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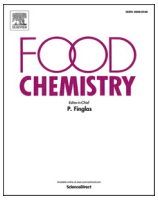

Extention and interlaboratory comparison of an LC-MS/MS multi-class method for the determination of 15 different classes of veterinary drug residues in milk and poultry feed

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ABSTRACT

An HPLC-MS/MS multi-class method for quantitation of 15 different classes of veterinary drug residues (>140 analytes) in milk and poultry feed was developed and validated. Accuracy criteria for routine laboratories were met for the majority of analytes, > 83 % in milk and between 50 and 60 % in chicken feed, with an apparent recovery of 60–140 %. Extraction efficiency criteria were met for >95 % of the analytes for milk and > 80 % for chicken feed. Intermediate precision meets the SANTE criterion of RSD < 20 % for 80–90 % of the analytes in both matrices. For all analytes with an existing MRL in milk, the LOQ was below the related MRL. Twenty-nine samples of commercial milk and chicken feed were analyzed within the interlaboratory comparison. No residues of veterinary drugs were found in the milk samples. However, the feed samples exhibited high levels of nicarbazin, salinomycin, and decoquinate.

1. Introduction

The United Nations (UN) reports that by 2050 there will be 9.8 billion global inhabitants (UN, n.d.). As a result, and according to the FAO, the expected growth of overall food production will need to rise by some 70 % between 2005/07 and 2050. It is important to emphasize that most of the future need in food production will occur in developing countries (FAO, n.d.). Climate change, which could lead to decreased agricultural productivity (Ortiz-Bobea et al., 2021) will accompany a rapid increase in global food production. In that respect, food safety and quality will rely on the ability to undertake reliable and accurate analysis.

Meat and cereals will be key commodities whose production is expected to rise. Consequently, the use of veterinary drugs will be unavoidable in the food and feed industry. Excessive and inappropriate use of veterinary drugs, carry-over from medicated to non-medicated animal feed, and fertilization with animal manure are just some pathways through which food and feed can become contaminated with veterinary drug residues. Therefore, veterinary drug residues will affect the environmental resistome and thus antimicrobial resistance (Perry & Wright, 2013). In addition, the globalization of trade combined with the absence of regulatory limits in some developing countries favors the entry of these substances into the European food and feed chain.

Multiclass analytical methods will likely play a pivotal role in ensuring food safety, with the emphasis likely to be on the sensitivity and robustness of the method to determine whether the broad range of different target components complies with current regulations (EC, 37/2010), ideally in one analytical run.

During the past few years, LC-MS/MS has gained popularity as a tool for the analysis of veterinary drug residues, due to its ability to detect trace levels of a wide range of veterinary drugs belonging to different (sub)classes with high accuracy and precision. There was limited availability of LC-MS/MS methods for the analysis of residues of veterinary drugs in milk until 2010. The earliest method developed was from

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2008, for the quantification of 25 veterinary drugs in milk (Turnipseed et al., 2008). In the case of analysis of veterinary drugs in animal feed, there were no LC-MS/MS methods in the literature until 2010, and even then, the methods developed in earlier years included small numbers of veterinary drugs, usually from the same drug class (Hoff et al., 2020; Wille et al., 2010; Zhao et al., 2013), as opposed to large multi methods capable of measuring > 100 analytes in a single run. Furthermore, despite significant progress in instrument performance and the use of extraction/clean-up techniques such as dilute-and-shoot (DnS), analysis of > 120 veterinary drug residues across a broad range of food/feed remains a challenge for a single multi-residue LC-MS/MS method, with only a combination of methods meeting the requirement for this number of analytes in order to offer consumer protection.

Nevertheless, the development of LC-MS/MS multi-methods for the analysis of a large number of veterinary drugs in food matrices such as milk and feed is a challenging task, mainly due to the broad spectrum of chemical and physical properties of these compounds. This diversity then leads to a number of analytical obstacles, such as differences in sample preparation and extraction methods, matrix interference, and the need for optimized analytical conditions for each drug. Besides that, the initial technical aspects such as the management of veterinary drug standards, play a critical role in ensuring their stability (Desmarchelier et al., 2018; Kenjeric et al., 2021). All of this hampers the development and implementation of a method that is efficient and also economically feasible, while including a large number of different veterinary drugs. Despite the obstacles, this study successfully developed and validated an LC-MS/MS multiclass method for the analysis of veterinary drug residues in chicken feed and milk. Additionally, the method was transferred to another laboratory where it was cross-validated, and an interlaboratory comparison on real samples was conducted. The final method covers > 140 analytes across > 15 drug classes, with the application of this method, demonstrated on 10 different milk and chicken feed matrices.

It was hypothesized that the previously developed LC-MS/MS multiclass method described by Steiner et al., 2020, would be applicable to a wide range of food and animal feed samples, and would also be suitable for detecting veterinary drugs that had yet to be included. To challenge the robustness and accuracy of the method, five different samples with potential differences in matrix effects were assessed, following the advice given in EC 808/2021 to examine relative matrix effects. Findings of which were then proved through the validation of the method across two laboratories.

2. Materials and method

2.1. Chemicals and samples

In the present study, 172 pharmacologically active substances coming from 18 different classes of veterinary drugs were assessed. Due to the diverse stability of the particular veterinary drug, the method was successfully validated for 144 compounds, 15 different classes (Aminogylcosides, Amphenicols, Beta Lactams, Cephalosporines, Glycopeptides, Dihydrofolate reductase inhibitors, Macrolides, Nitroimidazole, Penicilines, Pleuromutilines, Polyether ionophores, Polymixin, Polypeptide, Quinolones, Tetracyclines) together with some additional compounds. A list with all tested substances classified based on the drug class to which they belong is available in supplementary material Table 3. Powdered reference standards were purchased from Sigma-Aldrich (Vienna, Austria), Dr. Ehrenstorfer (Augsburg, Germany), and the European Union Reference Laboratory (Berlin, Germany), or were obtained as gifts from different research groups. LC-MS gradient-grade acetonitrile and methanol, as well as MS-grade glacial acetic acid (p.a.) and ammonium acetate, were purchased from Sigma-Aldrich (Vienna, Austria).

