021/808/EU and is currently used in a public health laboratory involved in veterinary medicines residue surveillance activities.

1. Introduction

Polypeptide antibiotics possess high efficiency against many microorganisms, and are used as antimicrobials in livestock farming, but also in humans. Bacitracin (BCT), polymyxin B (PMB) and colistin (CST) are representative compounds of this group, with molecular masses ranging from 1000 Da to 2000 Da.

BCT is isolated from strains of *Bacillus subtilis* and *Bacillus licheniformis*. It was initially reported as a single component, but actually, it is a combination of several species, being bacitracin A (BCTA) the major component. In terms of chemical structure, BCTA consists of a heptapeptide ring connected to a pentapeptide side chain (see Fig. S1 from supplementary information (SI)) (Sin, & Wong, 2003). BCTA exhibits a potent therapeutic activity against gram-positive cocci and bacilli. BCTA is administered in veterinary practices and humane medicine.

PMB and CST, otherwise known as polymyxin E, belong to the polymyxin class of antibiotics, and are produced by Bacillus polymyxa (Shaheen, Li, Ross, Vederas, & Jensen, 2011). PMB consists of a series of linear side-chain cyclopeptides. The predominant forms of PMB are polymyxin B1 (PMB1) and polymyxin B2 (PMB2) (Satlin, & Jenkins, 2017). CST is composed of a cyclic heptapeptide linked to a side chain of three amino acids and a fatty *N*-acyl residue. The two major forms of CST, which differ in the fatty acid residue, are named colistin A (CSTA) and colistin B (CSTB) (see Fig. S1 in SI).

Polymyxins are strong agents against infections caused by gram-negative bacteria. CST is used for livestock animals, especially pig. However, the medical use of polymyxins in humans is quite limited, due to their nephrotoxicity, but also to neurotoxicity and hypersensitivity reactions. Nevertheless, due to the marked increase of gram-negative bacteria resistance to antimicrobial drugs, polymyxins are currently considered last resort agents (Nang, Azad, Velkov, Zhou, & Li, 2021). In this context, it is a priority to preserve the usefulness of these antimicrobials.

The maximum residue limits (MRL) regulated in the European Union (EU) for BCT and CST (Commission Regulation (EU) 37/2010, 2009) in several food matrixes are listed down in Table S1 (Supplementary material). There is no MRL established for PMB for any food-producing species, and thus PMB should be considered as not allowed in veterinary practices (Regulation (EC) 470/2009, 2009); so, methods for the analysis of residues of PMB in food samples should be as sensitive as

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possible. In the EU frame, member states are required to implement residue monitoring plans (Regulation (EU) 2017/625, 2017) to detect incidences of possible misuse of allowed and not allowed veterinary medicines in livestock animals. Meanwhile, non-EU countries exporting to the EU are requested to put up their own residue monitoring plans, which should provide a comparable level of assurance in terms of food safety. In this context, analytical methods must be reliable and able of quantifying low concentration levels of polypeptide antibiotics in food samples of animal origin.

In recent years, some analytical methods for the determination of polypeptide antibiotics in food samples have been reported (Kaufmann, & Widmer, 2013; Boison,Lee & Matus, 2015; Zhang,et al., 2015; Fu, et al., 2018; Lee,Matus,Gedir,& Boison, 2011; Xu, et al., 2012; Wan, Ho, Sin, & Wong, 2006; Liu, et al., 2019; Wu, Turnipseed, Andersen, & Madson, 2020). The extraction procedure normally involves the use of an acidic hydro-organic mixture (methanol or acetonitrile). Thereafter, to remove matrix components, a clean-up step by solid phase extraction (SPE) is usually applied (Saluti,et al., 2018; Zhang,et al., 2015; Sin,et al., 2005). The extracts are then analyzed by liquid chromatography using C18 columns and mobile phases based on acetonitrile or methanol and water containing some acid. Some authors have proposed the use of ion pairing or the Hydrophilic Interaction Liquid Chromatography (HILIC) mode (Saluti, et al., 2018). Detection is performed by mass spectrometry (MS), usually with triple quadrupole mass spectrometers (Kaufmann, & Widmer, 2013; Kumar, et al., 2021; Liu, et al., 2019; Song, et al., 2022).

The greatest challenge in the analysis of polypeptides in biological matrices is the coelution of isobaric interferences, which impair the quantification at low concentration levels. In this scenario, the performance provided by triple-quadrupole instruments appears to be somewhat limited due to the poor selectivity of the low resolution of quadrupoles. Current high resolution mass spectrometry (HRMS) instrumentation, such as time of flight and Orbitrap based spectrometers, has proven suitability and high performance in routine quantitative analysis of complex samples (Grund, Marvin, & Rochat, 2016; Gavage, Delahaut, & Gillard, 2021; Kaufmann, 2020; Yan, Zhang, Zhou, Li, & Feng, 2022; Wu, Turnipseed, Andersen, & Madson, 2020). Mass resolution and mass accuracy are the key concepts in HRMS. Both play a crucial role in providing the selectivity required to overcome the interferences from isobaric compounds, as extremely narrow mass extraction windows can be applied to the target ions, and interference ions can be resolved (Eliuk, & Makarov, 2015). Therefore, LC-HRMS is especially suitable for challenging analytical issues, such as the quantification of low concentration levels of polypeptides in biological matrices (Saluti, et al., 2018; Wu, Turnipseed, Andersen, & Madson, 2020), with endogenous isobaric mass interferences similar in nature to the analytes, which are extremely difficult to remove by conventional clean-up procedures.

Here we present a method based on LC-HRMS for the analysis of bacitracin, polymyxin B and colistin that meets the demanding requirements of European regulations concerning veterinary drugs residue analysis, and which is suitable for laboratories with high workloads.

2. Experimental

2.1. Reagents, solvents and consumables

BCTA VETRANAL was obtained from Merck (Darmstadt, Germany); PMB sulfate (70 % PMB1 and 16 % PMB2) was obtained from USP (Rockville, MD, USA); CST sulfate salt was obtained from Sigma-Aldrich (Merck, Darmstadt, Germany), was estimated to be 28 % CSTA and 72 % CSTB after measuring peak areas ($n = 79$) of both species assuming that their MS signal is equivalent.

Individual stock solutions (1000 mg/L) were prepared by accurately weighing a proper amount of analytical standard into a 10 mL volumetric flask, subsequently dissolved and diluted to volume with ultrapure water. 20 mg/L stock solutions were prepared by dilution from the 1000 mg/L one in 0.2 % formic acid in water. Stock solutions containing all the analytes (5 mg/L and 0.4 mg/L) were prepared by transferring appropriate amounts of 20 mg/L stock solutions and diluting to appropriate volumes with 0.2 % formic acid in water. A spiking solution containing BCT and PMB at 0.8 mg/L and CST at 3.0 mg/L was prepared by a proper dilution with 0.2 % formic acid in water.

Water used in this study was purified in-house by a Milli-Q Integral water purification system (Merck, Darmstadt, Germany). Formic acid (88–91 %) and sulfuric acid (95–98 %) analytical grade, and acetonitrile and methanol LC-MS grade were supplied by Sigma-Aldrich (Merck, Darmstadt, Germany).

Mobile phase A was 0.2 % formic acid in water, while mobile phase B was 0.2 % formic acid in acetonitrile. Both mobile phases were filtered through 0.22 µm prior to use.

SPE cartridges used for sample clean-up were Bond Elut Plexa (200 mg, 6 mL) from Agilent (Santa Clara, CA, USA). The extracts were filtered using Millex syringe filters (0.22 µm) from Merck, before injection into the UHPLC-HRMS system.

2.2. Instrumentation

UHPLC: The instrument is composed of a Vanquish Flex (binary pump, autosampler, and column selector) from Thermo Fisher Scientific (Germering, Germany).

