Evaluation of hydrophilic interaction liquid chromatography–tandem mass spectrometry and extraction with molecularly imprinted polymers for determination of aminoglycosides in milk and milk-based functional foods

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A R T I C L E   I N F O

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HILIC
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A B S T R A C T

An analytical method for the determination of eleven aminoglycosides in different types of milk and milk-based functional products has been optimized and validated. A hydrophilic interaction chromatography (HILIC) column was proposed for the separation of analytes by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). A commercially molecularly imprinted polymer has been used for the solid phase extraction of the analytes, in order to achieve high selectivity in the sample treatment. The proposed method was characterized for different types of milk (whole cow milk, skimmed cow milk, whole goat milk) and functional dairy products, such as follow-on milk, Omega 3-enriched milk and isoflavones-enriched milk. Matrix effect was studied in the different matrices, being lower than 15% in all cases, showing that the proposed procedure provided very clean extracts. Limits of quantification in the range 4.2–49 µg kg\(^{-1}\) were estimated, well below the maximum residue limits established by the European Union. Recoveries ranged from 70% to 106% with RSD lower than 13%, in compliance with the current legislation. The combination of HILIC to solve the difficulties of the separation of these very polar compounds in reverse phase with the use of MIPs for sample treatment and MS/MS detection provided a very sensitive, highly selective, robust and useful method for identification and quantification of aminoglycoside residues in different types of milk and milk-based products.

1. Introduction

It is well known that aminoglycosides (AGs) are classes of antibiotics widely used in veterinary practice to treat bacterial infections of animals in livestock farming and bovine milk production [1]. However, the misuse of antibiotics in husbandry practices can generate the presence of residues of these compounds in foods of animal origin, such as milk. In general, nephrotoxicity, ototoxicity, and neuromuscular blockade are the main toxic effects in the human being [2]. More specifically, streptomycin (STP) and gentamicin (GNT) are primarily vestibulotoxic, causing dizziness, ataxia, and/or nystagmus [3]. On the other hand, amikacin (AMK), neomycin (NEO), dicydolactamcytin (DHS) and kanamycin (KNY) are primarily cochleotoxic, causing permanent hearing loss [4]. As a consequence, several organizations have invested resources to address these negative effects of the presence of AGs residues in food through of awareness campaigns [5,6]. Moreover, bearing in mind this matter of concern and in order to protect consumer health, the European Union (EU) has controlled their use in veterinary medicine thought the council regulation 470/2009/EC [7]. In addition, the EU has set maximum residue limits (MRLs) in milk between 100 and 200 µg kg\(^{-1}\) for these compounds [8]. Thus, the development of very sensitive methods to determine AGs in foods of animal origin at trace levels is mandatory.

Several methods have been proposed for the determination of these antibiotics using spectrophotometric, immunochemical, microbiological and chromatographic techniques [1,9]. Among them, liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI) has been selected as the better choice to obtain an unambiguous identification and quantification of AGs in a wide range of samples [10–13]. However, these compounds have a high polarity due to the presence of some amino and hydroxyl groups in their structure [14]. So, this polarity is a drawback for their analysis by LC,
as they are scarcely retained in reverse-phase columns. This poor retention could be overcome by the use of ion-pair reagent (such as trifluoroacetic acid, heptafluorobutyric acid or pentafluoropropanionic acid) in the mobile phase [11,13,15]. Nevertheless, these mobile phase additives can affect the performance of MS such as suppression of analytes and contamination of the ion source. Another encouraging recent development has been the use of hydrophilic interaction chromatography (HILIC) for the analysis of these compounds coupled to MS [10,12,14,16,17]. The main advantage of this approach is that polar compounds show good solubility in the aqueous mobile phase used in HILIC, which overcomes the drawbacks of the poor solubility often encountered in reverse-phase chromatography [18]. Other key point is the use of high organic content mobile phase, which offer readily compatibility with MS. Taking into account these characteristics, HILIC methodology could be an attractive alternative to the widely used reverse-phase chromatographic separations for the analysis of AGs and other antibiotics [19].