2.2. Instrumentation

The analytical platform used in this study was a modified version of Steiner et al., 2020. Briefly, a QTrap 5500 MS/MS system (Sciex, Foster City, CA, USA) equipped with a Turbo V electrospray ionization (ESI) source was coupled to a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini C18-column, 150 × 4.6 mm i.d., 5 µm particle size, equipped with a C18 security guard cartridge, 4 × 3 mm i.d. (both Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode with a flow rate of 1 000 µL/min. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid (10:89:1, v/v/v; eluent A) and (97:2:1, v/v/v; eluent B) respectively. For further purification of reverse osmosis water, a Pure-lab Ultra system (ELGA Lab Water, Celle, Germany) was used. After an initial hold time of 2 min at 100 % A, the proportion of B was increased linearly to 50 % within 3 min. A further linear increase of B to 100 % within 9 min was followed by a hold time of 4 min at 100 % B, then the column was re-equilibrated at 100 % A for a further 2.5 min. The injection volume was set at 5 µL.

ESI-MS/MS was performed in scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarity across two separate chromatographic runs. The settings of the ESI source were as follows: source temperature 550 °C, curtain gas 30 psi (206.8 kPa of max. 99.5 % nitrogen), ion source gas 1 (sheath gas) 80 psi (551.6 kPa of nitrogen), ion source gas 2 (drying gas) 80 psi (551.6 kPa of nitrogen), ion-spray voltage −4.5 kV and +5.5 kV respectively, collision gas (nitrogen) - medium. The column temperature was set at 25 °C. The target cycle time was 1000 ms, the MS pause time was 3 ms, and the detection window width was 60 s in the positive and negative ESI modes respectively. According to the SANTE/11312/2021 validation guidelines, two MRM transitions per analyte are acquired for accurate confirmation along with the retention time (RT). A list with MRM transitions of all substances included in the method is available in supplementary material in Table 2.

For the cross-validation and interlaboratory comparison of poultry feed samples, the LC-MS system used was the same as described above where the methodology was initially developed with some differences between the equipment used in both laboratories. The LC system used in the partner laboratory was an ExionLC AD System (SCIEX), and no guard cartridge was used with the LC column. Additionally, the analysis was conducted using fast polarity switching, resulting in only one analytical run, with the settling time between polarities set at 10 ms.

2.3. Calibration solutions

Respecting the solubility of compounds, six different solvents were used for the preparation of the stock standard solutions: water, methanol, methanol + water (1:1, v/v), methanol + dimethyl sulfoxide (DMSO) (1:1, v/v), water + acetonitrile (1:1, v/v) and 1 mM sodium hydroxide in methanol. Overall, six intermediate standard mixtures (each at 10 ppm) were prepared by combining individual stock solutions dissolved in the same solvent. All solutions were stored at −20 °C. A detailed overview related to the preparation of the 172 individual stock solutions and 6, respectivley 3 intermediate mixtures is given in the supplementary material in Table 3.

2.4. Spiking of samples and calibration curve

The final working standard solution was freshly prepared prior to the experiment. This was obtained by mixing 600 µL of the multianalyte mix (prepared by pooling the appropriate amount of 6 intermediate mixes) with 400 µL of diluent, ACN: H20 (50:50, v/v). The milk matrix (980 and 900 µL) was spiked with 20 µL (low spiking level) and 100 µL (high spiking level) of the freshly prepared working standard solution. The chicken feed matrix (0.5 g) was spiked with 25 µL (low spiking level) and

125 μL (high spiking level) of the same working standard solution. The spiking levels were chosen to cover the MRL of veterinary drugs and to match the linear range of the calibration curve. External neat calibration was performed by serial dilutions of the final working standard solution with the diluent, 1:10, 1:30, 1:100, 1:300, and 1:1000. For spiking after extraction (spiked extracts), 500 μL of the blank extract was mixed with the appropriate volume of spiking solution (20 μL for milk and 31 μL for chicken feed), then the diluent was added up to the 1 mL mark.

2.4.1. Samples

Models of five artificial chicken feed samples were prepared in-house by mixing different proportions of soy, distillers' dried grain with solubles (DDGS), rapeseed, and maize as described by Steiner et al. (14). Heterogeneous individual raw samples were provided by the following companies: LVA (Klosterneuburg, Austria), Bipea (Paris, France), Biomin (Getzersdorf, Austria), and Garant-Tiernahrung (Pöchlarn, Austria). The composition of artificial chicken feed samples is described in Supplementary materials, Table 1**.** Milk samples with different fat contents of 0.5, 0.9, 1.5, 3.2, and 3.5 %, respectively, were purchased at a nearby store.

2.5. Chicken feed sample preparation optimization

To optimize the sample preparation process and minimize matrix effects, different clean-up procedures were tested for the chicken feed matrix, with the dilute and shoot approach used as a control. Sodium chloride, anhydrous $MgSO_4$, primary secondary amine (PSA), and C18 sorbent used for the clean-up procedure were purchased from Sigma-Aldrich (Vienna, Austria). Five gram of each chicken feed samples was extracted with a 20 mL acidic solvent mixture of acetonitrile:water: acetic acid (79:20:1, v/v/v) to assess the feasibility of a combined method for antibiotics and mycotoxins, with the latter requiring acidic conditions in order to efficiently extract the fumonisins (Meister, 1999). The dilute and shoot control (I) was prepared using one aliquot(1 mL) from each sample. A 5 mL aliquot of each of the five chicken feed extracts underwent purification using QuEChERS. To 5 mL of extract of each sample, 0.5 g of sodium chloride together with 2 g of anhydrous $MgSO_4$ was added. After which, one aliquot (1 mL) of this modified extract was kept to evaluate QuEChERS clean up only (II), while two separate aliquotes of 1 mL each underwent further clean-up treatment with 25 mg (PSA) (III) and 25 mg C18 sorbent (IV) in order to remove sugars, non-polar interfering substances, and pigments. To assess the precipitation of lipid components from the feed matrix, another set of samples was extracted and QuEChERS was applied one day in advance, with the extract frozen overnight (V), then thawed, diluted, spiked, and injected the next day. All of the manipulated extracts, including the dilute and shoot as control, were subsequently spiked, diluted 1 + 1, and injected.

2.6. Milk sample preparation optimization

Three different concentration levels: 30, 100, and 300 ppb were prepared with four different dilution solvents: (I) milk without any dilution, (II) milk/extraction solvent (1:1, v/v), (III) milk/acetonitrile: water (1:1, v/v) (1:1, v/v). As a control, acetonitrile: water (1:1, v/v) was used. The relative response was calculated by comparing the peak area of acetonitrile: water (1:1, v/v) with the peak area of the same level obtained from other tested solvents.