HRMS: The high-resolution mass spectrometer is a Q Exactive Benchtop, a hybrid Quadrupole-Orbitrap equipped with a heated-electrospray ionization probe (HESI II). TraceFinder 4.1 and Xcalibur 4.0, also from Thermo Fisher Scientific (Bremen, Germany), were used for data acquisition.

For sample preparation, a multi-tube vortex VWR-DVX-2500 (Radnor, PA, USA), a laboratory centrifuge Hettich Rotanta 460R (Tuttlingen, Germany), a vertical agitator Agytax from AgytaxLab (Madrid, Spain), a Turbovap nitrogen evaporator from Biotage (Uppsala, Sweden) and an ultrasonic bath from Selecta (Abrera, Barcelona, Spain) were used.

2.3. Samples

Three hundred eleven muscle samples from various animal species (bovine, porcine, ovine, rabbit) were collected in slaughters by official inspectors, in the frame of the veterinary drug residues control plan in Catalonia (Spain) during the period 2017 to 2021. Samples were stored immediately on reception at −20 °C in numbered plastic jars until analysis. Samples were properly homogenized before analysis using a laboratory blender.

All blank samples used to validate the procedure had been previously analyzed to confirm the absence of polypeptide antibiotics.

2.4. Procedures

2.4.1. Extraction

Five grams of homogenized sample was weighed into a 50 mL centrifuge tube. Afterwards, 18 mL of extraction solution (methanol: water (1:3)) was dispensed into the centrifuge tube, followed by addition of 2 mL of sulfuric acid 0.5 mol/L. The centrifuge tubes were tightly capped and placed in a vortex mixer for 30 s. The tubes were then transferred into an ultrasonic bath and left for 5 min. Thereafter, centrifugation was performed at 4000 rpm at 10 °C for 10 min. The supernatant was collected into a separate 50 mL centrifuge tube and was set aside. A second extraction step was performed by adding 20 mL of methanol: water (1:3) to the remaining residue and repeating the same steps as mentioned above. The supernatant was then mixed with the previously collected one.

2.4.2. Clean-up

SPE Bond Elut Plexa (200 mg, 6 mL) polymeric cartridges were conditioned with 3 mL of methanol and 3 mL of water, followed by loading of the sample extract. Rinsing was then performed three times with 3 mL of water. Afterwards, the retained analytes were eluted with 2 mL of SPE elution solution (0.24 % formic acid in methanol: water (14:3)) while adjusting the vacuum to obtain a flow rate of approximately two drops per second. The eluent was collected into an evaporation tube, subsequently placed in an evaporator, with the water bath temperature set at 40 °C. Evaporation to total dryness was achieved by passing a gentle stream of nitrogen for approximately 1 h. The extract residue was re-dissolved with 1 mL of mobile phase A, mixed by vortex for 30 s, and ultrasonicated for 5 min. The extract solution was then filtered (0.22 μm) and collected into a vial ready for injection into the UHPLC-HRMS system.

2.4.3. *LC separation*

A gradient using 0.2 % formic acid in water (mobile phase A) and 0.2 % formic acid in acetonitrile (mobile phase B), at a flow rate of 0.4 mL min^{-1}, was used to separate the polypeptide antibiotics in a Kinetex C-18 (100 x 2.1 mm, 1.7 μm) column from Phenomenex (California, USA). The column oven temperature was maintained at 40 °C, and the injection volume was 15 μL. The gradient program was as follows (time in min, %B): (0,10), (1,10), (1.5,20), (3.5,20), (4,90), (6.5,90), (7, 100), (9, 100), (9.1, 10), (11,10). Total run time was 11 min.

2.4.4. *HRMS detection*

The H-ESI was operated in positive mode. The sheath gas flow rate (N_2) was set to 45 (arbitrary units, au), while the auxiliary gas flow rate was maintained at 10 (au). The spray voltage was established at + 3.0 kV, and S-lens RF level was fixed to 80 (au). The capillary temperature was maintained at 250 °C, while the auxiliary gas heater temperature was 380 °C.

According to elution profile, two time functions were created and different MS working modes were used for each one. Single ion monitoring (SIM) was selected as the operation mode with an isolation window at 4.0 *m/z* for the first time function (0–3.5 min), where all analytes elute, except BCTA. For the second time function (3.5–6 min), where BCTA elutes, the PRM mode was selected. Automatic gain control (AGC) for the C-Trap was set at $3{\cdot}10^6$ ions and the Orbitrap resolution was set to 70,000 (*m/z* 200, Full Width at Half Maximum (FWHM)). The multiplexing (MSX) tool, which allows simultaneous detection of ions was also used. In the (0–3.5) min range, MSX was set to 12 (au) to allow the detection of target ions eluting in this time window. In the second time function MSX was set at 3 (au). Triply-charged ions were chosen for quantification, while doubly-charged and quadruple-charged ions were monitored for confirmation, except in the case of BCTA, for which a doubly charged fragment at *m/z* 669.3367 was selected as confirmation ion 2 (Table 1).

Daily mass calibration of Orbitrap was performed using Pierce LTQ Velos ESI Positive ion calibration solutions (Thermo Fisher Scientific, Rockford, IL, USA) to guarantee a working mass measurement accuracy (MMA) of at least 5 ppm.

2.4.5. *Calibration*

The quantification of polypeptide antibiotic residues in muscle samples was achieved by calibrating with matrix-fortified standards (MFSs). This involved the addition of appropriate amounts of spiking solution containing the analytes to blank muscle samples. Five MFSs were prepared each session to build calibration curves. The resulting nominal concentration ranges in the MFSs were the following: BCTA from 20 to 100 μg kg^{-1} and 50 to 250 μg kg^{-1} in the case of rabbit samples; CSTA from 30 to 150 μg kg^{-1}; CSTB from 45 to 225 μg kg^{-1}; PMB1 from 16 to 80 μg kg^{-1} and PMB2 from 4 to 20 μg kg^{-1}. After 10 min, the MFSs were submitted to the whole analytical procedure. Quantification by using MFSs calibration provides concentration values corrected by extraction recoveries of the analytes, and at the same time potential matrix effects on MS detection are overcome.

3. Results and discussion

3.1. *LC-HRMS*

According to European Regulation 2021/808, 4 or 5 identification points (IPs) must be achieved to confirm the presence of permitted or banned substances, respectively. Our goal was to achieve 5 identification points (IPs) for all analytes, regardless of the MRL. When using HRMS, 1.5 IPs are obtained per ion, in addition to the IP obtained due to the chromatographic separation (Regulation (EU) 2021/808, 2021). Therefore, to achieve 5 IPs, at least 3 ions per analyte should be characterized.

As reported in previous works (Kumar et al., 2013), when using an Orbitrap instrument, different strategies can be applied to develop a confirmatory method for the analysis of veterinary drugs. In this sense, parallel reaction monitoring (PRM) or single ion monitoring (SIM) are among the Q-Orbitrap different working modes that could be run. Both methods are based on the selection of ions in the quadrupole and this prevents the C-trap saturation and improves mass spectrometer capabilities. These working modes differ in that the PRM get the ions through the HCD cell (and therefore they are susceptible to be fragmented) whereas the SIM sends the ions from the C-Trap directly to the Orbitrap analyzer.