Another critical challenge in the monitoring of trace-level AGs in highly complicated matrices, such as milk, is the extraction and clean-up procedure. Different strategies, such as liquid extraction [11], solid phase extraction (SPE) [10,13,17,20–22], online SPE [23,24], disposable pipette extraction [25] and matrix solid phase dispersion [26] have been employed to AG extraction and clean-up. Generally, in this purification step particular attention is paid on the recovery efficiency. However, another relevant issue, when ESI-MS is used, is the matrix effect (ME). The presence of co-eluting interfering species may cause signal enhancement or suppression of the analytes of interest [27–29]. These MEs hamper the accuracy of the results, reducing laboratory throughput [30]. To overcome, minimize or compensate ME during quantitative analytical LC–MS measurements several strategies could be carried out [31], such as the application of specific clean-up protocols. In this sense, molecularly imprinted polymers (MIPs) used as sorbent in solid phase extraction (MISPE) can provide cleaner extracts because the strong and selective interaction between MIPs and target molecules, being of special interest for complex matrices. MIPs are synthetic materials with artificially produced recognition sites capable of specifically catch target molecules [32]. So, several matrix components are removed from the final extract, reducing the ME. This methodology has been successfully applied for the determination of AGs in honey samples, achieving excellent results in terms of recovery and ME [33,34].

The aim of this study was to develop a fast multi-residue method for routine analysis of 11 AG residues in different types of milk (whole cow pasteurized milk, skimmed cow pasteurized milk, whole goat pasteurized milk and milk-based functional foods, such as follow-on milk (for children 6–12 months), omega 3-enriched milk and isoflavones-enriched milk, using a recently commercially available MISPE. The use of this highly selective extraction sorbent combined with HILIC–based UHPLC–MS/MS allowed to obtain outstanding results in terms of sensitivity, selectivity, ME, precision and trueness.

2. Materials and methods

2.1. Reagents and materials

Due to the high absorption affinity of the AGs to polar surfaces and their high photosensitivity, polypropylene amber vessels (flasks, glass and vials) were used during sample preparation, storage, and injection. Ultrapure water (Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout the work. Methanol (MeOH), acetonitrile (MeCN) and n-hexane (LC-MS HiPerSolv grade) were supplied by VWR (Radnor, PA, USA). Formic acid (LC-MS grade, 99%) and heptafluorobutyric acid (HFBA, > 99.5%) were obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonium hydroxide (30%), potassium hydroxide (85%) and dichloromethane (stabilized with 20 ppm of amylene) were obtained from Panreac-Quimica (Barcelona, Spain). Potassium dihydrogen phosphate (99%) was purchased from Alfa Aesar (Haverhill, USA). Ammonium acetate (98%) and trichloroacetic acid (TCA) (99.5%) were supplied by Merck (Darmstadt, Germany). Vetranal grade analytical standards of Gentamicin sulfate (GENT, 63.9%), which was a mixture of GENT C1, GENT Clα and GENT C2C2a, Apramycin sulfate salt (APM, 95%), Paromomycin sulfate salt (PRM, 74%), Dihydrostreptomycin sesquisulfate (DHS, 98%), Spectinomycin dihydrochloride pentahydrate (SPC, 60.3%), Kanamycin acid salt (KAM, 68.9%), Amikacin sulfate salt (AM, 74%), Tobramycin sulfate salt (TOM, > 99.9%) and Streptomycin sulfate salt (STP, > 99.9%) were supplied by Fluka Analytical (Steinheim, Germany). Individual stock standard solutions of 3 g L⁻¹ were prepared by dissolving accurately weighed amounts in water and stored in the dark at 4 °C. They were stable for at least 2 months. Standard solutions containing all the AGs were freshly prepared by proper dilution of the stock standard solutions with MeCN:H₂O (25:75; v/v). These solutions were stored in plastic tubes at 2–4 °C and remained stable for up to 1 week.

MISPE cartridges (SupelMIP AGs SPE Column, 50 mg, 3 mL) supplied by Supelco (Bellefonte, PA, USA) were used for extraction and clean-up process. Nylon syringe filters, 0.22 mm×13 mm (Agela Technologies, New York, USA) were used for filtration of the sample extracts before injection into the UHPLC–MS/MS system.