2.7. Final sample preparation used for validation

The final sample preparation procedure was the dilute and shoot approach for both matrices, milk, and chicken feed. For chicken feed, 0.5 g of sample was extracted with 2 mL of solvent (1:4, w/v) with and without acetic acid throughout the whole study for every experiment conducted. In the case of milk, a 1 mL sample was extracted with 3 mL of ACN: H2O (80:20, v/v). The samples were then placed in a horizontal position on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and agitated for 90 min. This was followed by centrifugation on a GS-6 centrifuge (Beckman Coulter, Fullerton, CA, USA) at 3 500 rpm for 10 min. After this, 500 μL of the supernatant was aliquoted to an HPLC vial. Each aliquot was diluted with an equal amount of a solvent mix, acetonitrile: water (20:80, v/v) in the case of acid-free extraction and acetonitrile:water: acetic acid (20:79:1, v/v/v) for acidic extraction. After appropriate mixing, the final diluted extract was filtered using a 0.2 μm PTFE filter and 5 μL of the diluted extract was injected into the LC-MS/MS system without further pre-treatment. This additional filtration step was performed at our partner laboratory(QUB).

2.8. Data analysis

Calibration curves construction and peak integration were performed using MultiQuant 2.0.2 software (Sciex, Foster City, CA, USA). The calibration curves were constructed as linear, 1/x weighted. Evaluation of the data was performed in Microsoft Excel 2013. To produce the visual components, the open-access visualization tool Flourish, developed by Kiln Enterprises Ltd in London, UK, was used. Other method performance characteristics such as recovery, apparent recovery, repeatability, and matrix effects were evaluated at high and low spiking levels for both matrices. The recovery of the extraction step (RE), the apparent recovery (RA), and the signal suppression/enhancement (SSE) were calculated from the peak areas of the samples spiked before extraction, the samples spiked after extraction, and the neat solvent standards, respectively, as follows:

$$SSE(\%) = \text{area (sample spiked after extraction) / area (neat solvent standard).}$$

$$RE(\%) = \text{area (sample spiked before extraction) / area (sample spiked after extraction)} \times 100.$$

$$RA(\%) = \text{area (sample spiked before extraction)/ area (neat solvent standard)} \times 100.$$

2.9. Validation of the method

Validation of the method was performed following the SANTE/11312/2021 validation guideline. For method validation purposes, chicken feed and milk samples were spiked at two levels (with a difference factor of five) with the appropriate amount of the final working standard solution. Spiked feed samples were then left overnight at 4 °C to achieve solvent evaporation and to achieve equilibration between the matrix and analytes. Extraction of the spiked feed samples and post-extraction spikes was performed with and without the use of acid, while in the case of the milk matrix, only extraction without acid was chosen. The whole validation procedure was miniaturized in order to economize on the amount of standards required, however, the routine analysis uses a larger amount of sample: 5 g (or 20 g) extracted using 20 mL (or 80 mL) of solvent. To assess the within laboratory reproducibility (RSD_{WLR}), validation of the method was performed over three different days at the higher concentration level. The repeatability of the method was expressed as the relative standard deviation (RSD_r) calculated by spiking a set of five different samples per matrix. In each case, the five different test items (lots) of each commodity were spiked in quintuplicate on each day before extraction. Examination of the matrix effects, expressed as signal suppression/enhancement (SSE) and extraction

efficiencies (RE) was performed by fortification of diluted blank extracts of each matrix at the high concentration level. The limit of quantification (LOQ) and limit of detection (LOD) were determined according to the EURACHEM guide. On day 3, each of the five different samples per matrix were additionally spiked before extraction at the low concentration level (as well as spiked after extraction at the high level for determination of SSE/RE). An extensive validation data set is provided in supplementary data in Tables 6 and 7.

2.10. Interlaboratory comparison

After the development and validation of the method at IFA-Tulln/BOKU Vienna in Austria, the final method was transferred, implemented, and a full validation using the same protocol was performed at the ASSET/NML Centre for Excellence in Agriculture and Food Integrity at Queen's University Belfast (QUB). Samples of powdered milk and poultry feed were also exchanged between the two laboratories and an interlaboratory comparison was conducted according to ISO/IEC 17043:2010. There were 29 samples analyzed in total: 17 of which were poultry feed samples and 12 were powdered milk samples. A 5 g aliquot sample was transferred to a 50 mL falcon tube and subsequently extracted with 20 mL of ACN: H20 (80:20, v/v). Samples were then transferred to a shaker for 90 min and centrifuged for 10 min. Then, a 500 μL aliquot of the supernatant was taken and diluted with the same volume of diluent, ACN: H20 (20:80, v/v), with this done for both the milk powders and poultry feed samples.

3. Results and discussion

3.1. Method modification

As already mentioned the original method was developed for the quantification of biotoxins, pesticides, and veterinary drugs in a complex feed. This method was transferred and the MRM transitions of pesticides and biotoxins were removed, while the existing transitions of veterinary drugs were left. LC-MS/MS parameters of additional analytes of interest were optimized and their respective MRM transitions were included in the method, resulting in an overall number of 322 MRMs. The MRM cycle time was set to 1 *sec* and the target retention time width was set to 60 *sec* since the number of MRM transitions decreased, resulting in dwell times of $>$ 25 ms. When the method was transferred to the partner lab, the MRM cycle time was also set to 1 *sec*, however, this was split between both polarities as polarity switching was enabled.

3.2. Solubility and stability of standards

Veterinary drugs represent an extremely complex group of compounds in terms of their solubility, stability, polarity, etc. To be sure that the liquid standard is stable in storage solutions prepared in the laboratory, or in dilution solvent of a calibration curve, it is necessary to optimize the entire standard preparation process. Consequently, management of a high number of veterinary drug standards represents a laborious task. The first challenge in preparing liquid stock solutions of solid veterinary drug standards of high concentration in order to ensure stability over several years is finding a suitable solvent. The second challenge is to pool individual stocks into intermediate mixes to assure easier handling, with a maximum of 3 months of intermediate mixture stability. The third challenge is the preparation of a working standard solution that will again facilitate handling when there are $>$ 50 analytes. For all of the above, optimized data on the solubility of the analytes included in this method can be found in the supplementary materials in Table 3.