Preliminary experiments to identify the main ions and to set-up the conditions for HRMS detection with the Q-Orbitrap mass spectrometer were performed by direct infusion of individual stock solutions of polypeptide antibiotics at 0.2 mg/L. Full mass spectra acquisitions were performed in the range of 100 to 1500 *m/z* under positive mode. The infusion experiments revealed that ionization mostly led to multiple-positive-charged ions, due to the protonation of the amino moieties of the polypeptides structure (Wan, Ho, Sin, & Wong, 2006). Single-charged forms of the precursor ions were not observed in the spectra. As a matter of example, the HRMS spectrum of PMB1 is shown in Fig. 1. Double, triple and quadruple-charged species were identified. In terms of intensity the order was $[M + H_3]^{3+} > [M + H_4]^{4+} > [M + H_2]^{2+}$. PMB2, CSTA and CSTB showed the same pattern, but the case of BCTA was somewhat different because the ionization process did not produce quadruple charged ions. Thus, for CSTA, CSTB, PMB1 and PMB2 multiple charged $[M + H_2]^{2+}$, $[M + H_3]^{3+}$ and $[M + H_4]^{4+}$ ions could be registered in the inclusion list of the SIM mode to select them in the quadrupole and send these target ions directly into the Orbitrap mass analyzer, providing 5.5 IPs for each compound. For BCTA only $[M + H_2]^{2+}$and $[M + H_3]^{3+}$ could be included, and thus 4 IPs would be achieved with the SIM mode.

Fragmentation studies by direct infusion of standards were carried

Table 1
Formula, molecular exact mass and monitored ions for quantification and confirmation of the analytes of the study.

Compound	Acronym	Formula	Molecular exact mass (Da)	Quantification ion (*m/z*)	Confirmation ion 1 (*m/z*)	Confirmation ion 2 (*m/z*)
Bacitracin A	BCTA	$C_{66}H_{103}N_{17}O_{16}S$	1421.7489	$[M + H_3]^{3+}$ (474.9236)	$[M + H_2]^{2+}$ (711.8855)	$[M + H_2\text{-}C_5H_{11}N]^{2+}$ (669.3367)
Colistin A	CSTA	$C_{53}H_{100}N_{16}O_{13}$	1167.7656	$[M + H_3]^{3+}$ (390.5958)	$[M + H_4]^{4+}$ (293.1987)	$[M + H_2]^{2+}$ (585.3910)
Colistin B	CSTB	$C_{52}H_{98}N_{16}O_{13}$	1154.7499	$[M + H_3]^{3+}$ (385.9239)	$[M + H_4]^{4+}$ (289.6948)	$[M + H_2]^{2+}$ (578.3833)
Polymyxin B1	PMB1	$C_{56}H_{98}N_{16}O_{13}$	1202.7499	$[M + H_3]^{3+}$ (401.9239)	$[M + H_4]^{4+}$ (301.6948)	$[M + H_2]^{2+}$ (602.3832)
Polymyxin B2	PMB2	$C_{55}H_{96}N_{16}O_{13}$	1190.7499	$[M + H_3]^{3+}$ (397.2520)	$[M + H_4]^{4+}$ (298.1908)	$[M + H_2]^{2+}$ (595.3754)

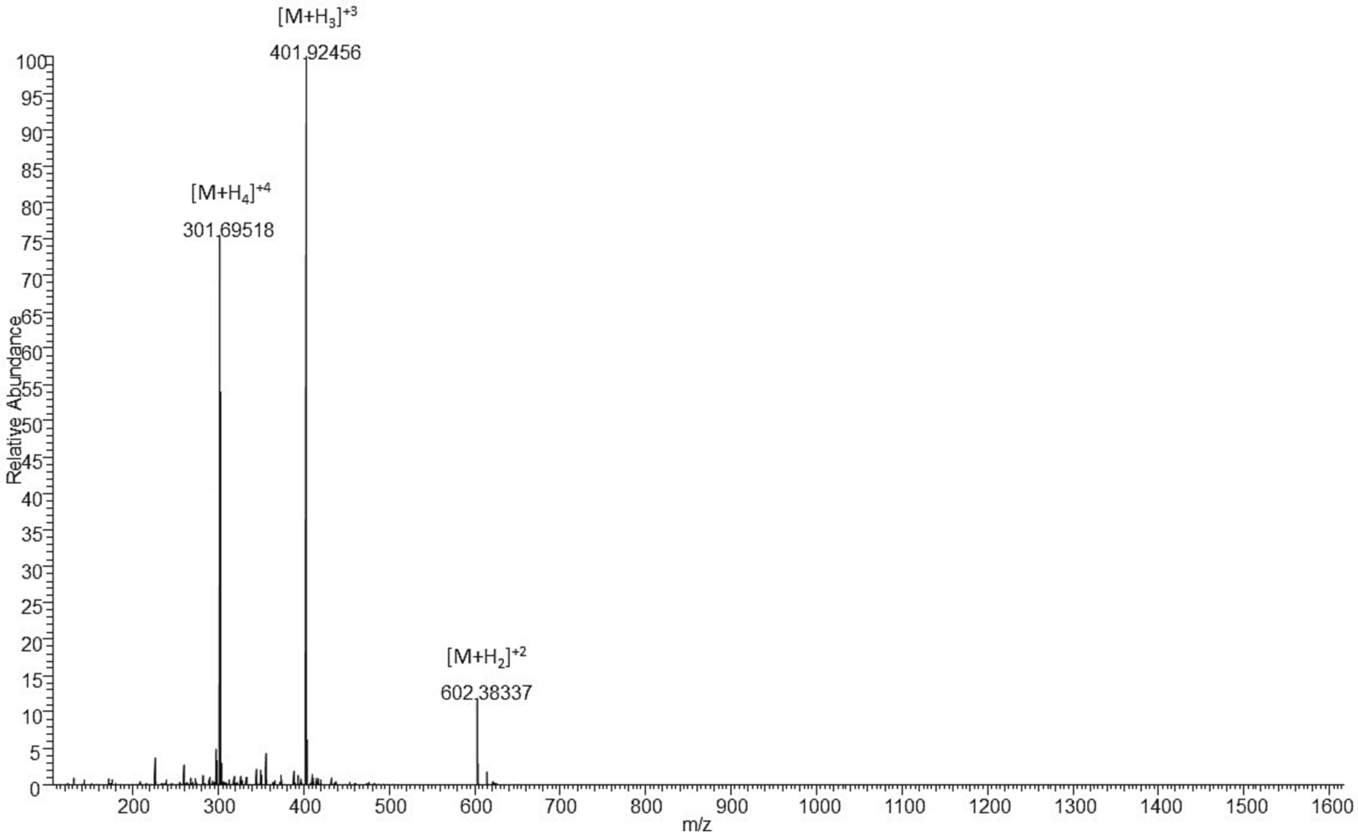

Fig. 1. Full Scan spectrum of Polymyxin B1 showing the multiple-positive-charged ions.

out to determine the optimal Normalized Collision Energy (NCE) values and to select product ions. Studies were performed at several NCE: 10, 20, 30 and 40 (au), and the following product ions were characterized: for BCTA, *m/z* 86.0964 (that corresponds to $C_5H_{12}N$), 199.0898 ($C_9H_{15}ON_2S$), 227.0850 ($C_{10}H_{15}O_2N_2S$) and the double charged 669.3367 ($C_{61}H_{94}O_{16}N_{16}S$) was obtained. For CSTA, PMB1 and PMB2