To prepare 50 mM potassium phosphate buffer pH 7.0, a proper amount of potassium dihydrogen phosphate was weighed and diluted with water; then the pH was adjusted to 7.0 with 50 mM potassium hydroxide.

2.2. Instrumentation

Separation was performed on an Agilent 1290 Infinity LC using a Kinetex HILIC column (100×2.1 mm, 1.7 µm) supplied by Phenomenex (Torrance, CA, USA). The mass-spectrometer measurements were performed on a triple quadrupole (QqQ) mass spectrometer API 3200 (AB Sciex, Darmstadt, Germany) with electrospray ionization (ESI). The instrumental data were collected using the Analysts Software version 1.5 with Schedule MRMTM Algorithm (AB Sciex).

MISPE was carried out on a Visiprep™ DL vacuum manifold (Supelco) for 12 cartridges. A centrifuge (Universal 320 model from Hettich, Leipzig, Germany), a vortex (Genie 2 model from Scientific Industries, Bohemia, NY, USA) and a pH-meter (Crison model pH 2000; Barcelona, Spain) with a resolution of ± 0.01 pH unit were also during the sample preparation procedure.

2.3. UHPLC–MS/MS analysis

Separation was performed in a HILIC column using a mobile phase consisting of 150 mM ammonium acetate containing 1% (v/v) formic acid (solvent A), and MeCN (solvent B) at a flow rate of 0.5 mL min⁻¹. The eluent gradient profile was as follow: 80% B at the beginning; 30% B at 2 min (held for 2 min); 5% B at 5 min (held for 5 min) and finally go back to the initial conditions at 12 min (held for 6 min). The temperature of the column was 35 °C and the injection volume was 20 μL.

The mass-spectrometer was working with ESI in positive mode under the multiple reaction monitoring (MRM) conditions shown in Table 1. The ionization source parameters were: dry gas temperature, 700 °C; curtain gas (nitrogen), 30 psi (207 kPa); ion spray voltage, 4000 V; collision gas, 5 and dry gas pressure (GS 1 and GS 2, both of them N₂) 50 psi (345 kPa).

2.4. Sample treatment procedure

A portion of 2g of milk (obtained from a local store), free of AGs was spiked at different concentration levels using the working standard solutions of AGs. After spiking and homogenizing in vortex, 250 μL of TCA (15%, m/v) were added for protein precipitation. Then the
mixture was homogenized by vortex and centrifuged at 9072 g for 5 min. The aqueous phase was collected and transferred to a falcon tube. Subsequently, 1 mL of n-hexane was added to remove the fatty components of the sample. The mixture was shaken during 1 min and centrifuged at 9072 g for 5 min. The pH of the final solution was checked to be 7.0 and adjusted with 50 mM potassium hydroxide, if necessary. Then, the final volume was adjusted to 5 mL with 50 mM potassium phosphate buffer pH 7.0. A 3 mL aliquot of this solution was loaded onto a SupelMIP AG SPE column (previously conditioned with 1 mL of MeOH and 1 mL of 50 mM potassium phosphate buffer at pH 7.0) at a flow rate of approximately 0.2 mL min⁻¹. After sample loading, the cartridge was washed with 3 mL of water at a flow rate lower than 0.5 mL min⁻¹. Subsequently, strong vacuum was applied for 5 min. Then, the MISPE cartridge was washed again with 1 mL of a mixture of dichloromethane: MeOH (50:50, v/v), After this washing step, a slight vacuum was applied for 10 s. Finally, the elution of the analytes was achieved using 1 mL of 1% (v/v) formic acid in MeCN:H₂O (20:80, v/v) with 5 mM HFBPA. Finally, 2 mL of MeCN:H₂O (20:80, v/v) were added to this final extract in order to make it compatible with the HILIC conditions. This extract was filtered and injected in the UHPLC-MS/MS system.