4. Sample preparation optimization

4.1. Milk sample preparation optimization

The testing of different dilution solvents for the extraction of milk was based on the knowledge gained from preliminary experiments on long and short-term stability findings conducted in the lab and by other authors (Desmarchelier et al., 2018; Kenjeric et al., 2021). Acid-free solvents have been shown to increase the storage stability of standard mixes and preliminary preparation of the calibration curve showed a slightly better response when using acetonitrile: water (1:1, v/v) as opposed to acetonitrile:water: acetic acid (49.5:49.5:1, v/v/v). The objective was to examine the impact of different dilution solvents on the relative response of the analyte in the presence of a milk matrix and thus check the most suitable solvent for extraction (and the option of injecting milk directly without any pretreatment). Furthermore, the aim was to understand the impact of the components of the milk matrix on the stability of the analytes in the sample vial and on the ionization efficiency, as well as to examine whether there is any sorption of non-polar compounds by fat globules in milk. The results indicate that the relative response was not significantly improved overall when the neutral dilution solvent was used, with only eprinomectin and sulfameter showing worse results when diluted with the presence of acid (see supplementary materials Table 4). This outcome may be attributed to the pH of the milk sample being 6.64, while the acetic acid used in the experiment is not a strong acid (with a pKa of 4.8). This suggests that milk may have a buffering capacity (Gaucheron, 2005), and therefore no significant change was seen between the solvents with and without the presence of acid. Additionally, other research studies have suggested that acidic conditions are not suitable for veterinary drug analysis (Fedeniuk & Shand, 1998; A. Stolker et al., 2000; A. A. M. Stolker & Brinkman, 2005). Therefore it was determined to perform sample preparation for milk samples under a neutral pH. Individual results of this study are available in the supplementary materials.

4.2. Chicken feed sample preparation optimization

Combining highly complex chicken feed and a wide range of chemical and physical properties of veterinary drugs represents a challenging task when developing an analytical method. The biggest obstacle in that regard are matrix effects. It has been stated by previous authors that the incorporation of a clean-up step in the sample preparation procedure can be advantageous in decreasing matrix effects (Greer et al., 2021; Mol et al., 2008; Stubbings & Bigwood, 2009). To test this, 4 different clean-ups were assessed, with the results compared against the general extraction approach utilized (Dilute and shoot, DnS). This was performed by spiking both the raw extract and the purified extracts after the related clean-up procedure had been performed (Quechers, modified Quechers (PSA and C18), and deep freeze). Clean-ups were tested on five different chicken feed samples and the results were evaluated for 56 selected analytes, with the results expressed as signal suppression/enhancement (SSE). A comparison of the percentage of analytes exhibiting an SSE range of (80–120 %) between the different clean-ups revealed no considerable improvement in reducing matrix effects (see supplementary materials table 5, Fig. 1). However, when differences in matrix effects within the drug classes were compared, it was observed that clean-up or simply acidic conditions were not an option for anthelmintics, coccidiostats, NSAID, and beta-lactams since signal suppression values were below 20 % for most of the analytes. Likewise, other authors confirmed that the avermectin, subgroup of anthelmintics shows better results in the absence of acid (Inoue et al., 2009). For coccidiostats, strong signal enhancement in feed was already reported by other authors (A. Stolker et al., 2000). These results were decisive for

the use of clean-up in the extraction of animal feed, as no substantial improvement was observed between the application of any clean-up and the dilute and shoot procedure, as demonstrated and detailed in the supplementary material Table 5.

5. Method validation

5.1. Method validation of milk and complex chicken feed

In order to evaluate the suitability of the developed multi-method for the purpose, validation was carried out according to the SANTE/11312/2021. The SANTE validation guideline is developed for the validation of pesticide residue methods in food and feed. Nevertheless, this guideline covers the feed matrix and thus can be correlated to the conditions that take place in our routine laboratory. Maximum residue levels for milk within the EU can be found in EC, 37/2010, but the challenge of determining maximum residue levels for feed has not yet been addressed. The closest to this purpose is regulation EC, 1831/2003 on additives used in animal nutrition, although it does not specify the MRL of certain drugs. As a result, low concentration levels that will be examined for both matrices were set based on EC, 37/2010. Due to the challenges associated with matrix-matched calibration, such as the lack of a matrix that is completely devoid of all target analytes, validation was conducted through external calibration using solvent-based standards. Until a few years previous, validation guidelines used different definitions of the term "recovery," particularly whether or not this term comprises matrix effects if calibration needs to be performed using solvent based standards due to the non-availability of true blank samples. Amendments in SANTE/11312/2021 give a clear-cut definition of recovery (also referred to as "absolute recovery", "extraction recovery" or simply "recovery"), whereas no specification was given on the acceptable extent of matrix effects (and thus of apparent recoveries). Thus, in this work both apparent recovery as well as recovery of extraction were calculated (although the range given in SANTE71131272021 applies only to the latter) and compared to the target range of 70–120 % and the extended range of 60–140 %. For the milk matrix, 72 % of analytes fall in the apparent recovery range of 70 to 120 %, and when the range from 60 to 140 % is applied > 83 % of the analytes fall in the desired range. For 88 % of the analytes, the recovery of the extraction was within the range of 70–120 %, while this figure increased to > 95 % if the range for routine analysis was applied. Apparent recoveries for chicken feed were within the desired range for 35 % of analytes when acidic extraction was applied. On the other hand, when neutral extraction conditions were used, this share of analytes went up by almost 15 %. When routine laboratory criteria were applied, this share of analytes was in the desired range for > 50 % of the analytes, indicating a significant difference between extraction conditions. Recovery of extraction was in the range of 70–120 % for 74 % of analytes when an acidic extraction was applied, and this figure increased by 9 % when neutral extraction conditions were used. If the criteria for routine laboratories are considered, then in both cases, > 80 % of the analytes fall into the desired range (Fig. 1).