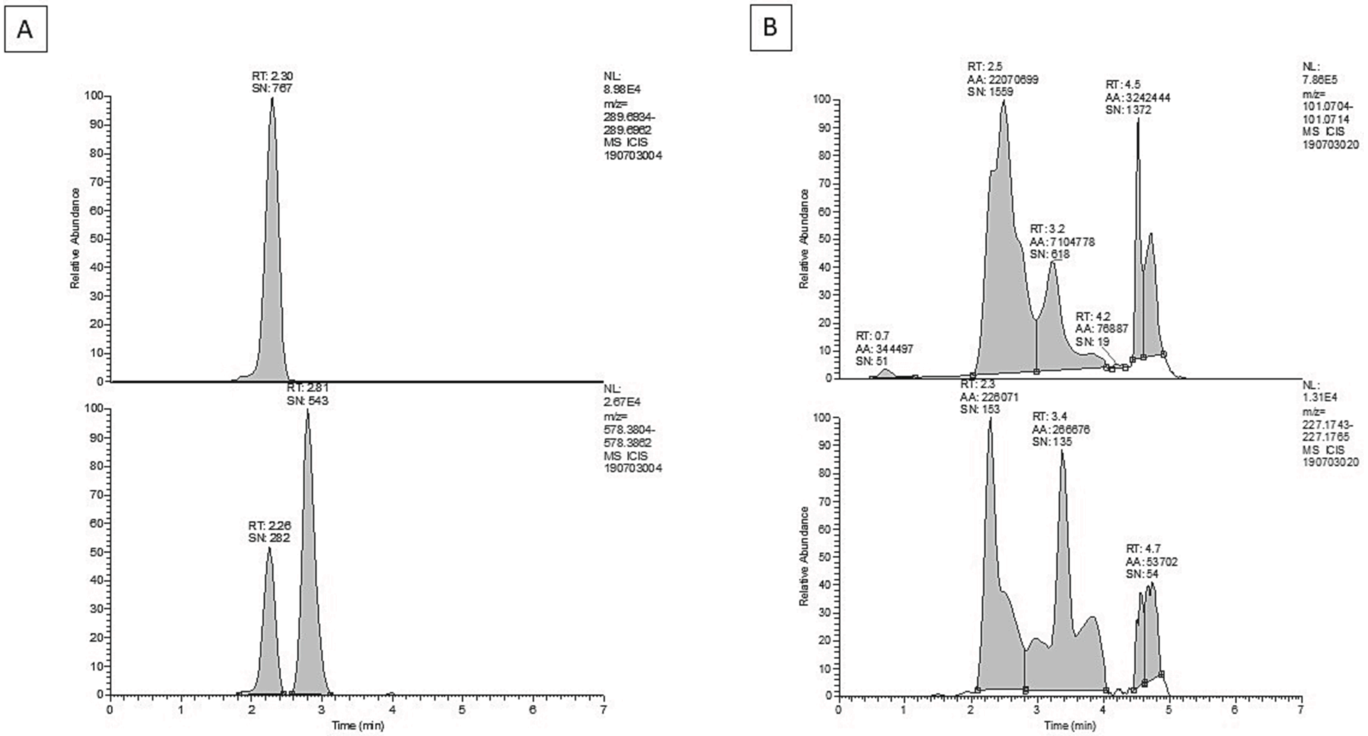

Fig. 2. Extracted chromatograms of CSTB for the injection of a spiked sample at LCL. Comparison of acquisition modes SIM (A) vs PRM (B). RT (CSTB) = 2.3 min.

common ions of *m/z* 241.1911 ($C_{13}H_{25}O_2N_2$) and 101.0709 ($C_4H_9ON_2$) were found, while for CSTB, ions of *m/z* 227.1754 ($C_{12}H_{23}O_2N_2$) and 101.0709 ($C_4H_9ON_2$) were detected.

Since fulfilling European legislation requires the detection of 3 ions, for the PRM mode it was necessary to detect precursor and product ions. For this reason, NCE was set at 10 (au).

When standards in solvent were injected in PRM mode using the optimized NCE conditions, good results in terms of sensitivity were obtained, but when injecting matrix extracts spiked with the standards at the same concentration levels, the isobaric coelutions around the target RT did not allow the confirmation with the selected fragments for compounds like CST. As a matter of example, Fig. 2 shows the case of CSTB, comparing the response of multiple charged ions acquired in SIM mode (A) vs fragment ions acquired using PRM mode (B). Trace chromatograms for product ions at *m/z* 101.0709 and 227.1754 show coeluting interferences from the matrix which prevent reliable confirmation of CSTB (MMA tolerance of 5 ppm), while in SIM mode no isobaric matrix interferences for the multiple charged ions occur. This selectivity improvement is partly due to the detection of ions of higher *m/z* values. Therefore, we concluded that for analytes eluting in the range 0–3.5 min, i.e., CSTA, CSTB, PMB1, PMB2, in the presence of matrix interferences, SIM mode is better than PRM mode, emulating the methods for dioxins and polychloro-biphenyls (PCBs) used for official controls (U. S. Environmental Protection Agency, Method 1613, 1994; Commission Regulation (EU) 2017/644, 2017.

However, the SIM mode was not suitable for BCTA, as no quadruple-charged ion of BCTA was detected and only the triple charged ion for quantification (*m/z* 474.9236) and the double charged ion for confirmation (*m/z* 711.8855) could be monitored. To fulfill European legislation, an additional confirmation ion is mandatory, so the PRM mode was used. Among the fragment ions mentioned above, the *m/z* 669.3367 was less interfered and had better S/N ratio. Fig. S2 shows the trace chromatograms for quantification (*m/z* 474.9236) and confirmation (*m/z* 711.8855 and 669.3367) ions for BCTA, free of interferences. Information on quantification and confirmation ions is summarized in Table 1.

All in all, the following strategy was set up: a) time sections were defined, first from 0 to 3.5 min and second from 3.5 to 6 min; b) the SIM working mode is used in the first time section, to detect and confirm the multiple charged ions of CSTA, CSTB, PMB1 and PMB2; c) the PRM working mode is used in the second time section for the detection and confirmation of BCTA. Finally, a total score of 5.5 IPs per analyte is achieved.

On the other hand, the Q-Orbitrap mass spectrometer can work from 17,500 to 140,000 resolution (200 *m/z*, FWHM). It should be also considered that in Orbitrap instruments resolution is inversely proportional to the ions' residence time in the analyzer, and therefore to the analysis time of each scan. A higher resolution will facilitate the separation between the analyte and possible matrix interferences, but in turn, it will decrease the number of scans per chromatographic peak. Balancing the interferences resolution and the scan rate, it was decided to use a resolution of 70,000 (obtaining a real resolution of around 50,000 at the *m/z* values of interest) as a compromise, necessary to achieve the separation of matrix compounds with very similar chemical characteristics compared to the compounds of interest. As an example, (Fig. 3), the extracted mass spectrum in the narrow mass range (1 Da) at 711 *m/z* at the retention time of BCTA of a LCL spiked porcine muscle sample obtained at 70,000 resolution is shown, where the observed ion 711.8855 *m/z* for BCTA is resolved and correctly identified and many other ions are separated. The working resolution (R) is also shown in Fig. 3, being of around 50,000 at the *m/z* of interest (Full Width at Half Maximum).

