

# LC-QTOF-MS evaluation of rabbit-specific peptide markers for meat quantitation

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## Abstract

Ten rabbit-specific tryptic peptide markers and one marker peptide specific to both rabbit and hare were evaluated for mass signal linearity in binary meat mixtures using liquid chromatography-quadrupole time-of-flight-mass spectrometry. Seven meat mixtures containing chicken and varying percentages of rabbit (1%, 5%, 10%, 30%, 60%, 90%, and 100%) were analyzed. Additionally, the signal linearity of twelve peptide markers for chicken meat was examined. The best candidate peptides for the quantification of meat content were selected. Five of eleven peptides for rabbit meat and five of twelve peptides for chicken meat showed good linearity ( $R^2 > 0.97$ ). The limits of detection and limits of quantification for these markers were in the range of 0.43–1.91% [w/w] and 1.44–6.38% [w/w], respectively. The method allowed determination of the percentage content of rabbit and chicken meat in two- and three-component meat mixtures with good accuracy. The preliminary quantification data provide a starting point for developing label-free and absolute quantification methods for rabbit and chicken meat using multiple reaction monitoring of peptide markers.

**Keywords:** Linearity, Liquid chromatography-mass spectrometry, Meat products, Peptide markers, Rabbit

## 1. Introduction

Meat fraud and adulteration have increased considerably in recent years, although food authenticity and correct labeling are required by many laws and regulations [1]. These unlawful practices are motivated by economic gain and have an enormous impact on public health. Undeclared meat can be a source of toxins, pathogens, and protein allergens responsible for severe allergic responses in consumers [2]. The most frequently reported types of fraud are the replacement of higher-value types of meat with inferior, cheaper, or undesirable alternatives, the presence of undeclared meats, and the replacement of meat with plant proteins. Most meat fraud is economically motivated, such as the addition of low-cost chicken to rabbit [3], pork to beef [4] or duck to lamb [5]. A small amount of adulteration might be due to unintentional cross-contamination during the

processing of different types of meat in the same facility [6]. Highly processed meat products are among the most prone to adulteration because the production of minced meat under high temperature conditions removes the morphological characteristics of muscle, making it difficult to identify the species of origin.

A minimum adulteration ratio of 1% (w/w) meat in a sample is considered intentional adulteration [7], and thus distinguishing intentional from accidental contamination requires a sensitive and reliable detection method. Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) provides a sequence-specific, protein-based approach of high sensitivity and selectivity adequate for the purpose. LC-MS/MS methods have high discrimination power and enable the simultaneous detection of multiple adulteration species in a single analysis. LC-MS/MS combines the high selectivity and efficiency of peptide separation by LC with peptide

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identification due to the high sensitivity and selectivity of mass spectrometry (MS). The potential of multi-methods using multiple reaction monitoring (MRM) or single-stage high-resolution MS methods for detecting meat adulteration below 1% (w/w) has been demonstrated. Li et al. [8] detected 0.5% (w/w) pork and beef in thermally processed samples. Von Bargen et al. [9] identified 0.24% (w/w) pork and horse meat mixed with beef. Montowska and Fornal [10] detected processed beef proteins at 0.8% (w/w) in commercial poultry frankfurters. Claydon et al. [7] found horse meat in corned beef at 0.5% (w/w) using a myoglobin peptide marker. Pan et al. [11] identified 0.5% (w/w) of four species in mixed meat. Montowska and Fornal [10,12] detected 1% (w/w) chicken and 1% (w/w) pork in a three-species meat mixture. All these results are based on the detection of the primary structures of proteins resistant to thermal processing, and thus these peptides can be used as species-specific markers for meat authentication. A list of 105 heat-stable species-specific peptide markers detected by LC-MS/MS originating from 11 types of meat (pork, beef, lamb, chicken, duck, goose, turkey, rabbit, buffalo, red deer, and horse) is available in our review paper [13].

Evaluating peptide markers by assessing their signal linearity is a critical step in quantification because not all peptide markers remain stable with acceptable linear correlation in meat mixtures [14–17]. For example, 49 heat-stable rabbit-specific peptides have been published to date, of which only one peptide, PHSHPALTPEQK ( $m/z$  447.9022<sup>3+</sup>) originating from the metabolic enzyme fructose-bisphosphate aldolase A isoform X1, was evaluated for its usefulness for quantification by Prandi et al. [18]. Rabbit meat is rich in protein and is regarded as healthy owing to its low allergenicity and excellent nutritional properties, including low fat and cholesterol levels [19,20]. The meat has also gained popularity and acceptance worldwide due to the absence of religious restrictions. Rabbit is more expensive than chicken, making it susceptible to fraudulent mislabeling.

In the present study, LC-quadrupole time-of-flight (QTOF)-MS was used to evaluate the linearity of the MS signals for rabbit meat peptide markers in binary meat mixtures of chicken with varying weight percentages of rabbit (1%, 5%, 10%, 30%, 60%, 90%, and 100%) to examine the suitability of markers for meat quantification. We selected ten rabbit-specific tryptic peptide markers and one tryptic peptide marker specific to both rabbit and hare which were the most frequently detected in highly processed meat products [3]. In addition, linearity testing was expanded to twelve peptide

markers for chicken, allowing us to monitor the abundance of chicken marker peptides in meat mixtures.

## 2. Materials and methods

### 2.1. Materials

LC-MS grade acetonitrile and methanol were supplied by Fisher Chemical (Waltham, MA, USA). Ultrapure water was obtained using a purification system (Millipore Direct-Q3-UV, Merck KGaA, Darmstadt, Germany). Ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), and formic acid (LC-MS grade) were obtained from Merck KGaA. MS grade lyophilized trypsin was purchased from Promega (Milford, MA, USA). DTT, IAA, and trypsin were dissolved in 50 mM aqueous ammonium bicarbonate solution. Reverse-phase extraction Sep-Pak C18 Plus short cartridges were purchased from Waters (Milford, MA, USA; sorbent weight 360 mg/0.7 mL).

### 2.2. Meat processing

Fresh rabbit (*Oryctolagus cuniculus*), chicken (*Gallus gallus*), and pork (*Sus scrofa*) were purchased from local retailers in March 2021 (Lublin, Poland). All meat samples were cut into 2-cm-thick slices and heat-treated separately by boiling in water at 100 °C for 30 min, and then they were cooled to room temperature and minced using a hand blender (MQ 7087, Braun, Kronberg, Germany). To avoid cross contamination, each meat was processed separately using a different blender container. The minced meats were immediately stored at –20 °C until use.