When co-eluting components interfere with the analytes of interest in the ionization process, matrix effects occur. This is manifested and expressed as signal suppression or enhancement (SSE). However, no proposal for classifying matrix effects could be found in the existing guidelines for the validation of analytical methods (FDA, 2018; EMA, 2011). Furthermore, the lack of classification parameters for matrix effects and related concerns has been expressed by other researchers in the field (Sulyok et al., 2020). In this work, matrix effects are classified as soft (±20 %, 80–100 %, and 100–120 %), moderate (±20-50, 50–80 %, and 120–150 %), and strong (> ± 50 %, > 150 %, < 50 %) as described in Ferrer Amate et al., 2010. In chicken feed regardless of the extraction conditions used, tetracyclines were prone to signal enhancement, while quinolones showed signal suppression (Fig. 2, a). If results from both matrices are checked, the susceptibility of tetracyclines to signal suppression can again be observed, while with sulfonamides, signal suppression is observed in both matrixes. Overall, moderate and strong

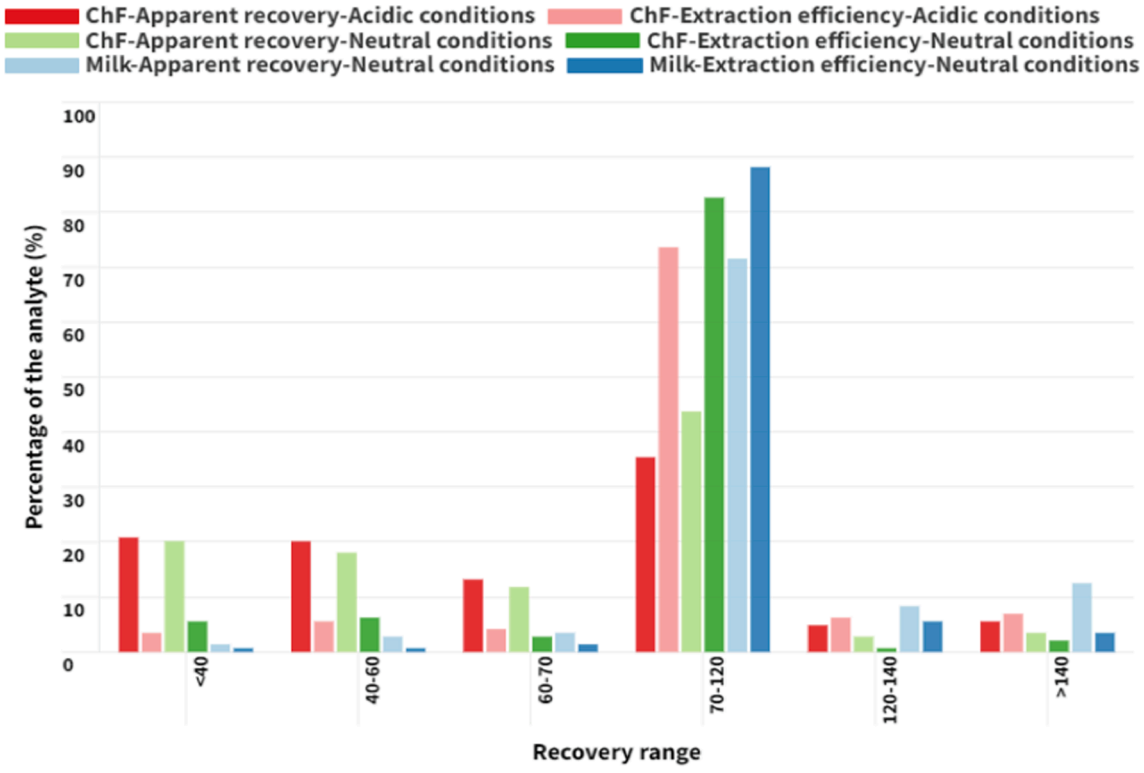

Fig. 1. Comparison of apparent recoveries (RA) and extraction efficiencies (RE) ranges with neutral (NC) and acidic (AC) extraction conditions obtained for chicken feed and milk matrix.

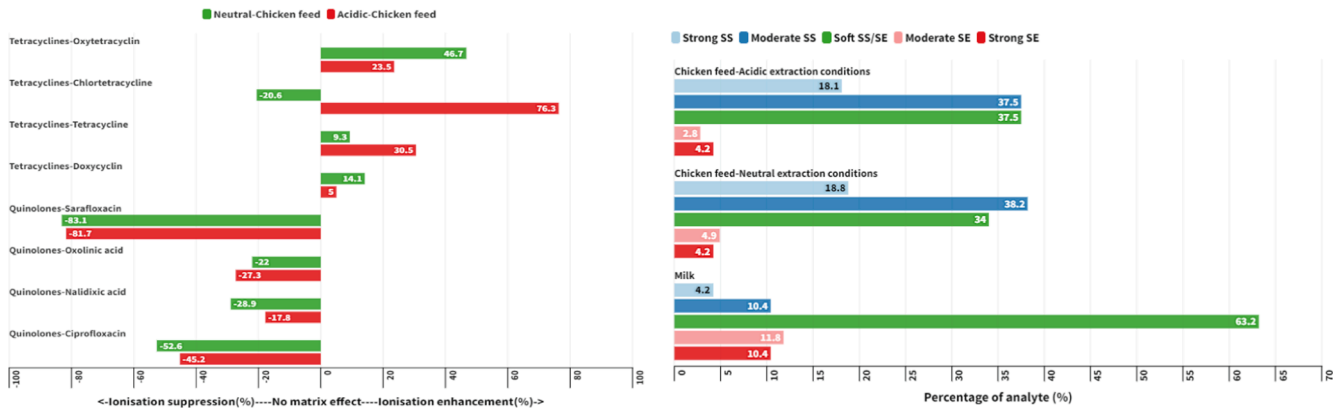

Fig. 2. A) Signal suppression/enhancement obtained for chicken feed between two different classes of drugs for the same matrix, B) Signal suppression/enhancement obtained for both investigated matrices.

signal enhancement for 22 % of analytes was observed in milk. In contrast, the high complexity of chicken feed caused strong and moderate signal suppression. Thus, within both extraction procedures, 55–57 % of the analytes were affected (Fig. 2,b). Similar results have been reported by another research group for the same type of matrix (Sulyok et al., 2020).