Apart from resolution, in order to optimize the analysis time of the mass analyzer, the multiplexing (MSX) parameter should be considered (Kumar, et al., 2013). MSX is used for the analysis of more than one ion in a single scan by accumulating different ions in the C-Trap, which

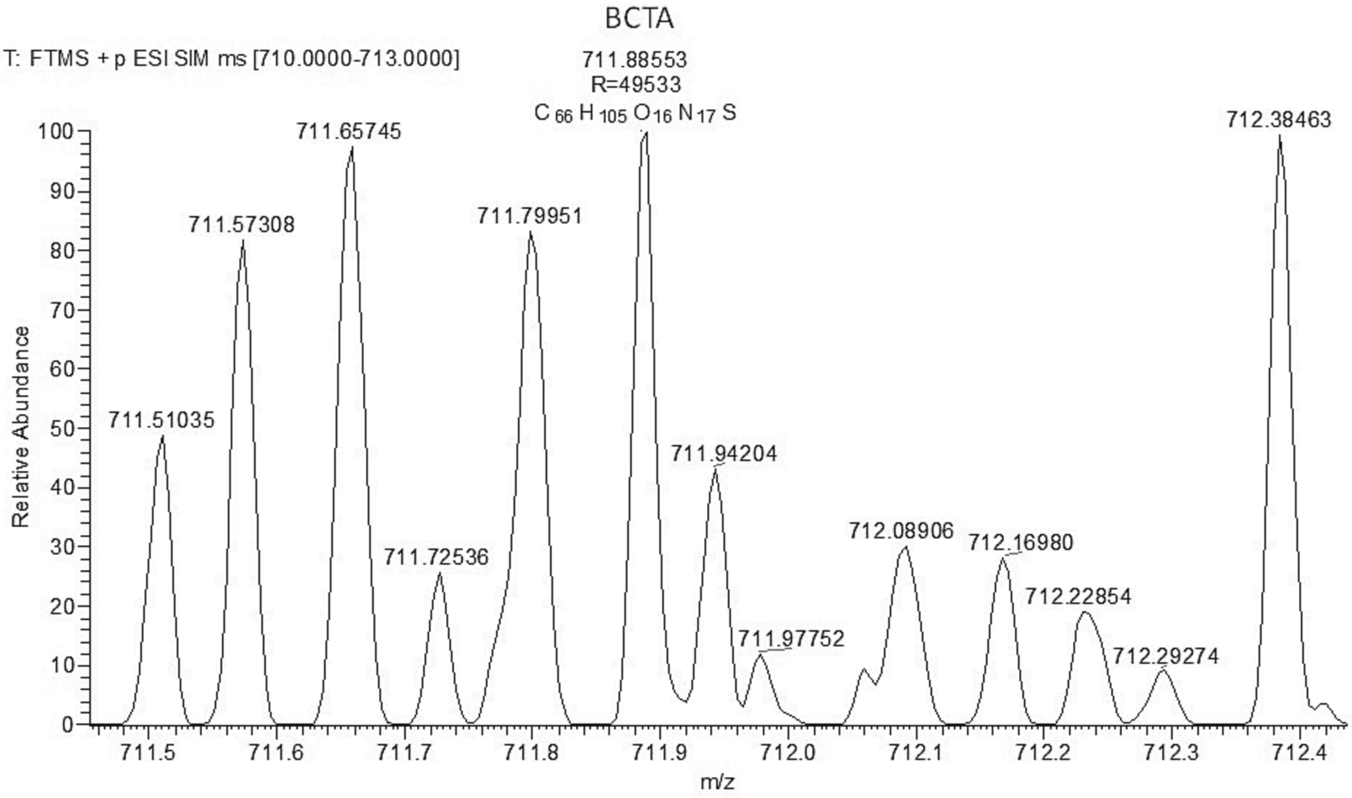

Fig. 3. Extracted mass spectrum at 711 *m/z* (at the retention time of BCTA) of a LCL spiked porcine muscle sample. The observed 711.8855 *m/z* for BCTA is correctly identified.

enables their simultaneous determination. In the study, MSX value was set at 12 (au) in the first time segment, between 0 and 3.5 min, where CSTA, CSTB, PMB1, and PMB2 elute. In the second time segment, starting at minute 3.5, MSX value was set at 3 (au) for BCTA detection.

The chromatographic separation of polypeptide antibiotics is usually performed with C18 columns and water-methanol or water-acetonitrile acidic mobile phases, to prevent the interaction of amino groups with residual silanols, which leads to tailing of chromatographic peaks. Ion-pair chromatography with mobile phases containing trifluoroacetic acid has also been described but, according to our experience, trifluoroacetic acid contaminates the system and makes necessary a thoroughly cleaning of the chromatographic equipment before running other methods, with significant instrument downtime.

In this work the performance of two stationary phases with big molecules such as the analytes was assessed. We carried out initial experiments comparing the separation obtained with a Kinetex C18 (100 x 2.1 mm x 1.7 μm) column and with a Hypersil Gold aQ C18 (100 x 2.1 mm x 3 μm) using mobile phases based on acetonitrile and water mixtures containing formic acid. Results showed that Hypersil Gold gave broader peaks for all analytes, confirming the suitability of the core–-shell technology present in the Kinetex column to achieve better peak resolution and peak shape. Finally, the Kinetex C18 column, 0.2 % formic acid in water (mobile phase A) and 0.2 % formic acid in acetonitrile (mobile phase B) were selected. Fig. 4 shows a chromatogram obtained at the final gradient conditions (detailed in section 2.4.3). CSTA, CSTB, PMB1, and PMB2 elute between 2 and 3.5 min, showing some overlapping due to the high similarity of the chemical structures of the compounds, but peaks are resolved by HRMS. On the other hand, retention time for BCTA is 4.75 min. The total time for a chromatographic run, including column re-equilibration, only takes 11 min, which is very suitable for laboratories with high sample load.

3.2. Extraction and clean-up

The organic solvents commonly used for the extraction of polypeptide antibiotics are methanol (Boison, Lee, & Matus, 2015; Xu, et al., 2012) and acetonitrile (Zhang, et al., 2015; Sin, Ho, Wong, Ho, & Ip, 2005). Each has its own pros and cons. For example, acetonitrile provides cleaner extracts, due to protein precipitation (Rúbies, Guo, Centrich, & Granados, 2016), but methanol provides higher extraction recoveries. On the other hand, when using methanol, emulsions may be formed, which makes the centrifugation and clean-up steps tedious and time-consuming. The addition of acid to the extraction solution is a common practice in residue analysis involving animal tissues. It aids liberating the analytes from the matrix and facilitates protein precipitation. It may also help to prevent the formation of emulsions. Methanol in acidic conditions was chosen as the extraction solvent to be used in this study due to its higher extraction recoveries. The results involving the use of formic acid were not very promising since turbid solutions were obtained, and the solid phase extraction step became very cumbersome, to the point that some solid phase extraction cartridges were totally blocked. Kaufmann and Widmer (2013) reported sulfuric acid as a suitable reagent for the acidification of the methanolic solution, providing less turbid extracts than other acids. We checked the effect of

