

1 **Relative quantification of pork and beef in meat products using global and**  
2 **species-specific peptide markers for the authentication of meat composition**

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4 Katarzyna Nalazek-Rudnicka <sup>a</sup>, Ilona Kłosowska-Chomiczewska <sup>b</sup>, Jens Brockmeyer <sup>c</sup>,  
5 Andrzej Wasik <sup>a</sup>, Adam Macierzanka <sup>b,\*</sup>

6  
7 <sup>a</sup> Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology,  
8 Narutowicza 11/12, 80-233 Gdańsk, Poland

9 <sup>b</sup> Department of Colloid and Lipid Science, Faculty of Chemistry, Gdańsk University of Technology,  
10 Narutowicza 11/12, 80-233 Gdańsk, Poland

11 <sup>c</sup> Department Food Chemistry, Institute for Biochemistry and Technical Biochemistry, University of  
12 Stuttgart, Allmandring 5B, 70569 Stuttgart, Germany

13

14 \* Corresponding author.

15 E-mail address: [adam.macierzanka@pg.edu.pl](mailto:adam.macierzanka@pg.edu.pl) (A. Macierzanka).

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22 Declarations of interest: none

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41 Abbreviations:

42 B, beef; BC, beef content; GSM, global and specific marker(s); P, pork; PC, pork content.

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45 **Abstract**

46 We used **global and species-specific peptide markers** for **a relative** quantitative determination of pork  
47 and beef in raw and processed meat products made of the two meat species. Four groups of products  
48 were prepared (i.e., minced raw meats, sausages, raw and fried burgers) in order to represent products  
49 with different extents of food processing. In each group, the products varied in the pork/beef proportions.  
50 All products were analysed **by multiple reaction monitoring mass spectrometry (MRM–MS)** for the  
51 **presence/concentration of pork- and beef-specific** peptide markers, as well as global markers – **peptides**  
52 widely distributed in muscle tissue. The combined MRM-MS analysis of pork-specific peptide  
53 HPGDFGADAQGAMSK, beef-specific peptide VLGFGH and global marker LFDLR offered the most  
54 reliable validation of declared pork/beef compositions across the whole range of meat products. **Our**  
55 **work suggests that a simultaneous analysis of global and species-specific peptide markers** can be used  
56 for composition authentication in commercial pork/beef products.

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58 **Keywords:**

59 Meat authentication, Peptide markers, Global markers, MRM-MS, Pork, Beef

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## 61 1. Introduction

62 Meat fraud remains a global food problem, with new cases coming to light on a regular basis (Alikord,  
63 Momtaz, Keramat, Kadivar, & Rad, 2018) (Zia, Alawami, Mokhtar, Nhari, & Hanish, 2020). This can  
64 contribute to a significant decrease in consumers' confidence in the food industry and food quality  
65 regulators, and **may** damage the whole food supply chain, from farmers to retailers. In 2018, the EC  
66 launched the Knowledge Centre network (EC Knowledge for Policy, 2018) in order to improve the quality  
67 and safety of food available in the European single market, and to counteract food fraud, including  
68 adulterations in the meat sector. Consequently, there has been a need for developing reliable analytical  
69 methods for the authentication of meat products.

70 Meat authentication methods are usually based on either genomic or proteomic analyses. The genomic  
71 approach often utilises the polymerase chain reaction (PCR) for qualitative (conventional PCR) or  
72 quantitative (real-time PCR) analysis (Wang et al., 2020). Although PCR can detect as little as 0.0001%  
73 (w/w) of undeclared meat in food (Hird et al., 2006), the method can be inaccurate when analysing  
74 processed meat products and complex foods. This is because the high processing of meat such as  
75 canning or autoclaving usually results in extensive DNA fragmentation. Moreover, the DNA-based  
76 methods are not tissue-specific; they cannot differentiate between e.g., chicken meat versus egg white,  
77 or beef versus bovine milk (Montowska & Fornal, 2019).