5.2. Method precision

The recommendation is to test repeatability and reproducibility on the basis of "identical/same test material" (EMA, 2011). Our recent studies, however, demonstrated that also relative matrix effects have an impact on the methods precision and thus on the overall uncertainty budget (Stadler et al., 2018). The relative effects of the matrix were investigated for the first time in the paper of Matuszewski et al., 2003 and represent variations between different lots of the same matrix. Hence, in order to also study the influence of lot-to-lot variation on the precision of our new methods, our validation efforts also included the analysis of individual test samples, with five different 'lots' of each commodity used. Five tested chicken feed samples consisted of different proportions of the same grains and were spiked at a high concentration level over three days. Milk samples differed in the percentage of fat content, and the spiking procedure remained the same. Samples were evaluated according to the criterion for precision and within laboratory reproducibility of RSD $<$ 20 %, as stated in the SANTE validation guideline. The repeatability of the apparent recovery (RA), extraction efficiencies (RE), and matrix effects (SSE) were calculated based on RSD values obtained from five individual samples spiked at a high level on the same day. The median value obtained from the results of the RA for milk is 5, while the median of RE and SSE results were between 4 and 6, indicating that the precision of the method was similarly impacted by both of these parameters (Fig. 3). Results of apparent recoveries for

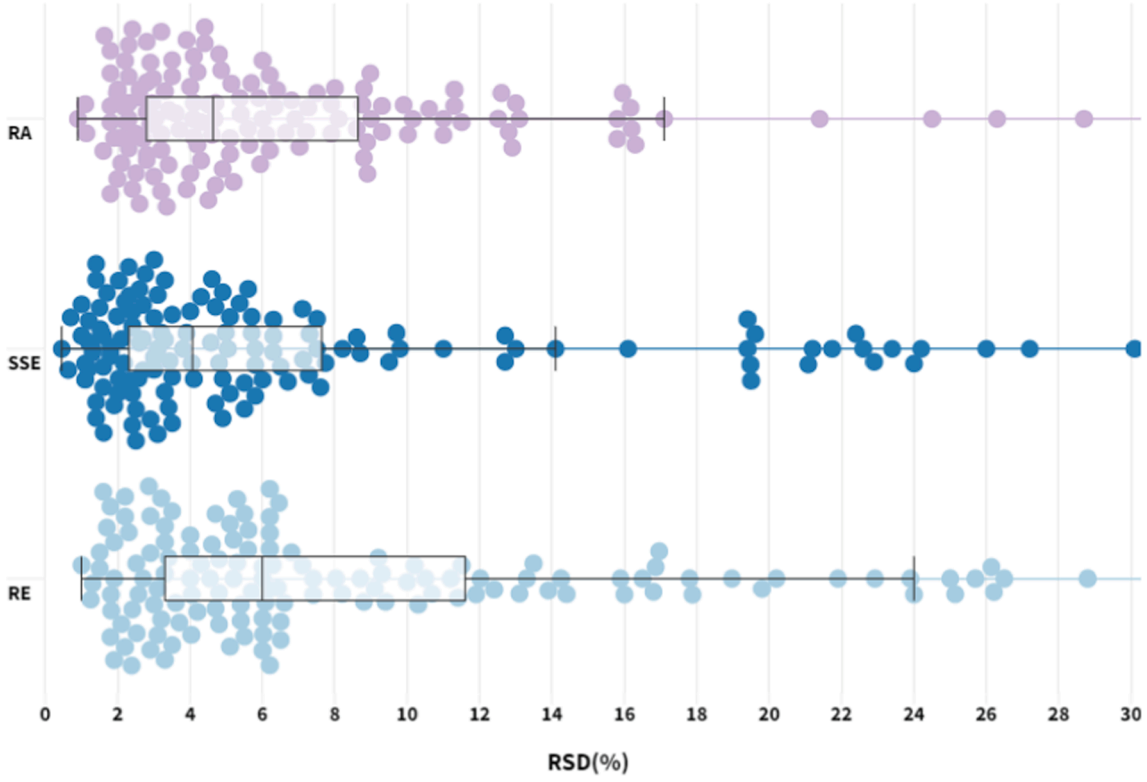

Fig. 3. Relative standard deviation (RSD) of apparent recoveries (RA), extraction efficiencies (RE), and signal suppression enhancements (SSE) obtained for milk matrix under repeatability conditions.