3. Results and discussion

3.1. Optimization of chromatographic separation and MS/MS detection

For the analysis of the target antibiotics by UHPLC-MS/MS, an MRM method was developed. Individual optimization for each AG (0.5 mg L⁻¹ in 0.1% (v/v) aqueous formic acid solution: MeCN (50:50, v/v)) was conducted using an external syringe pump connected to the mass spectrometer. During analyte infusions, Declustering Potential (DP), Entrance Potential (EP), Collision Cell Entrance Potential (CEP), Collision Gas (CAD), Collision Energy (CE) and Collision Exit Potential (CXP) of the two most abundant transitions were also optimized. The detailed optimized parameters and MRM transitions are shown in Table 1. Protonated molecular ions [M+H]+ were found for most of the studied compounds in ESI positive mode and were selected as precursor ions [35]. Only in the case of SPC and STP, the highest peak corresponded to the water adduct [M+H+H₂O]+, probably due to the unusual structural feature of these compounds, in which the carbonyl group is hydrated in an aqueous solution [36]. Two product ion transitions were set up (Table 1), the most intense one was used as quantification ion (Q) and the following was considered as confirmation ion (I). The MS/MS experiments were performed in scheduled MRM mode, with a target scan time for each MRM transition of 0.2 s, which provided 15 data points per peak.

As stated in the introduction, the use of an HILIC column is mandatory in order to increase chromatographic retention of polar antibiotics and to achieve higher MS sensitivity. Kinetex HILIC column based on an un-bonded silica phase was selected, as it can provide satisfactory results in terms of relative retention factor, selectivity and peak shape, according to Kumar et al. [20]. First of all, the mobile phase composition was evaluated. This step plays an important role in LC–ESI–MS/MS because it influences in the ionization efficiency and the separation quality [36]. Buffer salts such as ammonium acetate and ammonium formate are commonly used to improve peak shape and the ionization of the compounds. Thus, these buffer salts were evaluated using a concentration of 100 mM in solvent A. No strong differences were observed between both buffers in terms of peak shape. However, the sensitivity was slightly better when ammonium acetate was used, in agreement with previous studies [37,38]. Thus, the concentration of ammonium acetate was checked from 50 to 200 mM and the peak shapes improved up to 150 mM. Above this value, a higher ionic strength had a modest effect in the peak quality, so 150 mM of ammonium acetate (solvent A) was selected. The effect of the formic acid concentration was also studied using different percentages of formic acid (0–2%, v/v) in solvent A. A 1.5% formic acid (v/v) concentration provided sharp and symmetrical peaks due to minimized silanol interactions. So, the final composition of the mobile phase was 150 mM ammonium acetate containing 1% (v/v) formic acid (solvent A) and MeCN (solvent B). The gradient was optimized to get the best separation and peak shape in the shortest time. In order to delay the elution of the most polar AGs, it was necessary to start using 20% of solvent A. The rest of gradient program was as follow: 70% A at 5 min (held for 5 min) and finally go back to the initial conditions at 12 min (held for 6 min). The flow rate was studied from 300 to 600 µL min⁻¹ and finally 500 µL min⁻¹ was selected as a compromise between signal, peak shape and run time. The column temperature was studied between 25 °C and 55 °C, selecting 35 °C as optimum.

Sample solvent nature was investigated and optimized in terms of sensitivity and peak shape. The sample solvents tested were MeCN; MeCN:H₂O (80:20); MeCN:H₂O (60:40); MeCN:H₂O (40:60); MeCN:H₂O (20:80) and H₂O. It was observed that the higher the percentage of MeCN, the lower the sensitivity. This fact may be due to the poor solubility of AGs in this organic solvent. However, the use of 20% MeCN allowed an improvement in the peak shape for most of the AGs. Injection volume was evaluated from 5 to 20 µL (full loop). The maximum injection volume was used, obtaining the best sensitivity without losing peak efficiency.