78 In this light, the proteomic authentication methods seem to be a more promising solution. However, it is  
79 the **application of** qualitative proteomic methods that is most often reported in the scientific literature.  
80 They have been used to detect the presence of undeclared species of meat in products containing more  
81 than one type of meat (Montowska, Alexander, Tucker, & Barrett, 2014) (Ruiz Orduna, Husby, Yang,  
82 Ghosh, & Beaudry, 2017) (von Bargaen, Brockmeyer, & Humpf, 2014) (von Bargaen, Dojahn, Waidelich,  
83 Humpf, & Brockmeyer, 2013) (Watson, Gunning, Rigby, Philo, & Kemsley, 2015), as well as for  
84 distinguishing between high- and low-quality meats and meat products (Hou et al., 2020) (Nalazek-  
85 Rudnicka, Kłosowska-Chomiczewska, Wasik, & Macierzanka, 2019). Quantitative applications of  
86 proteomic authentication methods are scarce. Sentandreu et al. (Sentandreu, Fraser, Halket, Patel, &  
87 Bramley, 2010) as well as Montowska and Fornal (Montowska & Fornal, 2019) performed absolute  
88 quantification of meat using isotope-labelled peptides. The two groups of researchers were able to  
89 detect at least 0.5% (w/w) chicken meat in mixtures with pork, or at least 0.8% (w/w) chicken in mixtures  
90 with veal, respectively. The inaccessibility of some isotope-labelled peptides and high cost of analysis  
91 have been identified as limitations of **such absolute quantification** methods. Therefore, **they have** only  
92 been suggested to serve as a final confirmation of any adulteration that had been identified with other,  
93 presumably cheaper, methods (Montowska & Fornal, 2019).

94 **Li et al. (Li et al., 2021) have recently developed a LC–MS/MS internal standard method for quantifying**  
95 **pork content in meat products by analysing pork-specific peptides derived from carbonic anhydrase III.**  
96 **The limit of detection of the method was assessed to be as low as 0.1% (w/w) for peptide GGPLTAAYR,**  
97 **with over 80% recovery in processed pork (simulated meatballs with the pork contents varying from**  
98 **16.2% to 84.8%). The recovery of selected pork-specific peptides in commercial products was found to**



99 decrease, in general, with the increasing abundance of proteins from different sources (e.g., soy,  
100 chicken, beef). The authors did not propose any detailed numerical method. Prandi and co-workers  
101 investigated UHPLC/ESI-MS methods for the identification and quantification of meat species in  
102 Bolognese sauce. The researchers were able to detect at least 2% pork in beef matrix (Prandi et al.,  
103 2017) and, in a separate study (Prandi et al., 2019), as little as 0.2-0.8% of peptide markers specific to  
104 eight different meat species. Pan et al. (Pan, Chen, Chen, Huang, & Han, 2018) developed a parallel  
105 reaction monitoring (PRM) Orbitrap-MS method that enabled the detection of peptides specific for pork  
106 in quantities corresponding to as little as 0.5% pork in four-component meat mixtures (i.e., with chicken,  
107 sheep and beef). Montowska and Fornal (Montowska & Fornal, 2017) applied a nano-LC-Q-TOF-  
108 MS/MS for spectral matching quantitation. The authors were able to detect at least 1% (w/w) of pork  
109 and 1% (w/w) of chicken in ternary meat mixtures with turkey, as well as 0.8% (w/w) of beef in  
110 commercial poultry frankfurters. Feng et al. (Feng et al., 2021) have developed a LC-MS/MS method  
111 for the quantification of five meat species in their mixtures. The detection limit reported by the authors  
112 was 1%.