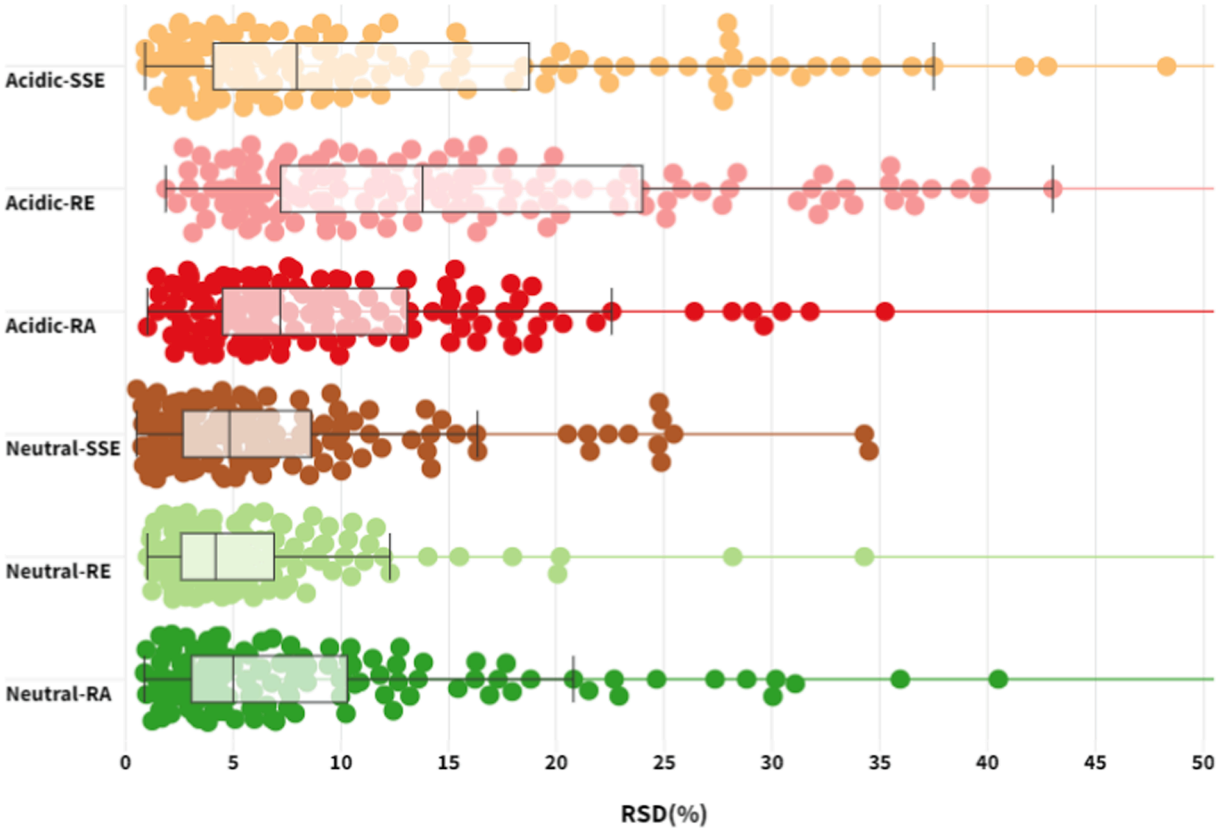

Fig. 4. Repeatability (RSD_r) of apparent recoveries (RA), extraction efficiencies (RE), and signal suppression enhancements (SSE) obtained for chicken feed matrix under neutral and acidic extraction conditions.

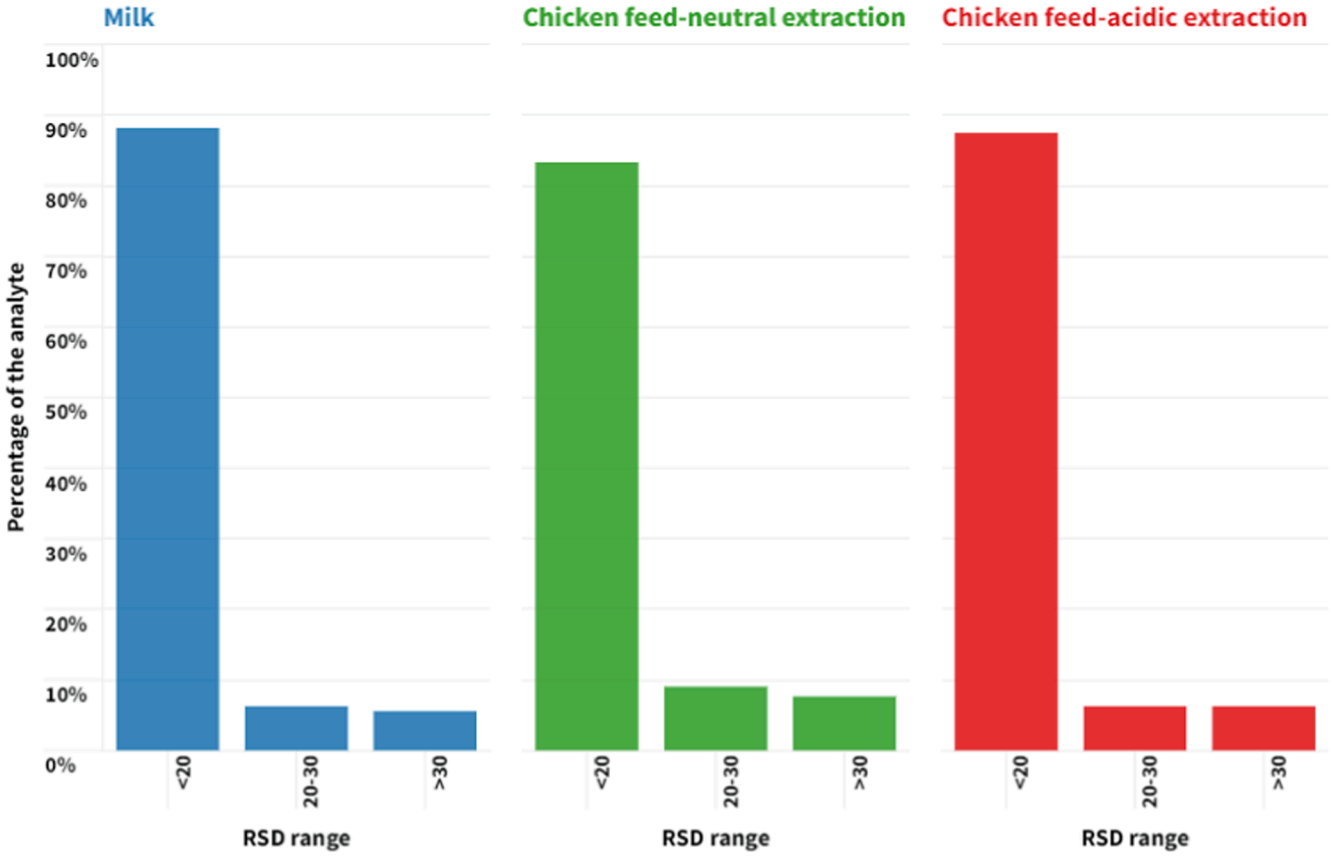

Fig. 5. Within laboratory reproducibility obtained for both investigated matrices.

chicken feed extracted under neutral and acidic conditions give a median of 5 and 7 %. Without significant changes, the median of RE and SSE results was between 4 and 5 % for neutral extraction conditions and between 7 and 8 % for acidic extraction conditions. The results (Fig. 4) reveal that only a small share of compounds exceed the RSD criterion of 20 %, although individual test samples were used instead of technical replicates from a single sample, making the results more robust. However, feed extracted under neutral conditions showed slightly better results than the one extracted under acidic conditions. Consequently, these results indicate that use of only one extraction procedure (neutral), could possibly be used for the analysis of both mycotoxins and residues of veterinary drugs. However, one caveat of using a neutral extraction solvent is that the extraction efficiency (RE) of the fumonisins is compromised. However, this should not be an issue as even with a lower RE, the lowest level permitted for fumonisins in feed is 5 ppm (5000 ppb), and even with a lower RE of even 50 %, this would still be easily detected. Further results obtained for the milk again highlighted the hypothesis of the reduction of signal enhancement by the milk matrix for certain classes of veterinary drugs and confirmed the feasibility of the use of matrix-matched calibration in routine laboratories.