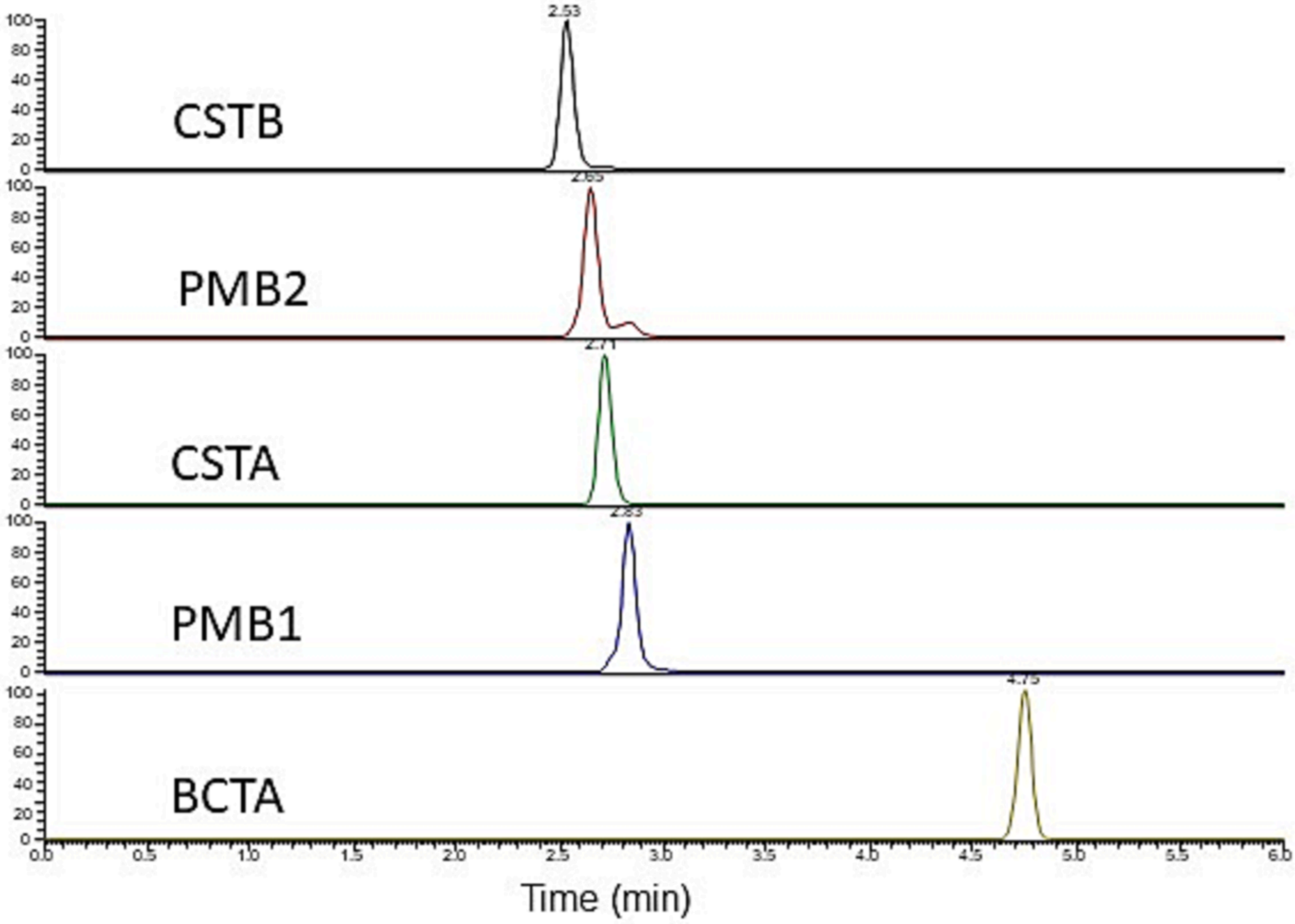

Fig. 4. Extracted ion chromatogram of $[M + H]^{3+}$ CSTB, PMB2, CSTA, PMB1 and BCTA standards in solvent using a Kinetex C18 (100 x 2.1 mm x 1.7 μm) column with optimized gradient conditions.

sulfuric acid at several concentration levels in the methanolic solution (0.1, 0.5 and 1.0 mol/L). Extracts from animal tissues, obtained with 0.1 mol/L sulfuric acid were very turbid, but turbidity decreased as acid concentration increased. However, no relevant differences were observed on the MS signals of the analytes for the different concentration levels of sulfuric acid, except for BCTA, which signal showed some decrease as the concentration of sulfuric acid increased. This fact was attributed to the instability of BCTA in acidic conditions (Kaufmann, & Widmer, 2013). Taking into consideration these findings, methanol containing 0.5 mol/L sulfuric acid was selected for the extraction procedure, as a compromise solution.

Solid-phase extraction in the reversed phase mode with polymeric materials is commonly employed for the clean-up of the extracts. However, since polypeptides exhibit physical and chemical properties which are very similar to endogenous animal tissues components, it is quite difficult to extensively isolate the analytes from the bulk of the matrix, and the chromatograms of the extracts often show peaks that elute very close, or even overlap, to those of the analytes.

We examined two types of polymeric-based SPE cartridges for the clean-up of porcine muscle extracts: OASIS HLB and Bond Elut Plexa, and the applicability of the cartridges was gauged in terms of analyte response and reproducibility of results. After sample loading, the cartridge was cleaned with water and the elution of the analytes was assayed with a mixture of methanol: water (82:18) with 0.2 % of formic acid solution. The performance of both cartridges was quite similar, except for BCTA which gave higher MS response with Bond ElutPlexa. On the other hand, no significant differences were observed in terms of reproducibility. Bond Elut Plexa cartridges were chosen, and the detailed procedure for the SPE step is described in extraction section.

Mean extraction absolute recoveries of the whole process, including the clean-up step, were: BCTA 92 %, CSTA 47 %, CST B 53 %, PMB1 87 % and PMB2 81 %. The matrix effects on the MS signals were investigated by comparing signals from solutions containing the analytes dissolved in solvent with those of analytes dissolved in matrix extracts. All analytes were affected by signal suppression, and therefore calibration with matrix-fortified standards is mandatory to obtain reliable quantification results. In particular, the MFSs approach overcomes matrix effects, and additionally the concentration values are corrected by extraction recoveries.

3.3. Validation

Porcine muscle was selected as the main matrix in the validation process because it is one of the most analyzed matrices in routine work in our laboratory. Blank samples from cattle, sheep and rabbits were also included in selectivity and linearity experiments (sections 3.3.1 and 3.3.3, respectively).

The validation was based on the performance criteria and other requirements of analytical methods for residues of pharmacologically active substances according to Regulation 2021/808 (Regulation (EU) 2021/808, 2021). In the design of the validation tests, it was considered that in the EU the MRL for CST in muscle is 150 μg kg^{-1} for all food-producing species, while for BCT the MRL in muscle is defined only for rabbit. (150 μg kg^{-1}), and there are no defined MRLs for PMB.

3.3.1. Selectivity

The selectivity of the method was investigated by analyzing muscle blank samples from beef, lamb, pork and rabbit. According to the resulting chromatograms, no interferences were observed at the retention time of the analytes considering a mass error lower than 5 ppm. As shown in Fig. 3, these results highlighted the crucial role of HRMS detection, which allows to resolve possible isobaric interferences that coelute with the analytes, unlike low resolution mass spectrometers (triple quadrupole), which do not offer sufficient selectivity to overcome this drawback.

3.3.2. Lowest calibration level (LCL)

The lowest calibration levels (LCL) were experimentally determined from spiked porcine muscle blank samples. Thus, a study was performed to assess the lowest concentration of each analyte that can be measured fulfilling the identification and confirmation criteria, i.e. 20 % permitted tolerance for relative ion intensities between confirmation and quantification ions. Also, a second criterion was applied: deviation to the relative response factor calculated from the calibration curve must be lower than 20 % (Wenzl, Haedrich, Schaechtele, Robouch, & Stroka, 2016).

The LCL were: BCTA 20 μg kg^{-1}, CSTA 30 μg kg^{-1}, CSTB 45 μg kg^{-1}, PMB1 16 μg kg^{-1}, PMB2 4 μg kg^{-1}. All LCL were included in the range (0,1–0.5) x MRL, as defined in the 808/2021 Regulation. BCTA trace chromatograms of a porcine sample spiked with BCTA at 20 μg kg^{-1} are shown in Fig. S2.

3.3.3. Linearity

The linearity of the method was evaluated based on the calibration with MFSs at five concentration levels starting at the LCL. The linearity ranges for each compound are shown in Table 2. Good results were achieved, with correlation coefficients higher than 0.99. In addition, % RSD for response factors in the calibration curve were evaluated and found below 20 % for all analytes.

On the other hand, blank samples of bovine, ovine and rabbit muscle were spiked in triplicate (BCTA 20 μg kg^{-1}, CSTA 30 μg kg^{-1}, CSTB 45 μg kg^{-1}, PMB1 16 μg kg^{-1}, PMB2 4 μg kg^{-1}) and analyzed. Bovine and ovine samples were correctly quantified with a calibration curve built with MFSs made from blank porcine samples, which indicates equivalence of matrices. For all the matrices and analytes the ion ratio criterion for confirmatory purposes was fulfilled, with variation of the ion ratios in the 1–14 % range. Thus, since these matrices show an equivalent behavior, they can be analyzed by using a single matrix-fortified calibration, which is very convenient. However, for rabbit samples the quantification with the calibration curve built with MFSs from pork muscle was not successful, which points out that the analysis of rabbit samples requires calibration with MFSs from rabbit muscle.