113 There has also been some development in non-MS methods. Seddaoui and Amine (Seddaoui & Amine,  
114 2021) developed a sensitive, portable immunoassay method for detecting and quantifying pork in binary  
115 mixtures with beef. The method, which is based on a colorimetric assay performed with a smartphone,  
116 was claimed to allow for detection of as little as 0.01% of pork in meat mixtures within only 30 minutes,  
117 which made it suitable for on-site inspections. Recently, Yamasaki et al. (Yamasaki et al., 2022) applied  
118 a sandwich enzyme-linked immunosorbent assay (s-ELISA) with SDS-supported extraction to quantify  
119 pork in pork/beef binary mixtures. The method allowed for detecting 1% (w/w) of pork in mixtures with  
120 raw and heated beef. Sezer et al. (Sezer, Bjelak, Velioglu, & Boyaci, 2021) reported on the determination  
121 of species-specific proteins and peptides by using laser induced breakdown spectroscopy (LIBS). The  
122 researchers combined LIBS with principal component analysis (PCA) or partial least squares (PLS)  
123 analysis to verify and quantify beef adulterations with pork or chicken. They analysed bulk proteins as  
124 well as their fractions. The limit of detection calculated for the LIBS-PLS using bulk proteins indicated a  
125 possibility to detect adulterations of beef with as low as 2.48% of chicken or 3.89% of pork in binary  
126 meat mixtures. Jiang et al. (Jiang, Ru, Chen, Wang, & Xu, 2021) used a near-infrared hyperspectral  
127 imaging combined with a PLS regression and PCA to investigate adulterations of ground pork with  
128 offal. The calculated limit of detection of the method indicated the potential to detect ca. 7.5%  
129 adulterations in analysed pork samples.

130 The above are good examples of quantitative methods with relatively low limits of detection. They are,  
131 however, based solely on the detection of species-specific proteins and/or peptide markers. This can  
132 present a limitation in investigating adulterations of meat products because such methods might be  
133 unable to detect the presence of a non-typical proteinaceous material, e.g., insect proteins. For a sole  
134 application of species-specific marker peptides, the source of fraudulent blending needs to be known or  
135 at least be suspected. Thus, only a limited number of potential contaminations or undeclared ingredients  
136 can be analysed. Conversely, the use of global protein markers - widely distributed in vertebrate and/or  
137 invertebrate muscle tissue - might allow, in combination with species-specific marker peptides, for the

138 authenticity control without prior knowledge of potential undeclared species. Even more importantly, the  
139 relative quantitation of declared species might be possible using this approach as the ratio between  
140 species-specific peptide marker(s) and global marker(s) can be used to determine the **relative** quantity  
141 of a specific meat species in its mixture with other meat species. Therefore, the aim of our study was to  
142 develop a quantitative method that allows for **a relative determination of** a composition of meat products  
143 (both raw and processed) containing different types of meat (pork-and-beef products were used in this  
144 study). For this purpose, the peptide markers specific for pork and beef, as well as the global peptide  
145 markers specific for animals, were detected in a range of different pork/beef products using mass  
146 spectrometry, and quantified in multiple reaction monitoring (MRM) mode. We hypothesise that by  
147 analysing different combinations of **species-specific and global** peptide markers **in raw and processed**  
148 **meat products with known pork and beef contents** several algorithms can be created and verified in  
149 order to find those that most accurately reflect the true composition of products containing pork and beef  
150 at various proportions. This **might allow for developing a relative quantification method of verifying** pork  
151 and beef contents declared in mixed meat products **that contain the two meat species.**

152

## 153 2. Material and methods

### 154 2.1. Materials

155 Acetonitrile (ACN, LC-MS grade), methanol (MeOH, LC-MS grade), urea, thiourea,  
156 tris(hydroxymethyl)aminomethane (TRIS), trypsin (T0303, type IX-S, 13,000–20,000 BAEE units/mg  
157 protein), dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma Aldrich (St. Louis,  
158 USA). Analytical grade hydrochloride, acetic acid and formic acid (FA) were obtained from POCH  
159 (Gliwice, Poland). Ultrapure water was prepared using a HLP<sub>5</sub> system (Hydrolab, Wiślina, Poland).

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### 161 2.2. Preparation of meat products

162 Beef round (B) and pork ham (P) were used in this study to prepare four different types of mixed meat  
163 products containing various pork-to-beef (P/B) proportions (i.e., pork and beef contents varied from 0  
164 to 100%, Table 1). The meat products were analysed in two different comparative sets in order to  
165 investigate whether any applied food processing affected the ability of the studied analytical procedure  
166 for quantitative determination of relative pork and beef contents in the products:

167 1. Sausages, made with different proportions of pork and beef, were analysed against mixtures of raw,  
168 minced pork and beef with corresponding proportions of the two meat species (Table 1). The sausages  
169 were custom made by a commercial meat processing company in Pomeranian Voivodeship (Poland)  
170 using a procedure that is conventionally applied in sausage manufacturing. The production process  
171 involved separate grindings of pork and beef through a  $\Phi$  3.5 mm steel sieve, followed by mixing the  
172 two meats together at different, strictly defined P/B proportions (w/w), and with addition of small  
173 quantities of flavourings (less than 0.5 wt% of the total meat content; Table 1). After the sausages had  
174 been formed by stuffing meat mixtures into casings, they were smoked for 4 h at 25 °C. A reference set  
175 of mixtures of minced raw meats, with corresponding P/B proportions, was prepared using samples of  
176 the very same pork and beef cuts (beef round and pork ham) as those that had been used for the  
177 production of the sausages. Both, the sausages and the mixtures of raw meats were stored at –80 °C  
178 prior to analysis (Montowska & Fornal, 2017) (Sentandreu et al., 2010). Preparation and analysis of all  
179 individual sausages and their corresponding mixtures of raw pork and beef (Table 1) were done in  
180 triplicate (n = 3).

181 2. Raw burgers, made with different proportions of minced pork and beef, were analysed against fried  
182 burgers with corresponding P/B proportions. In order to prepare the raw burgers, fresh pork and beef,  
183 marketed by Lidl Poland and purchased locally (Gdansk, Poland), were minced separately using a meat  
184 grinder (Zelmer, ZMM4050B, Poland) equipped with a  $\Phi$  5.0 mm steel sieve. In the next step, the two  
185 different types of meat were mixed together at different proportions (w/w), and with addition of small  
186 quantities of salt and pepper (less than 0.5 wt% of the total meat content; Table 1). The burgers were  
187 stored at –80 °C (Montowska & Fornal, 2017) (Sentandreu et al., 2010). In order to check the impact of  
188 frying on the stability of peptide markers analysed in this study, the raw burgers were defrosted at room  
189 temperature (RT) and subjected to thermal processing, i.e., frying in hot (190 °C) rapeseed oil (refined  
190 oil, ZT Kruszwica S.A., Kruszwica, Poland) until well-done burgers were obtained. This required frying  
191 for 7 min, over which the temperature inside burgers reached 80 °C. The burgers prepared according

192 to this procedure have been referred to as 'fried burgers' throughout the paper. Preparation and analysis  
 193 of all individual raw and fried burgers listed in Table 1 were done in triplicate (n = 3).

194 Table 1 summarises the compositions and the processing conditions of the meat products used in the  
 195 study.

196

197 Table 1. Meat products prepared and analysed in this study

Meat product	Sample name/number	Declared relative content of pork (wt%)	Declared relative content of beef (wt%)	Additives	Processing type
Mix of minced raw meats	M1	100	0	-	Grounding ( $\Phi$ 3.5 mm, sieve), mixing
	M2	90	10		
	M3	70	30		
	M4	50	50		
	M5	30	70		
	M6	10	90		
	M7	0	100		
Sausages	S1	100	0	salt, pepper, garlic	Grounding ( $\Phi$ 3.5 mm, sieve), mixing, stuffing into casing, cold smoking (25 °C, 4h)
	S2	90	10		
	S3	70	30		
	S4	50	50		
	S5	30	70		
	S6	10	90		
	S7	0	100		
Raw burgers	Bur1	100	0	salt, pepper	Grounding ( $\Phi$ 5.0 mm sieve), mixing
	Bur2	90	10		
	Bur3	75	25		
	Bur4	50	50		
	Bur5	25	75		
	Bur6	10	90		
	Bur7	0	100		
Fried burgers	FBur1	100	0	salt, pepper	Frying on the day of analysis in hot oil (190 °C) for 7 min
	FBur2	90	10		
	FBur3	75	25		
	FBur4	50	50		
	FBur5	25	75		
	FBur6	10	90		
	FBur7	0	100		

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### 199 **2.3. Sample preparation for HPLC-MS/MS analysis**