### 2.3. Preparation of meat mixtures

Meat mixtures were prepared from cooked and minced meats as follows. Calibration samples (S1–S7) were prepared from chicken and rabbit meat; the content of the latter was 1%, 5%, 10%, 30%, 60%, 90%, or 100%. On a separate day, two- and three-component meat samples containing 5% or 50% (w/w) rabbit meat were prepared using meat derived from different carcasses from these used for the calibration samples. Samples containing two species (B5, B50) were prepared by weighing appropriate amounts of chicken and rabbit meat. Samples containing three species (T5, T50) were prepared by weighing appropriate amounts of chicken and pork to obtain samples containing equal amounts of these species, together with 5% or 50% (w/w) rabbit meat. The meat mixture compositions

are presented in Supplementary materials Table S1 (the Supplemental Content can be found here: [https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3403&context=journal&type=additional&preview\\_mode=1](https://www.jfda-online.com/cgi/viewcontent.cgi?filename=1&article=3403&context=journal&type=additional&preview_mode=1)).

#### 2.4. Homogenization

All samples (0.3 g) were homogenized in 1 mL 100 mM ammonium bicarbonate using a laboratory ball homogenizer (Mini-Mill Pulverisette 23, Fritsch GmbH, Idar-Oberstein, Germany) with 5-mm-diameter balls. The grinding bowl oscillated at 3000 oscillations/min for two cycles of 1 min. The homogenates were dried under vacuum using a miVac Duo concentrator (Genevac Ltd., Ipswich, UK).

#### 2.5. In-solution trypsin digestion

Dried samples (5 mg) were rehydrated for 1 h in 50 mM ammonium bicarbonate (100  $\mu$ L), and then the proteins were reduced with 200 mM DDT at 56 °C for 1 h and alkylated using 200 mM IAA for 30 min in the dark at room temperature. The remaining IAA was neutralized by adding 200 mM DTT and incubated at room temperature for 30 min. Next, the samples were digested in ammonium bicarbonate solution containing 0.083  $\mu$ g/ $\mu$ L trypsin at 37 °C for 18 h, and then the insoluble materials, salts, and excess reagents were removed by reverse-phase solid-phase extraction using Sep-Pak C18 Plus cartridges. The column was equilibrated with solvent B (65% acetonitrile, 35% Milli-Q water, and 0.1% formic acid), and then with solvent A (98% Milli-Q water, 2% acetonitrile, and 0.1% formic acid). The digested sample was added to the cartridge, washed with solvent A, and then the peptides were eluted with solvent B. The purification solvents were removed under vacuum using the miVac Duo concentrator. Prior to LC-QTOF-MS analysis, the dried peptides were reconstituted with 5% acetonitrile in Milli-Q water containing 0.1% formic acid.

#### 2.6. LC-QTOF-MS analysis

LC-QTOF-MS analyses were performed using a high-performance liquid chromatograph (1290 Infinity, Agilent Technologies, Santa Clara, CA, USA) coupled to an accurate mass QTOF mass spectrometer (6550 iFunnel, Agilent Technologies) equipped with a ion source (Jet Stream, Agilent Technologies). The chromatographic separations were conducted using an RRHD Eclipse Plus analytical column (Agilent Technologies;

2.1  $\times$  150 mm, 1.8  $\mu$ m) at a flow rate of 0.3 mL/min. The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient elution program was as follows: 0–2 min, 3% B; 2–40 min, to 35% B; 40–45 min, to 40% B; 45–50 min, to 90% B; 50–55 min, 90% B; and a 5 min post-run at 3% B. The injection volume was 10  $\mu$ L and the column temperature was maintained at 40 °C. The mass spectrometer was operated in positive electrospray ionization mode (ESI+) with the following settings: ion source gas, N<sub>2</sub>; ion source gas temperature, 250 °C; ion source gas flow rate, 14 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 250 °C; sheath gas flow rate, 11 L/min; and capillary voltage, 3500 V. The nozzle voltage was 1000 V and the fragmentor voltage was 400 V. Positive ions were acquired in the range 100–1700 *m/z* in MS scan mode and in auto MS/MS mode; for MS scan mode the scan rate was 1.5 scans/s and for the auto MS/MS mode scan rates of 9 scans/s for MS and 7 scans/s for MS/MS were applied. Internal mass calibration was enabled by using the reference ions at *m/z* 121.0509 and 922.0098. The data were recorded with Agilent MassHunter Data Acquisition B.09.00 software and processed using Agilent MassHunter Qualitative Analysis B.07 software (Agilent Technologies). Peptide annotations were performed by MS/MS ions matching using Spectrum Mill MS Proteomics Workbench (Agilent Technologies) with the following parameters: trypsin enzyme; NCBI protein database (76640 entries for *Gallus gallus* and 53133 entries for *Oryctolagus cuniculus*, downloaded on March 26, 2021); two missed cleavages; 5 ppm precursor ion mass tolerance; 50 ppm product ion mass tolerance; maximum 6+ precursor charge; and carbamidomethylation as fixed modification. The results were validated at 1.2% of the false discovery rate across each LC run using Spectrum Mill Auto Thresholds Strategy and Peptide Mode.

#### 2.7. Linearity evaluation

Linearity studies were based on the peak areas of the extracted ion chromatograms for the marker peptides. Protein digests of the binary meat mixtures containing various amounts of rabbit and chicken at the concentrations of 1:99, 5:95, 10:90, 30:70, 60:40, 90:10 and 100:1 (rabbit: chicken w/w) were analyzed in triplicate. Calibration curves were generated for 10 rabbit-specific tryptic peptide markers and one tryptic peptide marker specific to both rabbit and hare. In addition, 12 tryptic peptides were selected for chicken meat. Linearity was assessed by evaluating the correlation coefficients

( $R^2$ ) and by analyzing the residuals. The acceptance criterion for the correlation coefficients was set at 0.97 for  $R^2$ . The limit of detection (LOD) and the limit of quantification (LOQ) were determined for peptides which met the acceptance criterion for linearity. LOD and LOQ were calculated based on signal-to-noise ratios of 3 and 10, respectively.