Finally, to obtain the maximum response, the ionization source parameters were evaluated. Curtain gas (nitrogen) was tested between 20 (138 kPa) and 35 psi (241 kPa) and finally 30 psi (207 kPa) was selected as optimum. Turbo V ion source temperature was evaluated between 300 and 750 °C, achieving a satisfactory solvent evaporation at 700 °C. The response of the ion spray voltage was checked from 5000 to 5500 V. However, the response was not improved when this voltage was increased and finally 5000 V was selected as optimum. Nitrogen nebulizer gas (Gas 1) and nitrogen heater gas (Gas 2) pressure were

| Table 1 |
|-------------------------|--------|--------|--------|-------------------------|
| Precursor ion | Rt (min) | DP (V) | EP (V) | CXP (V) | Product ion | CE (V) | CXP (V) |
| AM          | 586.2   | 1.7    | 66     | 4.5   | 24 | 163.0 (Q) | 45 | 6 |
| APM         | 540.2   | 2.1    | 66     | 6.0   | 22 | 217.0 (Q) | 35 | 6 |
| DHS         | 584.2   | 1.6    | 76     | 9.0   | 20 | 263.0 (Q) | 39 | 8 |
| GENT        | 464.3   | 2.5    | 51     | 4.5   | 16 | 322.0 (Q) | 21 | 6 |
| C2a         | 160.0   | 1.0    | 51     | 4.5   | 16 | 160.0 (I) | 29 | 4 |
| GENT        | 478.3   | 2.6    | 66     | 5.0   | 20 | 322.0 (Q) | 21 | 6 |
| GENT        | 450.3   | 2.5    | 41     | 4.5   | 18 | 322.0 (Q) | 21 | 6 |
| C1a         | 157.0   | 1.0    | 51     | 4.5   | 16 | 160.0 (I) | 29 | 4 |
| KAM         | 485.2   | 1.9    | 41     | 4.5   | 16 | 163.1 (Q) | 35 | 6 |
| PRM         | 616.3   | 2.0    | 66     | 8.5   | 20 | 163.1 (Q) | 47 | 6 |
| SPC         | 351.2   | 1.3    | 41     | 4.5   | 14 | 333.0 (Q) | 23 | 6 |
| STP         | 600.2   | 1.5    | 121    | 10.0  | 22 | 98.0 (I)  | 45 | 4 |
| TOM         | 468.5   | 2.0    | 41     | 6.5   | 16 | 163.1 (Q) | 33 | 4 |

Rt: Retention Time; DP: Declustering Potential; EP: Entrance Potential; CEP: Collision Exit Potential; I: Transition employed to complete the identification.
optimized at the same time, obtaining the best signals when both parameters were set to 50 psi (345 kPa).

3.2. Optimization of MISPE

Milk and milk based products are complicated samples that present a high content in polyunsaturated fatty acids, minerals, vitamins, proteins and salts. So, in order to remove the main matrix components, the sample treatment is mandatory. The use of MISPE simplifies the extraction of AGs from these complicated matrices, providing a higher selectivity and reducing sample manipulation. Initially, the protocol proposed by Supelco for the SupelMIP AGs SPE Columns for the determination of Neomycin, GENT C1, DHS, STP, Genetici (G418-2), AM, TOM, KAM, APy, Hygroymycin, Puromycin and SPC in honey samples was followed with some modifications [39], using 2 mL of whole cow milk as representative sample. First of all, the use of TCA for precipitation of proteins and inhibition of protein binding of the analytes was checked [40,41]. Thus, 1 mL of different concentrations of TCA solutions (15%, 25% and 50%, m/v) was added to the sample, and 15% of this agent showed to be enough to precipitate all proteins. Then, a study of the TCA volume was carried out (from 100 to 1000 µL). Volumes lower than 250 µL were insufficient to obtain a complete precipitation. On the other hand, the precipitation efficiency was similar from 250 µL, so 250 µL of 15% TCA was selected as optimum value. The original protocol proposed by Supelco included four consecutive washing steps: (1) 3 mL of water; (2) 1 mL of 0.1% (v/v) ammonium hydroxide; (3) 1 mL of a mixture of MeCN:H2O (40/60, v/v); and (4) 1 mL of a mixture of dichloromethane:MeOH (50/50, v/v). However, in the case of milk samples satisfactory recoveries (average recovery, 84%) and very low MEs (average, 5%) were obtained using only steps (1) and (4), achieving an important simplification of the sample treatment. Finally, the concentration of HFBA in the elution step was studied between 0 and 10 mM. A concentration of 5 mM was enough to obtain satisfactory extraction efficiency for all AGs. The final MISPE procedure is described in detail in Section 2.4.