200 Raw burgers, sausages and the mixtures of minced raw meats were defrosted at RT on the day of  
 201 analysis. The casing was removed from sausages prior to taking samples. Fried burgers were analysed  
 202 immediately after cooling down to RT. Sampling was done from below the surface, after the fried crust  
 203 had been removed. Samples (1 g) were taken and placed in a plastic 50-mL centrifuge tubes, and the  
 204 extraction buffer (10 mL; 6 M urea, 1 M thiourea, 50 mM TRIS, pH 8.0) added. The mixtures were  
 205 homogenized (2 min, 9600 rpm) using an Ultra-Turrax (IKA, Poland), followed by centrifugation at 4 °C  
 206 for 60 min at 10,733g. Clear supernatants were collected, transferred to plastic 50-mL centrifuge tubes  
 207 and vortexed. Subsequently, the extracts were centrifuged at 4 °C for 3 min at 1,315g in order to  
 208 suppress foam that could have formed during the previous step. TRIS stock solution (400 mM, pH 7.8)



209 was used for preparation of the reducing and alkylating agents applied in the following steps. Aliquots  
210 (100  $\mu$ L) of the extracts were transferred to 1.5-mL reaction tubes and 5  $\mu$ L of reducing agent (100 mM  
211 TRIS, 200 mM DTT) added in order to reduce disulfide bonds in analysed proteins. The resulting  
212 samples were incubated at RT for 1 h. Next, 20  $\mu$ L of alkylating agent (100 mM TRIS, 200 mM IAA) was  
213 added. Samples were then incubated in dark for 1 h to alkylate the resulting thiol groups. Subsequently,  
214 20  $\mu$ L of the reducing agent was added again and the samples incubated at RT for 1 h. The extracts  
215 were finally diluted with water (775  $\mu$ L per sample) and digested using trypsin solution (200 ng/ $\mu$ L trypsin,  
216 100 mM TRIS) at 37 °C overnight. Next day, the trypsin was inactivated by adding 5  $\mu$ L acetic acid, and  
217 then the extracts were cleaned-up and enriched using Strata-X 33  $\mu$ m SPE cartridges filled with  
218 60 mg/3 mL polymeric reversed-phase material (Phenomenex, Macclesfield, UK). The cartridges were  
219 activated with 2 mL of MeOH followed by 2 mL of 1% (v/v) aqueous solution of FA. Afterwards, the  
220 extracts were loaded into the cartridges. The cartridges were then washed with 2 mL of the 1% (v/v) FA.  
221 Finally, the peptides were eluted with 2 mL of the MeOH:water mixture (9:1 v/v, containing 1%, v/v, FA)  
222 into 12-mL glass test tubes. Subsequently, the solvents were completely evaporated under a stream of  
223 nitrogen at 40 °C. Prior to the chromatographic analysis, the extracts were reconstituted with 100  $\mu$ L of  
224 the ACN:water mixture (3:97, v/v, containing 0.1% v/v FA), vortexed for 30 s, and transferred to 250- $\mu$ L  
225 inserts. If needed, inserts were placed in 1.5-mL eppendorf tubes and centrifuged to suppress foam that  
226 might have formed (3000 rpm, 30 s). The sample preparation procedure has been summarised in the  
227 Supplementary Material (Fig. S1).

#### 228 **2.4. Multiple reaction monitoring mass spectrometry (MRM-MS) instrumentation**

229 Peptides specific for pork (P1, P2) and beef (B1, B2) derived from myoglobin (Mb) specific for a given  
230 meat species (Sentandreu & Sentandreu, 2014) (Watson et al., 2015) (Montowska et al., 2014)  
231 (Montowska, Alexander, Tucker, & Barrett, 2015). The selection of three different global peptide markers  
232 (G1, G2, G3) was based on a recent study that used shotgun proteomics followed by database search,  
233 and found the peptides represented highly conserved amino acid sequences in the muscle proteome of  
234 numerous vertebrate and invertebrate species (Brümmer, Murr, & Brockmeyer, 2022). The amino acid  
235 sequences of the species-specific markers and the global markers as well as MRM-MS conditions are  
236 given in Table 2.