### 2.8. Stability of marker peptides

Short-term stability of samples was assessed by comparing peptide signals obtained for freshly prepared samples and for samples stored for 24 h at 10 °C. Evaluations were conducted on the 1% and 100% (w/w) rabbit meat samples.

Long-term stability of the 1% and 100% (w/w) rabbit meat samples was assessed by comparing peptide signals in freshly prepared samples to those obtained for samples stored for 7 days at –20 °C.

Stability was calculated according to Eq. (1).

$$\text{Stability}(\%) = 100 \frac{\text{Peptide marker peak area at 24 h/7 days}}{\text{Peptide marker peak area at 0 h}} \times 100\% \quad (1)$$

## 3. Results and discussion

### 3.1. Selection of peptides

Ten rabbit-specific peptides and one tryptic peptide marker specific to both rabbit and hare were selected to evaluate their usefulness for quantifying rabbit meat content. We chose the top eleven heat-stable tryptic peptide markers confirmed previously by us in complex meat matrix samples of highly processed sterilized and roasted pâté-type products with a declared rabbit meat content [3]. For chicken meat, we selected tryptic peptides reported previously as suitable for chicken meat authentication [7,8,10,12,17,18,21,22] and experimentally confirmed in our laboratory (i.e., were detected in complex meat matrix samples from products with declared chicken meat content [3]). Theoretical isotopic masses and the amino acid sequences of the selected for study peptides along with the protein names and NCBI protein accession numbers are presented in Table S2 in Supplementary materials.

All the selected peptide markers were detected in the analyzed meat mixtures using bottom-up LC-QTOF-MS/MS method with a retention time of  $\pm 0.2$  min and an accurate peptide mass error of  $< 5$  ppm. For the calibration samples (S1–S7), MS/MS ions were confirmed by Spectrum Mill

extraction and search algorithms with scored peak intensity in the range of 62.3–100% (Table 1). Chromatograms of the eleven selected heat-stable peptides for rabbit (including one for both rabbit and hare) and the twelve selected heat-stable peptides for chicken present in thermally processed binary meat mixtures (B50) are shown in Fig. 1.

### 3.2. Assessment of specificity

All the 23 tryptic peptide markers were checked for their suitability for differentiating rabbit and chicken meat. BLAST verification (accessed 2 May 2021) showed that of the 11 rabbit and 12 chicken markers, 2 and 8 peptides, respectively, were present in proteins from other species, of which 1 and 7 peptides, respectively, showed homologies to other consumed species. The benefit of including in the methods peptides that show homology to closely-related species in addition to species-specific peptides is to increase the reliability of detecting a particular meat, especially when present at low amounts in complex food mixes. These peptides can be considered as supporting peptides that together with species specific peptides enhance species detection. The Clustal Omega protein multiple sequence alignment of the corresponding peptides and their homologies with other consumed species are shown in Figure S1 (Supplementary materials). The list of homologies is given in Supplementary materials (Tables S3 and S4). Finally, we confirmed 10 out of 11 and 5 out of 12 tryptic peptides as rabbit- and chicken-specific markers, respectively, i.e. they are not present in proteins of other commonly consumed species.

Our verification of chicken meat is consistent with the experimental and BLAST verification of 40 amino-acid sequences for chicken meat presented by Häfner et al. [23]. Out of 40 chicken peptides, 23 showed homologies to other consumed species according to the NCBI protein database and seven showed homologies to those confirmed experimentally. None of the 40 chicken peptides were detected in rabbit meat by experimental and BLAST verification.

To confirm the utility of the 23 selected peptides for distinguishing rabbit and chicken experimentally, the presence of each peptide in pure chicken and rabbit meat was checked. None of the rabbit peptides were detected in chicken proteins and none of the chicken peptides were detected in rabbit proteins by experimental and BLAST verification, showing that all the selected peptides can be used as markers for unambiguous LC-MS/MS discrimination of rabbit from chicken meat.



Table 1. Linearity evaluation results for twenty-three heat-stable peptide markers for rabbit and chicken meat. The peptides with  $R^2 > 0.97$  are in bold.