3.3.4. Precision

Considering the MRL for CST and the LCL for BCT and PMB, three concentration levels were defined for each compound in the precision study, values ranging from 4 to 150 μg kg^{-1} (Table 2). For each validation level, six replicates on three separate days were carried out. Repeatability (within-day precision), was determined from experiments ($n = 6$) carried out in the same day and repeated three times, whereas reproducibility (between day precision) was calculated from data ($n = 18$) obtained in three different working days. It can be noticed that within-day and between-day precision values, expressed in terms of % RSD, are quite similar (Table 2). The calculated %RSD ranged from 5.2 % to 16.8 %, and did not exceed the value calculated by the Horwitz equation; thus the criterion of 2021/808/EU Regulation for precision was fulfilled.

3.3.5. Trueness

Since there are no available certified reference materials, trueness was assessed by determining the recovery of blank porcine muscle samples spiked at three concentration levels. Experiments were done in different days, until 18 replicates were obtained. Results for trueness, were within 100 ± 10 % (Table 2), and thus the criterion set 2021/808/EU for confirmatory methods was fulfilled.

3.3.6. Decision limit (CCα)

Regarding the CCα calculation, different situations arise, depending on the legislated MRL. As expressed in Table S1 (Supplementary material), CST has a legislated MRL for all food-producing species, BCT has MRL only for bovine milk and rabbit, and PMB lacks MRL.

In 2021/808/EU Regulation, different approaches for CCα

Table 2
Quality parameters obtained during the validation of the method (muscle).

Compound	Linearity range (µg kg^{-1})	Concentration level (µg kg^{-1})	Precision (RSD%) (within day/between days)	Trueness (%)	CCα (µg kg^{-1})	CCβ (µg kg^{-1})	Extended Uncertainty (%)	LCL (µg kg^{-1})
Bacitracin A	20–100	20	14.0/16.8	98	5	*7*	33	*20*
		60	13.4/13.5	100				
		90	12.1/15.6	102				
Bacitracin A (rabbit)	50–250	150	12.4/12.7	90.3	178	205	25	50
Colistin A	30–150	30	5.2/7.8	96	170	189	21	30
		90	7.0/10.7	99				
		150	7.0/6.8	106				
Colistin B	45–225	45	11.0/15.2	94	157	164	30	45
		60	6.2/9.7	99				
		135	9.7/9.3	100				
Polymyxin B1	16–80	16	9.4/13.2	93	5	8	27	16
		30	7.7/9.5	104				
		50	6.2/7.0	106				
Polymyxin B2	4–20	4	8.7/12.2	95	2	3	24	4
		6	7.0/7.1	103				
		12	7.1/7.5	102				

calculation are described. For compounds with an established MRL, CCα can be calculated analyzing blank samples fortified with the analyte at the MRL level, and CCα is determined as the MRL plus k times the standard uncertainty. Gauss distribution (one sided, 95 %) or *t*-distribution (one-sided, 95 %, and the corresponding degrees of freedom) can be applied for k value. We applied this approach for CST (pork muscle) and for BCT (rabbit muscle). Results are shown in Table 2.

When there is no MRL established, 2021/808/EU regulation also proposes different approaches to determine CCα. We applied the calibration curve procedure, which is based on ISO 11843 standard regulation (ISO 11843). Thus, for Bacitracin and Polymixin B calibration curves with MFSs (pork muscle) were built at low concentration levels, and the CCα values were determined from the corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the intercept. Results, shown in Table 2, indicate that the method is suitable for residue analysis, i.e. it is able to detect and confirm low concentrations of the analytes.

3.3.7. Measurement of uncertainty

According to SANCO/2004/2726/rev 4-December 2008 (SANCO/2004/2726, 2008), the combined measurement uncertainty (u_c) of a method can be adequately estimated by within-laboratory reproducibility values. Subsequently, the extended uncertainty (U) can be calculated by multiplying a coverage factor, k, to the combined measurement uncertainty. For most applications, a value of $k = 2$ is used, representing an interval with a confidence level of approximately 95 %. Table 2 lists down for each analyte the extended uncertainty values obtained from the validation study at the different validated concentrations using $k = 2$.

3.4. Analysis of muscle samples

The new method has been successfully applied to more than 300 samples from bovine, porcine ovine and rabbit species. All analyzed samples were compliant with EU regulation. Very few samples contained detectable levels of either bacitracin or colistin, and in every case, the levels were below the established MRLs. As an example, Fig. S3 shows extracted ion chromatograms of a porcine muscle sample containing colistin. Currently, this method is used in the laboratory for routine analysis of polypeptide antibiotics.

4. Conclusions

A reliable analytical method has been proposed for the determination of polypeptide antibiotic residues in animal muscle tissue. The HRMS-SIM acquisition mode provides proper sensitivity and selectivity for polymyxins, while the HRMS-PRM mode is used for bacitracin. Using multiple-charged ions, analytes can be successfully confirmed with mass errors below 5 ppm. The method avoids drawbacks such as matrix interferences in MS detection, while overcoming sensitivity limitations.

The suitability of the method has been assessed in daily laboratory work with the analysis of large numbers of samples, and no additional maintenance of the mass spectrometer was required. The new method has been applied to more than 300 official control muscle samples in the period 2017–2022. Bacitracin and colistin have been detected in very few samples, always below MRL.

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CRediT authorship contribution statement

Antoni Rúbies: Conceptualization, Writing – review & editing. **Iñaki Beguiristain:** Formal analysis, Writing – original draft. **Jojo Tibon:** Investigation, Validation. **Nuria Cortés-Francisco:** Methodology, Writing – review & editing. **Mercè Granados:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138481.

References

Boison, J. O., Lee, S., & Matus, J. (2015). A multi-residue method for the determination of seven polypeptide drug residues in chicken muscle tissues by LC-MS/MS. *Analytical and Bioanalytical Chemistry, 407*(14), 4065–4078. https://doi.org/10.1007/s00216-015-8644-z

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32010R0037 (accessed November 10, 2022).

COMMISSION REGULATION (EU) 2017/644 of 5 April 2017 laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32017R0644&from=PL (accessed November 10, 2022).

Eliuk, S., & Makarov, A. (2015). Evolution of Orbitrap Mass Spectrometry Instrumentation. *Annual Review of Analytical Chemistry, 8*, 61–80. https://doi.org/10.1146/annurev-anchem-071114-040325

Fu, Q., Li, X., Zheng, K., Ke, Y., Wang, Y., Wang, L., Yu, F., & Xia, X. (2018). Determination of colistin in animal tissues, egg, milk, and feed by ultra-high performance liquid chromatography-tandem mass spectrometry. *Food Chemistry, 248*, 166–172. https://doi.org/10.1016/j.foodchem.2017.12.029

Gavage, M., Delahaut, P., & Gillard, N. (2021). Suitability of High-Resolution Mass Spectrometry for Routine Analysis of Small Molecules in Food, Feed and Water for Safety and Authenticity Purposes: A Review. *Foods, 10*, 601. https://doi.org/10.3390/foods10030601

Grund, B., Marvin, L., & Rochat, B. (2016). Quantitative performance of a quadrupole-orbitrap-MS in targeted LC-MS determinations of small molecules. *Journal of Pharmaceutical and Biomedical Analysis, 124*, 48–56. https://doi.org/10.1016/j.jpba.2016.02.025

ISO 11843. Capability of Detection, Part 1: Terms and Definitions; Part 2: methodology in the linear calibration case; Part 5: Methodology in the linear and non-linear calibration cases. https://www.iso.org/obp/ui/#iso:std:iso:11843:-1:ed-1:v1:en; https://www.iso.org/obp/ui/#iso:std:iso:11843:-2:ed-1:v1:en; https://www.iso.org/obp/ui/#iso:std:iso:11843:-5:ed-1:v1:en (accessed November 10, 2022).