3.3. Method characterization

The method was characterized in a wide range of milk samples and milk-based functional foods. Parameters such as linear dynamic range, limits of detection (LODs) and limits of quantification (LOQs), ME, precision (both inter- and intraday precision) and trueness were taking into account.

3.3.1. Calibration curves, LODs and LOQs

Procedural standard calibration curves were established at five different concentration levels (10, 25, 50, 100 and 150 µg kg$^{-1}$ for SPC and DHS; and 50, 100, 150, 200 and 250 µg kg$^{-1}$ for the rest) by spiking blank whole milk cow samples (as representative matrix) before the extraction process. Each level was prepared following the proposed MISPE procedure and injected in triplicate. Two product ions were selected, the most intense one was used as quantification ion (Q) and the following was considered as confirmation ion (I). These transitions together with the retention times were employed to ensure adequate analytes identification. Performance characteristics of the method are shown in Table 2. LODs and LOQs were calculated as the minimum analyte concentration yielding a S/N equal to three and ten, respectively. As can be seen, LOQs lower than the MRLs were obtained for all AGs. Therefore, the proposed method is adequate for the determination of very low levels of these residues in the selected matrix.

3.3.2. Precision study

Both repeatability (interday precision) and intermediate precision (intraday precision) were tested by application of the proposed MISPE-UHPLC-MS/MS method in whole cow milk samples spiked at two different concentration levels of AGs: 10 and 25 µg kg$^{-1}$ for DHS and SPC; and 50 and 100 µg kg$^{-1}$ for the rest. To check the repeatability, three samples were prepared and injected in triplicate on the same day, under the same conditions. Similar procedure was carried out in the evaluation of intermediate precision. Thus, during three consecutive days, one sample per day was prepared and injected in triplicate. The results, expressed as %RSD of peak areas, are shown in Table 3. Good precision (RSD lower than 12%) was obtained in all cases. So, it could be concluded that the obtained results are in agreement with the current demand [42].

3.3.3. Matrix effect

ME was estimated for each AGs in the different milk samples and milk-based functional foods (whole cow milk, skimmed cow milk, whole goat milk, follow-on milk, Omega 3-enriched milk and isoflavones-enriched milk). To evaluate this factor, the slope of matrix-matched calibration curves and the slope of external standard calibration curves were compared according to the following equation: $[(\text{calibration curve slope in matrix/ calibration curve slope in solvent})-1] \times 100$ [43]. The obtained data are shown in Table 4. ME was always lower than 15% which involves that the proposed sample treatment is enabled to remove co-extractants in all the studied matrices. Thus, MEs were negligible in all studied cases, so that the use of matrix-matched calibration would not be mandatory.

3.3.4. Trueness assessment

The trueness of the proposed method was assessed by recovery studies in the different types of milk samples spiked at two different concentration levels of each AG: 10 and 25 µg kg$^{-1}$ for DHS and SPC; and 50 and 100 µg kg$^{-1}$ for the rest. The absolute recoveries have been calculated by comparing the concentration of AGs in milk samples spiked before the MISPE procedure with the concentration in extracts of milk samples spiked after the MISPE procedure. Each sample was analysed in triplicate and injected three times. Blank samples were previously analysed to check the presence of AGs; none of them gave a result above the LOQs of the method. The recoveries were between 70% and 106% for all analytes except for TOM 45–65%) in all samples tested and also for APy, GENT C1a, KAM, PRM and TOM in Omega 3-enriched milk (recoveries lower than 70%). Regarding precision, satisfactory RSD% were obtained for all analytes in all samples (see Table 5), fulfilling current legislation [42].

A typical extracted ion chromatogram corresponding to a whole cow milk sample spiked with 25 µg kg$^{-1}$ for DHS and SPC and 100 µg kg$^{-1}$ for the rest and analysed by the proposed MISPE-UHPLC–MS/MS method is shown in Fig. 1.