Peptide sequence	Formula	tR ± SD (min)	Observed (m/z) Average ± SD	Theoretical (m/z)	SPI (%)	Regression equation	R <sup>2</sup>
<b>Rabbit</b>							
PHSHPALTPEQK	C <sub>59</sub> H <sub>92</sub> N <sub>18</sub> O <sub>18</sub>	3.68 ± 0.05	447.9037 ± 0.00028	447.9022	70.9–92.0	$y = 137313x + 515737$	0.9919
SSVFDVADPK	C <sub>43</sub> H <sub>68</sub> N <sub>10</sub> O <sub>14</sub>	11.82 ± 0.06	475.2544 ± 0.00018	475.2533	77.7–94.1	$y = 113945x + 351583$	0.9902
GDEVFTVTEGR	C <sub>51</sub> H <sub>80</sub> N <sub>14</sub> O <sub>20</sub>	14.88 ± 0.04	605.2936 ± 0.00020	605.2918	67.7–89.5	$y = 10833x + 121119^a$	0.9206
AFFGHYLYEVAR	C <sub>72</sub> H <sub>97</sub> N <sub>17</sub> O <sub>17</sub>	22.55 ± 0.05	491.5820 ± 0.00016	491.5828	63.5–77.0	$y = 22013x + 137309^a$	0.9809
LQLYSQFLGK	C <sub>57</sub> H <sub>89</sub> N <sub>13</sub> O <sub>15</sub>	23.35 ± 0.08	598.8380 ± 0.00022	598.8377	76.3–82.4	$y = 19797x + 18200^a$	0.9912
ALVFQPVTELQNQDTDFEHR	C <sub>101</sub> H <sub>154</sub> N <sub>28</sub> O <sub>32</sub>	25.10 ± 0.04	758.0524 ± 0.00008	758.0509	73.1–95.3	$y = 16588x + 331888$	0.9449
TLAFLFSGAQTGEEGGGGGK	C <sub>82</sub> H <sub>126</sub> N <sub>22</sub> O <sub>29</sub>	25.34 ± 0.09	942.4628 ± 0.00015	942.4609	73.6–90.3	$y = 24823x + 26024^a$	0.8982
VLAAFSEGLNHLNDNLK <sup>b</sup>	C <sub>78</sub> H <sub>125</sub> N <sub>21</sub> O <sub>24</sub>	24.35 ± 0.06	580.9837 ± 0.00015	580.9813	77.9–96.5	$y = 17241x + 242155^a$	0.9555
EFNAETFTFHADIC*TLPETER	C <sub>111</sub> H <sub>162</sub> N <sub>28</sub> O <sub>38</sub> S	25.87 ± 0.10	843.3874 ± 0.00026	843.3852	89.2–93.6	$y = 6979.9x + 50049^a$	0.8727
TLAFLFTGTA AAAEAEGGGK	C <sub>81</sub> H <sub>126</sub> N <sub>20</sub> O <sub>27</sub>	27.71 ± 0.08	906.4637 ± 0.00032	906.4626	71.4–100.0	$y = 23487x + 1295142$	0.7038
VLAAFSEGLSHLDNLK	C <sub>77</sub> H <sub>124</sub> N <sub>20</sub> O <sub>24</sub>	25.43 ± 0.05	571.9792 ± 0.00017	571.9777	82.4–96.8	$y = 16208x + 69286^a$	0.9722
<b>Chicken</b>							
ALGQNPTNAEINK <sup>c</sup>	C <sub>57</sub> H <sub>96</sub> N <sub>18</sub> O <sub>21</sub>	9.23 ± 0.04	685.3582 ± 0.00043	685.3571	72.2–92.0	$y = 308683x - 2319322$	0.8442
VAGAAALPC*APAVK	C <sub>54</sub> H <sub>93</sub> N <sub>15</sub> O <sub>15</sub> S	15.13 ± 0.08	612.8343 ± 0.00035	612.8314	75.3–96.9	$y = 31186x - 232982$	0.8486
AFEEAAEHFQPYVK <sup>c</sup>	C <sub>78</sub> H <sub>108</sub> N <sub>18</sub> O <sub>23</sub>	17.92 ± 0.05	555.9375 ± 0.00028	555.9351	70.8–94.3	$y = 29597x - 305454$	0.8367
IGIFTEDEEVSGR	C <sub>62</sub> H <sub>98</sub> N <sub>16</sub> O <sub>24</sub>	19.71 ± 0.06	726.3571 ± 0.00016	726.3542	72.0–96.1	$y = 36093x + 491279$	0.7249
MTEEEVEELMK <sup>c</sup>	C <sub>56</sub> H <sub>94</sub> N <sub>12</sub> O <sub>23</sub> S <sub>2</sub>	20.59 ± 0.07	684.3081 ± 0.00026	684.3071	75.4–94.3	$y = 207722x - 463178$	0.9820
EPADAMAAGAVEASFK <sup>c</sup>	C <sub>67</sub> H <sub>105</sub> N <sub>17</sub> O <sub>24</sub> S	20.59 ± 0.06	782.8714 ± 0.00027	782.8692	74.0–97.8	$y = 40202x + 49632$	0.9813
DLFDPVIQDR <sup>c</sup>	C <sub>54</sub> H <sub>84</sub> N <sub>14</sub> O <sub>18</sub>	23.20 ± 0.05	609.3134 ± 0.00025	609.3117	74.0–94.4	$y = 143644x - 612558$	0.9831
LDVPISGEPAPTITWK	C <sub>79</sub> H <sub>124</sub> N <sub>18</sub> O <sub>24</sub>	24.05 ± 0.04	855.4607 ± 0.00029	855.4590	75.9–96.7	$y = 17837x - 147339$	0.8818
TSDVDSVFFIR	C <sub>58</sub> H <sub>88</sub> N <sub>14</sub> O <sub>19</sub>	25.20 ± 0.05	643.3271 ± 0.00024	643.3248	70.5–98.9	$y = 20859x - 106094$	0.8980
GEMLDLQHGSLFLK <sup>c</sup>	C <sub>71</sub> H <sub>114</sub> N <sub>18</sub> O <sub>21</sub> S	24.63 ± 0.06	529.9471 ± 0.00015	529.9448	62.3–86.5	$y = 155873x + 161071$	0.9909
DQGTFFDFVEGLR <sup>c</sup>	C <sub>66</sub> H <sub>97</sub> N <sub>17</sub> O <sub>24</sub>	28.25 ± 0.05	756.8544 ± 0.00025	756.8519	72.1–91.8	$y = 200739x - 1210734$	0.9884
LAMQEFMVLPVGAASFHDAMR	C <sub>103</sub> H <sub>161</sub> N <sub>27</sub> O <sub>28</sub> S <sub>3</sub>	32.12 ± 0.06	774.3813 ± 0.00033	774.3795	77.0–94.2	$y = 295527x - 338945$	0.8996

C\* – carbamidomethylated cysteine; tR – retention time; SD – standard deviation; SPI – scored peak intensity; <sup>a)</sup> linearity range (5%–100%); <sup>b)</sup> also occurs in hare; <sup>c)</sup> also occurs in usually consumed species; see Table S1 (Supplementary materials).