Kaufmann, A. (2020). High-resolution mass spectrometry for bioanalytical applications: Is this the new gold standard?. Article e4533-n/a *Journal of Mass Spectrometry, 55*(9). https://doi.org/10.1002/jms.4533.

Kaufmann, A., & Widmer, M. (2013). Quantitative analysis of polypeptide antibiotic residues in a variety of food matrices by liquid chromatography coupled to tandem mass spectrometry. *Analytica Chimica Acta, 797*, 81–88. https://doi.org/10.1016/j.aca.2013.08.032

Kumar, P., Rúbies, A., Centrich, F., Granados, M., Cortés-Francisco, N., Caixach, J., & Companyó, R. (2013). Targeted analysis with benchtop quadrupole-orbitrap hybrid mass spectrometer: Application to determination of synthetic hormones in animal urine. *Analytica Chimica Acta, 780*, 65–73. https://doi.org/10.1016/j.aca.2013.04.017

Kumar, H., Kumar, D., Nepovimova, E., Oulkar, D., Kumar, A., Azad, R. M. R., Budakoti, S. K., Upadhyay, N. K., Verma, R., & Kuča, K. (2021). Determination of Colistin B in Chicken Muscle and Egg Using Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *International Journal of Environmental Research and Public Health, 18*, 2651. https://doi.org/10.3390/ijerph18052651

Lee, S. C., Matus, J. L., Gedir, R. G., & Boison, J. O. (2011). A validated LC-MS method for the determination of bacitracin drug residues in edible pork tissues with confirmation by LC-tandem mass spectrometry. *Journal of Liquid Chromatography and Related Technologies, 34*(20), 2699–2722. https://doi.org/10.1080/10826076.2011.593224

Liu, T., Zhang, C., Zhang, F., Nie, B., Yuan, F., Huang, H., & Li, H. (2019). Sensitive Determination of Four Polypeptide Antibiotic Residues in Milk Powder by High Performance Liquid Chromatography-Electrospray Tandem Mass Spectrometry. *Chromatographia, 82*(10), 1479–1487. https://doi.org/10.1007/s10337-019-03777-y

Nang, S. C., Azad, M. A. K., Velkov, T., Zhou, Q., & Li, J. (2021). Rescuing the Last-Line Polymyxins: Achievements and Challenges. *Pharmacological reviews., 73*, 679–728. https://doi.org/10.1124/pharmrev.120.000020

Regulation (EC) No. 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32009R0470 (accessed November 10, 2022).

Regulation (EU) 2017/625 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32017R0625 (accessed November 10, 2022).

Regulation (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32021R0808 (accessed November 10, 2022).

Rúbies, A., Guo, L., Centrich, F., & Granados, M. (2016). Analysis of non-steroidal anti-inflammatory drugs in milk using QuEChERS and liquid chromatography coupled to mass spectrometry: Triple quadrupole versus Q-Orbitrap mass analyzers. *Analytical and Bioanalytical Chemistry, 408*, 5769–5778. https://doi.org/10.1007/s00216-016-9679-5

Saluti, G., Diamanti, I., Giusepponi, D., Pucciarini, L., Rossi, R., Moretti, S., Sardella, R., & Galarini, R. (2018). Simultaneous determination of aminoglycosides and colistins in food. *Food Chemistry, 266*, 9–16. https://doi.org/10.1016/j.foodchem.2018.05.113

SANCO/2004/2726-rev 4-December 2008, Guidelines for the implementation of Decision 2002/657/EC, https://ec.europa.eu/food/system/files/2016-10/cs_vet-med-residues_cons_2004-2726rev4_en.pdf (accessed November 10, 2022).

Satlin, M. J., & Jenkins, S. G. (2017). Polymyxins. In J. Cohen, W. G. Powderly, & S. M. Opal (Eds.), *Infectious diseases* (4th ed., pp. 1285–1288). Elsevier.

Sin, D. W. M., Ho, C., Wong, Y. C., Ho, S. K., & Ip, A. C. B. (2005). Analysis of major components of residual bacitracin and colistin in food samples by liquid chromatography tandem mass spectrometry. *Analytica Chimica Acta, 535*(1–2), 23–31. https://doi.org/10.1016/j.aca.2004.11.063

Sin, D. W. M., & Wong, Y. C. (2003). Analytical methodologies for identifying a polypeptide antibiotic. *TrAC - Trends in Analytical Chemistry, 22*, 799–809. https://doi.org/10.1016/S0165-9936(03)01204-4

Shaheen, M., Li, J., Ross, A. C., Vederas, J. C., & Jensen, S. E. (2011). Paenibacillus polymyxa PKB1 produces variants of polymyxin B-type antibiotics. *Chemistry and Biology, 18*(12), 1640–1648. https://doi.org/10.1016/j.chembiol.2011.09.017

Song, X., Turiel, E., Yang, J., Martín-Esteban, A., & He, L. (2022). Determination of polypeptide antibiotics in animal tissues using liquid chromatography tandem mass spectrometry based on in-line molecularly imprinted solid-phase extraction. *Journal of Chromatography. A, 1673*, Article 463192. https://doi.org/10.1016/j.chroma.2022.463192

U.S. Environmental Protection Agency. (1994). Method 1613. Tetra- through octa-chlorinated dioxins and furans by isotope dilution HRGC/HRMS. https://well-labs.com/docs/epa_method_1613b_1994.pdf (accessed November 10, 2022).

Wan, E. C. H., Ho, C., Sin, D. W. M., & Wong, Y. C. (2006). Detection of residual bacitracin A, colistin A, and colistin B in milk and animal tissues by liquid chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry, 385*(1), 181–188. https://doi.org/10.1007/s00216-006-0325-5

Wenzl, T., Haedrich, J., Schaechtele, A., Robouch, P., & Stroka, J. (2016). Guidance document on the estimation of LOD and LOQ for measurements in the field of contaminants in feed and food. *Publications Office of the European Union*. https://doi.org/10.2787/8931

Wu, I. L., Turnipseed, S. B., Andersen, W. C., & Madson, M. R. (2020). Analysis of peptide antibiotic residues in milk using liquid chromatography-high resolution mass spectrometry (LC-HRMS). *Food Additives and Contaminants - Part A, 37*, 1264–1278. https://doi.org/10.1080/19440049.2020.1766703

Xu, Y., Tian, X., Ren, C., Huang, H., Zhang, X., Gong, X., Liu, H., Yu, Z., & Zhang, L. (2012). Analysis of colistin A and B in fishery products by ultra performance liquid chromatography with positive electrospray ionization tandem mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 899*, 14–20. https://doi.org/10.1016/j.jchromb.2012.04.028

Yan, X. T., Zhang, Y., Zhou, Y., Li, G. H., & Feng, X. S. (2022). Technical Overview of Orbitrap High Resolution Mass Spectrometry and Its Application to the Detection of Small Molecules in Food (Update Since 2012). *Critical Reviews in Analytical Chemistry, 52*, 593–626. https://doi.org/10.1080/10408347.2020.1815168

Zhang, D., Park, J. A., Kim, D. S., Kim, N. H., Kim, S. K., Cho, K. S., Jeong, D., Shim, J. H., El-Aty, A. M. A., & Shin, H. C. (2015). Simultaneous detection of bacitracin and polymyxin B in livestock products using liquid chromatography with tandem mass spectrometry. *Journal of Separation Science, 38*(14), 2371–2380. https://doi.org/10.1002/jssc.201500282