3.3.5. Comparison with other methods

A comparative overview of the main analytical performance characteristics of the proposed method with other published methods for the determination of AGs in milk samples are shown in Table 6. The results in terms of LOQs, recoveries and number of AGs simultaneously studied were similar or even better than those obtained by the other methods. Moreover, the amount of required sample in the proposed MISPE procedure (2 g) was usually lower. However, the most relevant issue was the lower MEs: as could be observed, the proposed method provided significantly lower ME results than the other methods. In fact, some of these methods needed two consecutive SPE to obtain satisfactory MEs. So, it can be concluded that MISPE provides greater cleanup than traditional SPE methods.

4. Conclusions

In the present study, the use of a HILIC column in UHPLC-MS/MS
### Table 2
Statistics and performance characteristics of the MISPE-UHPLC-MS/MS method for the analysis of AGs in whole cow milk.

<table>
<thead>
<tr>
<th>AG</th>
<th>Linear dynamic range ($\mu g \cdot kg^{-1}$)</th>
<th>Slope (SD)</th>
<th>Intercept (SD)</th>
<th>R² (%)</th>
<th>LOD ($\mu g \cdot kg^{-1}$)</th>
<th>LOQ ($\mu g \cdot kg^{-1}$)</th>
<th>MRL ($\mu g \cdot kg^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM</td>
<td>31.3–250</td>
<td>8202 (126)</td>
<td>−9116 (21,005)</td>
<td>0.995</td>
<td>9.4</td>
<td>31.3</td>
<td>NA</td>
</tr>
<tr>
<td>APM</td>
<td>38.5–250</td>
<td>1955 (29)</td>
<td>19,166 (4948)</td>
<td>0.990</td>
<td>11.5</td>
<td>38.5</td>
<td>NA</td>
</tr>
<tr>
<td>DHS</td>
<td>7.7–150</td>
<td>96,120 (164)</td>
<td>−121,633 (2738)</td>
<td>0.988</td>
<td>2.3</td>
<td>7.7</td>
<td>200</td>
</tr>
<tr>
<td>GENT C2C2a</td>
<td>45.3–250</td>
<td>2039 (32)</td>
<td>8766 (5330)</td>
<td>0.996</td>
<td>13.6</td>
<td>45.5</td>
<td>100</td>
</tr>
<tr>
<td>GENT C1</td>
<td>30.9–250</td>
<td>2567 (39)</td>
<td>−16,666 (6535)</td>
<td>0.998</td>
<td>9.3</td>
<td>30.9</td>
<td>100</td>
</tr>
<tr>
<td>GENT C1a</td>
<td>49.0–250</td>
<td>5544 (96)</td>
<td>56,900 (15,948)</td>
<td>0.989</td>
<td>14.7</td>
<td>49.0</td>
<td>100</td>
</tr>
<tr>
<td>KAM</td>
<td>45.5–250</td>
<td>9208 (113)</td>
<td>−51,566 (18,761)</td>
<td>0.993</td>
<td>13.6</td>
<td>45.5</td>
<td>150</td>
</tr>
<tr>
<td>PRM</td>
<td>47.6–250</td>
<td>3390 (27)</td>
<td>−3966 (4544)</td>
<td>0.994</td>
<td>14.3</td>
<td>47.6</td>
<td>NA</td>
</tr>
<tr>
<td>SPC</td>
<td>4.2–150</td>
<td>1588 (18)</td>
<td>−2606 (3051)</td>
<td>0.993</td>
<td>13.8</td>
<td>45.9</td>
<td>150</td>
</tr>
<tr>
<td>STP</td>
<td>45.9–250</td>
<td>2159 (16)</td>
<td>3833 (2681)</td>
<td>0.990</td>
<td>13.6</td>
<td>45.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

* MRL: Maximum residue limit in milk [8]; NA: Non-authorized in milk.

### Table 3
Precision expressed as %RSD of peak areas for spiked whole cow milk samples (three samples, injected in triplicate, n=9).