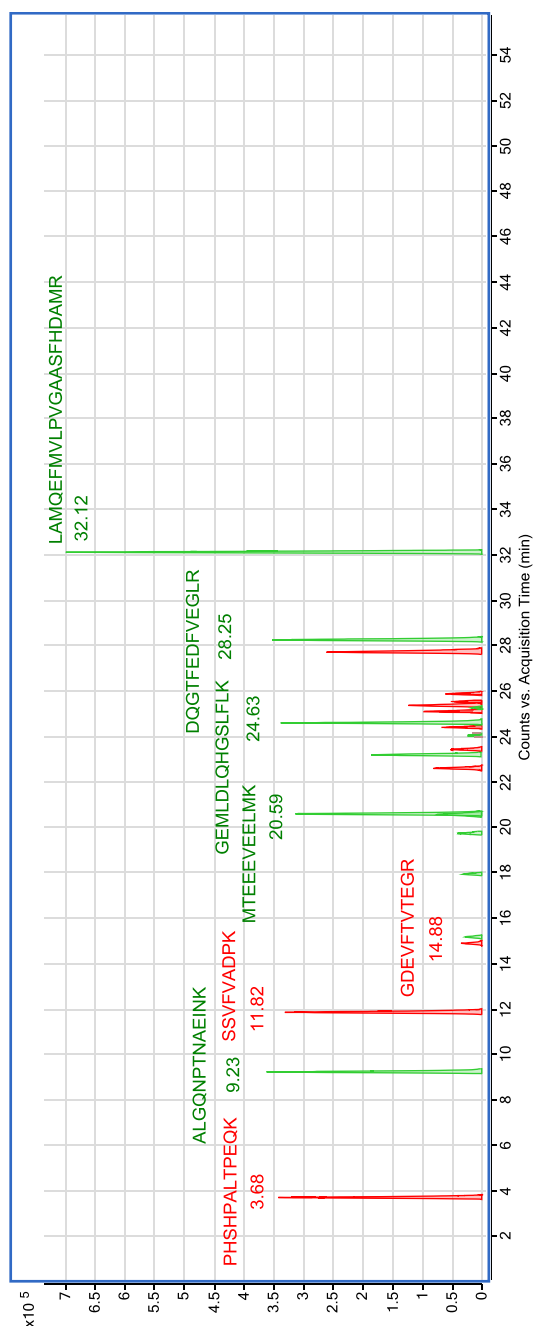


Fig. 1. LC-QTOF-MS/MS extracted ion chromatograms of 23 heat-stable marker peptides present in a binary meat mixture (B50). The 11 marker peptides for rabbit are shown in red and the 12 marker peptides for chicken are shown in green.

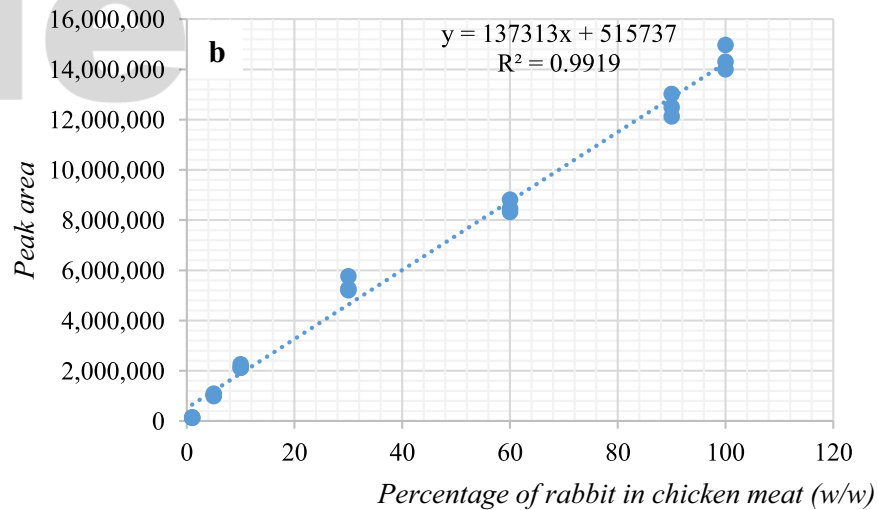
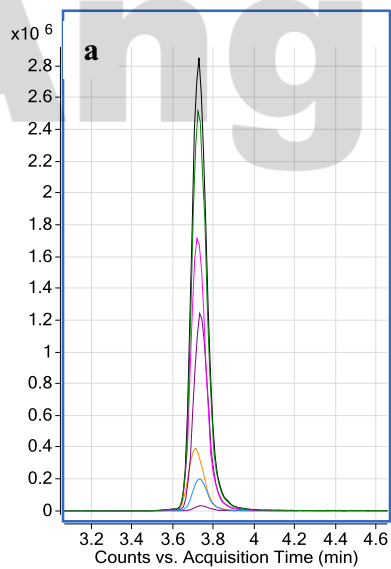
### 3.3. Evaluation of peptide marker linearity

The correlation between the peak area of the most abundant MS ion of each peptide and the percentage concentration of meat was evaluated in the range of 1%–100% (w/w) and 10%–99% (w/w) meat content for rabbit and chicken, respectively. However, for the six rabbit-specific peptides, GDEVFTVTEGR ( $m/z$  605.2918<sup>2+</sup>), AFFGHYLYEVAR ( $m/z$  491.5828<sup>3+</sup>), LQLYSQFLGK ( $m/z$  598.8377<sup>2+</sup>), TLAFLFSGAQTGEEGGGGK ( $m/z$  942.4609<sup>2+</sup>), VLAASFSEGLNHLNLIK ( $m/z$  580.9813<sup>3+</sup>), and EFNAETFTFHADICTLPETER ( $m/z$  843.3852<sup>3+</sup>), and for the one tryptic peptide for both rabbit and hare, VLAASFSEGLSHLDNLIK ( $m/z$  571.9777<sup>3+</sup>), linear regression was fitted over the range 5%–100% (w/w). We did not detect these seven tryptic peptides in calibration samples (S1) containing 1% rabbit meat. The results are summarized in Table 1. Five of the 11 peptides for rabbit meat and five of the 12 peptides for chicken meat showed good linearity, with  $R^2$  coefficients exceeding 0.97. The highest  $R^2$  values were found for the rabbit peptide, PHSHPALTPEQK ( $m/z$  447.9022<sup>3+</sup>), derived from metabolic enzyme fructose-bisphosphate aldolase A isoform X1, and for the chicken peptide, GEMLDLQHGSFLFK ( $m/z$  529.9448<sup>3+</sup>), derived from the L-lactate dehydrogenase A chain. Extracted ion chromatograms and calibration curves for these peptides are presented in Fig. 2. Calibration curves for the remaining rabbit and chicken peptides with  $R^2 > 0.97$  are presented in the Supplementary materials (Figure S2).

Of the rabbit peptides evaluated in this study, only PHSHPALTPEQK ( $m/z$  447.9022<sup>3+</sup>) has previously been used for quantifying rabbit meat. Prandi et al. [18] quantified the amount of rabbit meat (in %, w/w) in Bolognese sauce samples using PHSHPALTPEQK with a mass label of 447.8 ( $m/z$ ) and selected reaction monitoring transitions 447.8 → 234.8 and 447.8 → 314.0 as a rabbit-specific marker with a linear regression fitting of  $R^2 = 0.931$ , and LOD and LOQ values of 1.8% (w/w) and 5.5% (w/w), respectively. Of the five rabbit-specific peptides with  $R^2 > 0.97$ , three (PHSHPALTPEQK ( $m/z$  447.9022<sup>3+</sup>); SSVFVADPK ( $m/z$  475.2533<sup>2+</sup>), and AFFGHYLYEVAR ( $m/z$  491.5828<sup>3+</sup>)) were identified by us previously as the most reliable heat-stable markers for detecting rabbit meat in highly processed meat products [3].