The Within Laboratory Reproducibility (RSD_{WLR}) was calculated based on measurements obtained from five freshly spiked individual samples each day, overall 15 replicates over 3 days for each individual sample. For chicken feed extracted under acidic conditions and milk extracted under neutral extraction, 87–88 % of analytes are within the criteria of $RSD_{WLR} < 20$ %. With regards to the neutral extraction of chicken feed, the results were lower with 83.3 % of analytes falling into that range (Fig. 5). These results once again demonstrate that the difference between neutral and acid extraction is not pronounced overall but rather varies for individual drugs or drug classes. Despite this, the importance of this knowledge must not be understated as it indicates the potential of expanding the scope of the method to include mycotoxins, therefore reducing the need for two extracts to a single one as indicated earlier. On the other hand, matrix effects and extraction efficiencies remained unchanged over time, which is crucial for routine laboratories since matching standards with each individual matrix would be costly in terms of time and money.

5.3. LOD and LOQ

In the case of a multi-method with hundreds of analytes, the lowest calibration points near the limit of detection (LOD) would require extreme effort ((Sulyok et al., 2020; Wenzl et al., 2016). Therefore, the LOD and LOQ were calculated according to the EURACHEM validation guidelines. The standard deviation obtained at the low concentration level was multiplied by 10 for the calculation of LOQ (equal to RSD of 10 %) and by 3 for the LOD. The LOQ was below the MRL for 42 % of analytes, whereas, for 14 % of the analytes for which an MRL was indicated, the LOQ was higher than the MRL. For 44 % of the analytes, there were no given MRLs as these drugs were not intended for use in animals from which milk is produced (as shown in supplementary materials Table 6 and Fig. 2). Nevertheless, within that group, the LOQ for 87 % of the analytes was between 10 and 50 μg/kg. Obtained LOQs for analytes such as Chloramphenicol (prohibited) and Monesin, did not comply with the given limits, but still had a good signal peak intensity at low concentration levels. To avoid a significant overestimation of the LOQ, the calculation of LOQ for all the analytes was based on the signal-to-noise ratio as well (as shown in supplementary materials Table 5 and Figs. 3 and 4), even though it is not a recommendation of European reference laboratories. The LOD and LOQ of this method for chicken feed can be found in the supplementary materials Table 7, as MRLs for veterinary drug residues in animal feed are not available within regulations.

5.4. Interlaboratory comparison

For the purpose of the method cross-validation, the samples were exchanged between labs and analyzed 'blindly' to remove any bias. These results are indicated in the supplementary material (Table 8) and show a good overall correlation between the analytes detected between the laboratories. In order to statistically compare the results, a student two-tailed *t*-test was performed. The p-value was $0.1765 > 0.05$, indicating that the difference between the two sets of data between the two laboratories is not statistically significant. However, some of the analytes detected indicated a stronger difference in the values reported between the labs. To explain this, several facets of the methodologies were examined, with the main difference between methods being the use of fast polarity switching and therefore one chromatographic run in one lab compared to two separate runs in either mode in the other. However, this was ruled out as the cause of the differences between the results reported as the analytes indicating this were not at the same retention times (RTs), and it was also the case for analytes in both polarities. In order to investigate further, the measurement uncertainty ($U_{lot\text{-}to\text{-}lot}$) was calculated for each analyte from the results of the validations, with this calculated separately for each of the two labs involved, these results are available in the supplementary material Table 9. There were small differences in results reported between labs, the application of the $U_{lot\text{-}to\text{-}lot}$ negated the differences, with results overlapping within due to the measurement uncertainty. The application of Ulot-to-lot did not correct the differences in levels reported between those that showed significant differences in the calculated concentrations. To investigate further, poultry samples in which results were significantly different for some AMRs detected, were re-extracted in each laboratory in triplicate. This was done to check the sample's homogeneity and whether the levels detected in each sample would differ between replicate extracts, potentially due to 'hot spots' in the samples (Delatour et al., 2018). This appeared to confirm that the differences in levels reported for some of the AMRs detected were potentially due to hot spots within the samples, with this indicated through differences shown in the levels calculated from triplicate extractions of the same sample, such as monensin in one sample (503) and nicarbazin in two other samples (529 and 532). Furthermore, the method's robustness had been verified by the validation of the method, with the RSD for those compounds affected being below 20 %, further indicating that the variation was indeed due to the sample and not the methodology used.

6. Conclusion

A novel LC-MS/MS method has been developed to simultaneously determine up to >140 antimicrobial and antiparasitic compounds across 15 drug classes. The stability of the standards, the suitability of the dilution solvent in milk, and the effectiveness of clean-up procedures in the extraction of chicken feed were tested as part of this work. A marginal improvement in results was observed when neutral conditions for the extraction and dilution of both matrices were compared to acidic conditions, although these differences were more pronounced in certain drug classes. This indicates that expanding the scope of the existing method to include mycotoxins, which normally require acidic conditions, is feasible. For chicken feed, different clean-up procedures were tested and the results were compared to a simple dilute-and-shoot methodology. Nevertheless, none showed a significant reduction in the matrix effects (SSE) as was expected based on the literature review. Therefore, we concluded that the increased workload and the use of hazardous chemicals did not justify the use of an additional clean-up step. Method validation again revealed slightly better results when neutral extraction conditions were used for the extraction of chicken feed. Furthermore, for only 14 % of the analytes, the respective LOQs

obtained for milk were not within the MRLs. Although there are no regulations for MRL of veterinary drugs in chicken feed, the evaluated data demonstrate that over 80 % of the analytes had LOQs between 10 and 50 μg/kg. The developed and validated method has the potential to facilitate the generation of comprehensive datasets on the presence of antimicrobials, antiparasitics, and feed additives in milk and chicken feed. Despite strict regulations in developed countries such as the EU, the globalization of food systems and the lack of (enforced) regulations in developing countries, can still have a potentially severe impact on the risk of these contaminants entering the food and feed supply and thus promoting the growth of antibiotic-resistant bacteria. The method also meets the analytical requirements of end-user laboratories, even when dealing with complex samples as feed, and could be extended to include mycotoxins.

CRediT authorship contribution statement

Lidija Kenjeric: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Michael Sulyok:** Conceptualization, Methodology, Writing – review & editing. **Alexandra Malachova:** Conceptualization, Methodology, Writing – review & editing. **Brett Greer:** Conceptualization, Methodology, Writing – review & editing. **Oluwatobi Kolawole:** Formal analysis, Investigation, Methodology, Validation. **Brian Quinn:** Conceptualization, Methodology, Writing – review & editing. **Christopher T. Elliott:** Funding acquisition, Project administration, Resources, Writing – review & editing. **Rudolf Krska:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, 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.

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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.2024.138834.

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