<table>
<thead>
<tr>
<th>AG</th>
<th>Repeatability</th>
<th>Intermediate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level 1</td>
<td>Level 2</td>
</tr>
<tr>
<td></td>
<td>Level 1</td>
<td>Level 2</td>
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</tbody>
</table>

### Table 4
ME for all samples studied.

<table>
<thead>
<tr>
<th>AG</th>
<th>Whole cow milk</th>
<th>Skimmed cow milk</th>
<th>Whole goat milk</th>
<th>Follow-on milk</th>
<th>Isoflavones-enriched milk</th>
<th>Omega 3-enriched milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level 1</td>
<td>Level 2</td>
<td>Level 1</td>
<td>Level 2</td>
<td>Level 1</td>
<td>Level 2</td>
</tr>
</tbody>
</table>

### Table 5
Recoveries % and (RSD %) for different spiked samples (three samples, injected in triplicate, n=9).

<table>
<thead>
<tr>
<th>AG</th>
<th>Whole cow milk</th>
<th>Skimmed cow milk</th>
<th>Whole goat milk</th>
<th>Follow-on milk</th>
<th>Isoflavones-enriched milk</th>
<th>Omega 3-enriched milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level 1</td>
<td>Level 2</td>
<td>Level 1</td>
<td>Level 2</td>
<td>Level 1</td>
<td>Level 2</td>
</tr>
</tbody>
</table>

* Level 1: 10 µg kg⁻¹ for DHS and SPC, 50 µg kg⁻¹ for the rest.
* Level 2: 25 µg kg⁻¹ for DHS and SPC, 100 µg kg⁻¹ for the rest.
combined with MISPE has been presented as a reliable, selective and highly sensitive methodology for the simultaneous quantification and confirmation of 11 aminoglycosides in different types of milk (whole cow milk, skimmed cow milk, whole goat milk) and milk-based functional foods (follow-on milk, Omega 3-enriched milk and iso-avonies-enriched milk). The results showed that MISPE is a robust tool for extraction of AGs and sample clean-up, achieving ME lower than 15% in all cases. Calibration curves were established in the presence of matrix and the low LOQs obtained allowed determining the 11 AGs at concentrations lower than the limits established by current legislation for AGs in milk, with satisfactory precisions. In addition, trueness has been successfully evaluated, achieving good recoveries for all AGs, except for TOM in milk. The developed method is rapid, low solvent consumption and inexpensive providing good sensitivity. Thus, these results showed the suitability of this MISPE-UHPLC-MS/MS procedure for the monitoring of AGs residues in milk and milk-based products.

Acknowledgment

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Table 6
Comparison of the proposed method with other reported methods for the determination of AGs in milk samples.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample treatment</th>
<th>Number of AGs</th>
<th>LOQ (µg kg⁻¹)</th>
<th>Recovery (%)</th>
<th>Amount of sample</th>
<th>ME (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC-MS/MS</td>
<td>SPE (C18)</td>
<td></td>
<td>10</td>
<td>25–125</td>
<td>87–95</td>
<td>2.0 mL</td>
<td>–</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Consecutive SPE (C18-Weak cation exchange)</td>
<td>4</td>
<td>7.7–19.4</td>
<td>73.9–86.2</td>
<td>2.0 mL</td>
<td>–</td>
<td>[12]</td>
</tr>
<tr>
<td>Ion pair chromatography-MS/MS</td>
<td>Consecutive SPE (HLB-HLB)</td>
<td></td>
<td>13</td>
<td>–</td>
<td>64–108</td>
<td>4.0 mL</td>
<td>–</td>
</tr>
<tr>
<td>HILIC-MS/MS</td>
<td>SPE (Weak cation exchange)</td>
<td></td>
<td>14</td>
<td>2–152</td>
<td>91–114</td>
<td>6.0 g</td>
<td>(−81) to (−16)</td>
</tr>
<tr>
<td>HILIC -MS/MS</td>
<td>MISPE</td>
<td></td>
<td>11</td>
<td>4.2–49</td>
<td>70–106</td>
<td>2.0 g</td>
<td>(−15) to (1)</td>
</tr>
</tbody>
</table>

Fig. 1. Extracted ion chromatogram of a spiked whole cow milk sample applying the proposed method (25 µg kg⁻¹ for DHS and SPC, 100 µg kg⁻¹ for the rest).

References