All five rabbit-specific peptides with  $R^2 > 0.97$  were shown in the present study to have high quantitative potential, and thus can be used to detect rabbit meat at low concentrations. Their LOD and LOQ values were as follows: 0.43% and 1.44%

**I. PHSHPALTPEQK ( $m/z$  447.9022<sup>3+</sup>)**



**II. GEMLDLQHGSFLK ( $m/z$  529.9448<sup>3+</sup>)**

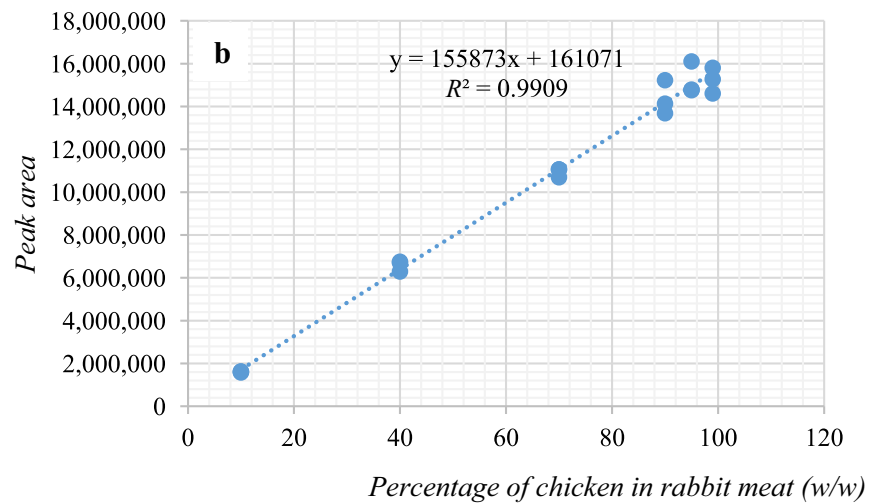
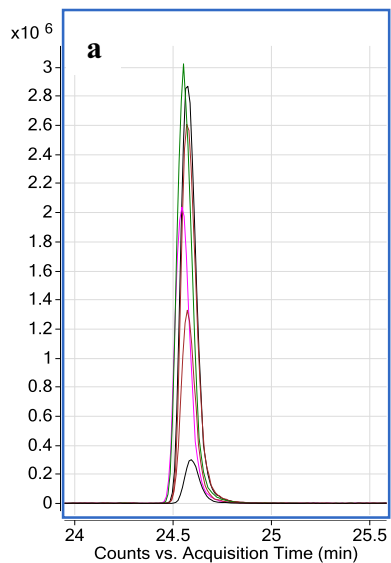


Fig. 2. Extracted ion chromatograms (a) and calibration curves (b) for (I) the rabbit peptide PHSHPALTPEQK ( $m/z$  447.9022<sup>3+</sup>) and (II) the chicken peptide GEMLDLQHGSFLK ( $m/z$  529.9448<sup>3+</sup>).

[w/w] (PHSHPALTPEQK ( $m/z$  447.9022<sup>3+</sup>)); 1.07% and 3.57% [w/w] (SSVFDVADPK ( $m/z$  475.2533<sup>2+</sup>)); 1.81% and 6.02% [w/w] (AFFGHYLYEVAR ( $m/z$  491.5828<sup>3+</sup>)); 1.87% and 6.25% [w/w] (LQLYSQFLGK ( $m/z$  598.8377<sup>2+</sup>)); and 1.91% and 6.38% [w/w] (VLAASFSEGLSHLDNLK ( $m/z$  571.9777<sup>3+</sup>)).

Of the chicken peptides evaluated in the current study, five ((MTEEEVEELMK ( $m/z$  684.3071<sup>2+</sup>) derived from myosin light chain 1/3, EPADAMAAGAVEASFK ( $m/z$  782.8692<sup>2+</sup>) derived from pyruvate kinase, DQGTFFDFVEGLR ( $m/z$  756.8519<sup>2+</sup>) derived from myosin light chain 1, and LDVPISGEPAPTVTWK ( $m/z$  855.4590<sup>2+</sup>) and TSDVDSVFIR ( $m/z$  643.3248<sup>2+</sup>) derived from myosin-binding protein C, were previously verified by MRM transitions [12,17,21,22]. Of these five peptides, three (EPADAMAAGAVEASFK ( $m/z$  782.8692<sup>2+</sup>), LDVPISGEPAPTVTWK ( $m/z$  855.4590<sup>2+</sup>), and DQGTFFDFVEGLR ( $m/z$  756.8519<sup>2+</sup>)) were previously used for absolute quantification (AQUA) of the abundance of chicken proteins in highly processed meat products [12,22]. The MRM transitions from the literature are presented in Table 2. Of the five chicken peptides listed in Table 2, we found that three ((MTEEEVEELMK ( $m/z$  684.3071<sup>2+</sup>), EPADAMAAGAVEASFK ( $m/z$  782.8692<sup>2+</sup>), and DQGTFFDFVEGLR ( $m/z$  756.8519<sup>2+</sup>)) were good candidate peptides for the quantification of chicken meat, with  $R^2$  coefficients above 0.97.

The chicken peptide, GEMLDLQHGSFLK ( $m/z$  794.930<sup>2+</sup>), derived from L-lactate dehydrogenase A

chain, had the highest linear fitting in the current study and was previously used for the detection of chicken proteins at 0.5% (w/w) in three-component meat mixtures in highly processed meat products [8].

### 3.4. Stability

The stability test results of five rabbit-specific peptide markers with  $R^2 > 0.97$  in binary meat mixtures for low (1%, w/w) and high (100%, w/w) rabbit meat content samples are shown in Table 3. All peptide markers remained stable through the study period. For short-term stability, the change in the MS signal was in the range of  $-4.93\%$  to  $8.75\%$  and  $-1.86\%$  to  $5.09\%$  for low and high rabbit meat content samples, respectively. For long-term stability, the change in the MS signal was in the range of  $-22.76\%$  to  $3.06\%$  and  $-17.93\%$  to  $3.81\%$  for low and high rabbit meat contents, respectively. The peptide LQLYSQFLGK ( $m/z$ , 598.8377<sup>2+</sup>) originating from glutathione S-transferase Mu 1, showed the poorest stability at a low rabbit meat content, with a loss of signal intensity of approximately 22% after storage for 7 days at  $-20^\circ\text{C}$ . The acceptance criteria set as  $< 10\%$  for the short-term stability met all five peptides, and  $< 15\%$  for the long-term stability did not meet only LQLYSQFLGK. When it is to be included in the developed methods the use of a stable isotope-labelled standard is highly recommended if laboratory analysis throughputs does not allow the

Table 2. MRM transitions for rabbit and chicken peptides obtained from the literature.

Species	Peptide	Precursor ion $m/z$	Product ion $m/z$	AQUA	LOD (w/w)	LOQ (w/w)	Ref.
Rabbit	PHSHPALTPEQK	447.8	234.8, 314.0	–	1.8%	5.5%	[18]
Chicken	MTEEEVEELMK	684.3	1236.6, 1135.5, 1006.5, 887.4, 748.4, 649.3	–	–	–	[21]
	EPADAMAAGAVEASFK	782.9	1152.6, 950.5, 879.5, 808.4, 581.3	+	1%	–	[12,17]
	LDVPISGEPAPTVTWK	855.5	1172.6, 899.5, 731.4, 1085.6, 434.2	+	1%	–	[12,17]
	TSDVDSVFIR	643.3	1097.6, 883.5, 582.3, 982.5, 681.4, 435.3	–	–	–	[17]
	DQGTFFDFVEGLR	756.9	1111.5, 964.5, 835.4, 720.4, 474.3, 244.1	+	0.5%	–	[21,22]

AQUA – absolute quantification; LOD – limit of detection; LOQ – limit of quantification.

Table 3. Short- and long-term stability of five rabbit-specific peptides with  $R^2 > 0.97$  measured in samples at low (1%, w/w) and high (100%, w/w) rabbit meat contents.

Peptide sequence ( $m/z$ )	1% rabbit meat (w/w)		100% rabbit meat (w/w)	
	Short-term stability [%]	Long-term stability [%]	Short-term stability [%]	Long-term stability [%]
PHSHPALTPEQK (447.9022 <sup>3+</sup> )	1.31	–7.07	5.09	–6.48
SSVFDVADPK (475.2533 <sup>2+</sup> )	8.75 <sup>a</sup>	3.06 <sup>a</sup>	0.91	–1.57
AFFGHYLYEVAR (491.5828 <sup>3+</sup> )	–4.93 <sup>a</sup>	–8.27 <sup>a</sup>	–1.86	3.81
LQLYSQFLGK (598.8377 <sup>2+</sup> )	2.24 <sup>a</sup>	–22.76 <sup>a</sup>	2.09	–17.93
VLAASFSEGLSHLDNLK (571.9777 <sup>3+</sup> )	–4.07 <sup>a</sup>	–9.08 <sup>a</sup>	1.54	–6.32

<sup>a</sup> Evaluated at a rabbit meat content of 5% (w/w).



analyze samples on the day of sample preparation or as a mitigation plan for mass spectrometer breakdown to compensate for signal loss.

### 3.5. Determination of the percentage of rabbit and chicken meat in two- and three-component meat mixtures

To demonstrate the applicability of the selected peptide markers for quantitative analysis, the percentage of rabbit and chicken meat content in two- (B5–B50) and three- (T5–T50) component meat mixture samples was determined. The samples were prepared in duplicate and analyzed with three replicate injections. The results and the mean  $\pm$  standard deviation are shown in Table 4.

The method detected the percentage of rabbit and chicken meat with good accuracy in an acceptable range of 80%–120%. A less satisfactory accuracy was observed for mixture B5 containing 5% rabbit meat (for rabbit: 3.54%  $\pm$  0.56 instead of 5%, trueness 71%). In three (B5, B50, and T50) of the four meat mixtures analyzed, the average percentage of chicken meat detected was lower than the percentage actually added, whereas in the other meat mixture, the average percentage of chicken meat detected was higher. Higher values were obtained for mixture T5 (for chicken: 56.11%  $\pm$  3.56 instead of 47.50%, trueness 118%). Considering the rabbit meat content obtained by different peptides, the best accuracy was observed for rabbit-specific SSVFVADPK ( $m/z$ , 475.2533<sup>2+</sup>) peptide marker (for mixture B5: 4.48%  $\pm$  0.15 instead of 5%, trueness 90%, for mixture T5: 5.40%  $\pm$  0.65 instead of 5%, trueness 108%, for mixture B50: 44.15%  $\pm$  1.21 instead of 50%, trueness 88%, for mixture T50: 48.81%  $\pm$  1.73 instead of 50%, trueness 98%). In three

(AFFGHYLYEVAR ( $m/z$ , 491.5828<sup>3+</sup>), LQLYSQFLGK ( $m/z$ , 598.8377<sup>2+</sup>), VLAAFSEGLSHLDNLK ( $m/z$ , 571.9777<sup>3+</sup>)) of the five rabbit-specific peptides analyzed, the percentage of rabbit meat detected was lower than the percentage actually added. The lowest values were obtained for rabbit-specific AFFGHYLYEVAR ( $m/z$ , 491.5828<sup>3+</sup>) peptide marker (for mixture B5: 3.12%  $\pm$  0.64 instead of 5%, trueness 62%, for mixture T5: 3.18%  $\pm$  0.75 instead of 5%, trueness 64%, for mixture B50: 38.31%  $\pm$  2.85 instead of 50%, trueness 77%, for mixture T50: 48.81%  $\pm$  1.73 instead of 50%, trueness 84%). Our results are in agreement with the results obtained by Prandi et al. [18]. Data for her group demonstrates the simultaneous detection and quantification of eight meat species in Bolognese sauce using species specific sequence approach. Of the 8 meat species analyzed, buffalo meat and red deer meat were found underestimated (1%  $\pm$  0.5 instead of 2%, trueness 50% and 2.3%  $\pm$  0.1 instead of 4%, trueness 58%), respectively [18]. They found similar observation, when the absolute values were low (low % of meat species added), the error percentage became high. For higher values (higher % of meat species added), the trueness improved. To minimize difference in accuracy, the stable isotope-labelled peptides for the quantification are highly recommended. An earlier study by Sentandreu et al. [22] and Montowska and Fornal [12] has demonstrated the use of the AQUA stable isotope peptide approach.

The main limitation of our findings arises from the fact that the results are based on trypsin digests of two- and three ingredient mixtures prepared from heat-treated meats with no additives. More complex matrixes, such as trypsin-digested highly processed meat products (e.g., pâtés, sausages), containing a larger number of peptides may affect the accuracy of

Table 4. Percentage content of rabbit and chicken meat determined in two- and three-component meat mixtures.

Peptide sequence ( $m/z$ )	B5	T5	B50	T50
Percentage (w/w) of rabbit meat added:	5.00%	5.00%	50.00%	50.00%
PHSHPALTPEQK (447.9022 <sup>3+</sup> )	3.45% $\pm$ 0.52	5.20% $\pm$ 0.19	41.39% $\pm$ 4.80	39.94% $\pm$ 1.70
SSVFVADPK (475.2533 <sup>2+</sup> )	4.48% <sup>a</sup> $\pm$ 0.15	5.40% <sup>a</sup> $\pm$ 0.65	44.15% $\pm$ 1.21	48.81% $\pm$ 1.73
AFFGHYLYEVAR (491.5828 <sup>3+</sup> )	3.12% <sup>a</sup> $\pm$ 0.64	3.18% <sup>a</sup> $\pm$ 0.75	38.31% $\pm$ 2.85	41.99% $\pm$ 3.13
LQLYSQFLGK (598.8377 <sup>2+</sup> )	3.52% <sup>a</sup> $\pm$ 0.30	3.43% <sup>a</sup> $\pm$ 0.29	39.56% $\pm$ 4.73	41.89% $\pm$ 1.05
VLAAFSEGLSHLDNLK (571.9777 <sup>3+</sup> )	3.13% <sup>a</sup> $\pm$ 0.73	3.94% <sup>a</sup> $\pm$ 0.81	36.63% $\pm$ 2.10	43.52% $\pm$ 3.41
Average percentage (w/w) of rabbit meat detected:	3.54% $\pm$ 0.56	4.23% $\pm$ 1.02	40.01% $\pm$ 2.90	43.23% $\pm$ 3.37
Percentage (w/w) of chicken meat added:	95.00%	47.50%	50.00%	25.00%
MTEEEVEELMK (684.3071 <sup>2+</sup> )	91.22% $\pm$ 1.31	54.57% $\pm$ 4.12	44.23% $\pm$ 2.86	26.89% $\pm$ 1.68
EPADAMAAGAVEASFK (782.8692 <sup>2+</sup> )	94.99% $\pm$ 2.71	60.09% $\pm$ 3.42	39.79% $\pm$ 1.15	20.99% $\pm$ 0.59
DLFDPVIQDR (609.3117 <sup>2+</sup> )	92.52% $\pm$ 2.58	52.19% $\pm$ 1.77	41.57% $\pm$ 2.11	24.75% $\pm$ 1.03
GEMLDLQHGSLFLK (529.9448 <sup>3+</sup> )	88.75% $\pm$ 3.48	59.68% $\pm$ 2.92	35.14% $\pm$ 1.48	20.16% $\pm$ 1.32
DQGTFFDFEGLR (756.8519 <sup>2+</sup> )	90.34% $\pm$ 1.36	54.00% $\pm$ 5.17	44.35% $\pm$ 1.85	21.85% $\pm$ 2.29
Average percentage (w/w) of chicken meat detected:	91.56% $\pm$ 2.35	56.11% $\pm$ 3.56	41.02% $\pm$ 3.80	22.92% $\pm$ 2.81

\*- Estimated.

quantification due to matrix effect. Matrix effect correction by adding stable isotope-labelled peptides for the quantification of pork in processed meat products was demonstrated by Li and his co-workers [24]. They proved that carbonic anhydrase 3, an enzyme with 3 unique thermostable pork peptides, exhibits excellent quantification ability. In contrast to other examined by them thermostable pork-specific peptides, EPITVSSDQMAK, GGPLTAAAYR, HDPSLLPWTASYDPGSAK of carbonic anhydrase 3 showed excellent quantification ability, good signal linearity and satisfactory recovery.

Basing on the results from current studies, MRM label-free or absolute quantification methods including the most promising markers can be developed and implemented in food testing laboratories for quantification of rabbit meat.

#### 4. Conclusion

A proteomics-based LC-QTOF-MS/MS method was used to evaluate 10 rabbit-specific tryptic peptide markers and one tryptic peptide marker specific to both rabbit and hare for quantification. The linearity of 12 chicken peptide markers was simultaneously evaluated. Of the 11 rabbit peptides, five peptides ((PHSHPALTPPEQK ( $m/z$  447.9022<sup>3+</sup>), SSVFVADPK ( $m/z$  475.2533<sup>2+</sup>), AFFGHLYEVAR ( $m/z$  491.5828<sup>3+</sup>), LQLYSQFLGK ( $m/z$  598.8377<sup>2+</sup>), and VLAASFEGLSHLDNLK ( $m/z$  571.9777<sup>3+</sup>)) had advantages in terms of good linearity and stability, with LOD and LOQ values in the range of 0.43%–1.91% [w/w] and 1.44%–6.38% [w/w], respectively. Of the chicken peptides evaluated, MTEEEVEELMK ( $m/z$  684.3071<sup>2+</sup>) derived from myosin light chain 1/3, skeletal muscle isoform, EPADAMAAGAVEASFK ( $m/z$  782.8692<sup>2+</sup>) derived from pyruvate kinase, DLFDPVIQDR ( $m/z$  609.3117<sup>2+</sup>) derived from creatine kinase M-type, GEMLDLQHGSLFLK ( $m/z$  529.9448<sup>3+</sup>) derived from L-lactate hydrogenase A chain, and DQGTFFEDFVEGLR ( $m/z$  756.8519<sup>2+</sup>) derived from myosin light chain 1, skeletal muscle isoform were identified as the most promising for quantification. Three of these five chicken peptides (i.e., MTEEEVEELMK, EPADAMAAGAVEASFK, and DQGTFFEDFVEGLR) have been used in previous studies for the determination of chicken content using either AQUA quantification or an MRM approach.

The findings presented in the paper will facilitate the selection of rabbit- and chicken-specific markers for accurate meat quantification by MRM methods to ensure high-sensitivity determinations.

#### Conflict of 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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