237 The HPLC-MS/MS analyses of the peptide markers were performed using a LCMS-8060 triple  
238 quadrupole mass spectrometer (Shimadzu, Japan) equipped with an electrospray ionization (ESI)  
239 source working in a positive multiple reaction monitoring (MRM) ion mode. The parameters of the ion  
240 source were set as follows: nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; interface  
241 temperature, 300 °C; desolvation line temperature, 250 °C; heat block temperature, 400 °C; and drying  
242 gas flow, 10 L/min. Each marker was monitored by four most intense MRM transition, with the exception  
243 of global marker G1, for which two most intense MRM transition were monitored. The source and MS  
244 parameters have been shown in Table 2. Data acquisition and analysis were accomplished with  
245 LabSolutions 5.85 software (Shimadzu, Japan). The chromatographic separation was done using an  
246 UPLC Nexera X2 System (Shimadzu) equipped with a LC-30AD binary pump, a DGU-20A5R degasser,  
247 a CBM-20A controller, a SIL-30AC autosampler and a CTO-20AC thermostated column oven. The



248 selection of separation conditions and the optimisation of the method were described previously  
 249 (Nalazek-Rudnicka et al., 2019). A Kinetex XB C-18 reversed-phase (RP) column (100 x 2.1 mm, 2.6  
 250 µm; Phenomenex, Torrance (USA) was used for separation of peptides. The separation conditions have  
 251 been summarised in Table S1.

252 Table 2. MRM transition parameters and conditions of the ESI source for detection of marker peptides  
 253 in meat samples  
 254

Protein	Uniprot ID	Peptide marker symbol and amino acid sequence	Protein/Peptide origin	Parent ion (m/z)	Fragments (m/z)	Q1 (V)	Collision energy (V)	Q3 (V)
Myoglobin	P02189	P1, GHPETLEK	Pork	455.7	490.2	-16	-23	-24
					716.3	-16	-18	-38
					147.1	-11	-24	-27
Myoglobin	P02189	P2, HPGDFGADA QGAMSK	Pork	744.8	619.4	-11	-18	-28
					234.1	-24	-34	-23
					1254.5	-22	-26	-46
Myoglobin	P02192	B1, VLGFHG	Beef	315.2	692.3	-22	-28	-26
					1351.6	-20	-28	-48
					417.2	-11	-10	-19
Myoglobin	P02192	B2, HPSDFGADA QAAMSK	Beef	766.8	213.2	-11	-15	-23
					530.3	-11	-12	-26
					360.2	-10	-17	-17
G1 (global marker)	-	G1, LFDLR	n/a, global marker	332.2	234.1	-24	-35	-10
					1298.6	-26	-26	-36
					706.4	-28	-31	-26
G2 (global marker)	-	G2, DIDDLELTLAK	n/a, global marker	623.3	1395.6	-20	-28	-40
					550.3	-11	-13	-18
					403.2	-11	-12	-17
G3 (global marker)	-	G3, HQGVMVGMG QK	n/a, global marker	586.3	1017.5	-10	-18	-21
					674.5	-12	-19	-31
					902.5	-11	-19	-17
G3 (global marker)	-	G3, HQGVMVGMG QK	n/a, global marker	586.3	787.5	-10	-20	-19
					906.5	-15	-22	-18
					750.4	-15	-24	-21
G3 (global marker)	-	G3, HQGVMVGMG QK	n/a, global marker	586.3	619.3	-13	-25	-23
					849.5	-13	-23	-25

255 Q1, quadrupole 1 pre-rod bias; Q3, quadrupole 3 pre-rod bias.

256

## 257 **2.5. Data processing**

258 Global and Specific Marker (GSM) algorithms were created to determine a relative quantitative  
259 composition of meat in analysed products from the MRM-MS data obtained. Pork (P) and beef (B)  
260 specific markers were divided into two groups: P1, B2 - group 1, and P2, B1 - group 2. The MS signal  
261 intensity of each marker was taken into account when classifying markers to a particular group, i.e.,  
262 markers P1 and B2 - higher intensity of signal; markers P2 and B1 - lower intensity of signal. The  
263 GSM algorithms were based on the ratios of peak areas obtained for the specific markers (P1,B2 or  
264 P2,B1) and the global markers (G1, G2 or G3). In total, six GSM algorithms (A-F) were developed  
265 using different combinations of peptide markers; group 1 algorithms: A (P1,B2/G1), B (P1,B2/G2), C  
266 (P1,B2/G3); and group 2 algorithms: D (P2,B1/G1), E (P2,B1/G2), F (P2,B1/G3) (Table S2).  
267 The analysed contents (%) of pork and beef (CPM and CBM, respectively) in each meat product were  
268 determined from the following equations:

$$269 \quad \text{CPM (\%)} = (P_x/G_x)_{A_y} / (P_x/G_x)_{A1} \times 100 \quad (1)$$

$$270 \quad \text{CBM (\%)} = (B_x/G_x)_{A_y} / (B_x/G_x)_{A7} \times 100 \quad (2)$$

271 where:

272  $P_x$ , peak area recorded for pork marker ( $P_1$  for marker P1,  $P_2$  for marker P2);  
273  $B_x$ , peak area recorded for beef marker ( $B_1$  for marker B1,  $B_2$  for marker B2);  
274  $G_x$ , peak area recorded for global marker ( $G_1$  for marker G1,  $G_2$  for marker G2,  $G_3$  for marker G3);  
275  $A_y$ , sample name/number ( $y = 1-7$ , see Table 1), where the declared beef or pork content ranges from  
276 0 to 100%, and for which relevant  $P_x$ ,  $B_x$  and  $G_x$  values should be selected for calculations ( $A_y$   
277 indicates an individual meat product name/number within any of the four different groups of products  
278 (Table 1));  
279  $(P_x/G_x)_{A1}$ , ratio of the peak area of pork marker to the peak area of global marker in a sample where  
280 the declared relative content of pork is 100% (see Table 1);  
281  $(B_x/G_x)_{A7}$ , ratio of the peak area of beef marker to the peak area of global marker in a sample where  
282 the declared relative content of beef is 100% (see Table 1).  
283

284 Finally, the relative pork content (PC) and the relative beef content (BC) in their binary mixtures were  
285 calculated for every sample of analysed meat products in the way that takes into account the MS  
286 signals obtained for both, pork and beef markers in each particular sample; as follows:

$$287 \quad \text{PC (\%)} = (\text{CPM} + (100 - \text{CBM})) / 2 \quad (3)$$

$$288 \quad \text{BC (\%)} = (\text{CBM} + (100 - \text{CPM})) / 2 \quad (4)$$

289  
290 The PC and BC values were obtained from three individual analyses carried out separately, according  
291 to Sections 2.3, 2.4 and 2.5, for three individual samples ( $n = 3$ ) of every meat product included in the  
292 study (Table 1). The values are presented as the mean  $\pm$  SD.

293 A GSM algorithm (Table S2) was considered efficient if the composition of a meat product determined  
294 with its use was consistent with the declared (true) composition (Table 1). The efficiency of each  
295 algorithm was evaluated using a statistical test, one sample t-test, where the mean relative content of  
296 a specific meat species obtained with a given algorithm (i.e., mean PC or BC) was compared to a

297 known value – the true **relative** content of pork or beef. *P*-values were calculated using MedCalc  
298 software version 20.011 (MedCalc Software Ltd, Ostend, Belgium). The *P*-values were used to identify  
299 algorithms, which returned results that did not differ significantly ( $P > 0.05$ ) from the true, declared  
300 compositions. Additionally, absolute errors (AEs) were calculated for the use of each GSM algorithm  
301 to estimate accuracy of the algorithms. AE values were obtained from a difference between the  
302 calculated **relative** content of a specific meat (i.e., **PC or BC**) and the declared, true content of that  
303 type of meat in every meat product tested. Efficient GSM algorithms were required to return  $AE \leq 10\%$ .

304 **The detection limit (DL) of the method utilising a selected GSM algorithm was calculated from the**  
305 **mean standard deviation values (SD; i.e.,  $DL = 3 \times SD$  (Magnusson & Örnemark, 2014)) that had been**  
306 **recorded for PC and BC in samples of the meat products that contained the lowest (10 wt%) declared**  
307 **contents of either pork or beef (i.e., samples marked with number '6' or '2', respectively; Table 1).**  
308

### 309 **3. Results and discussion**

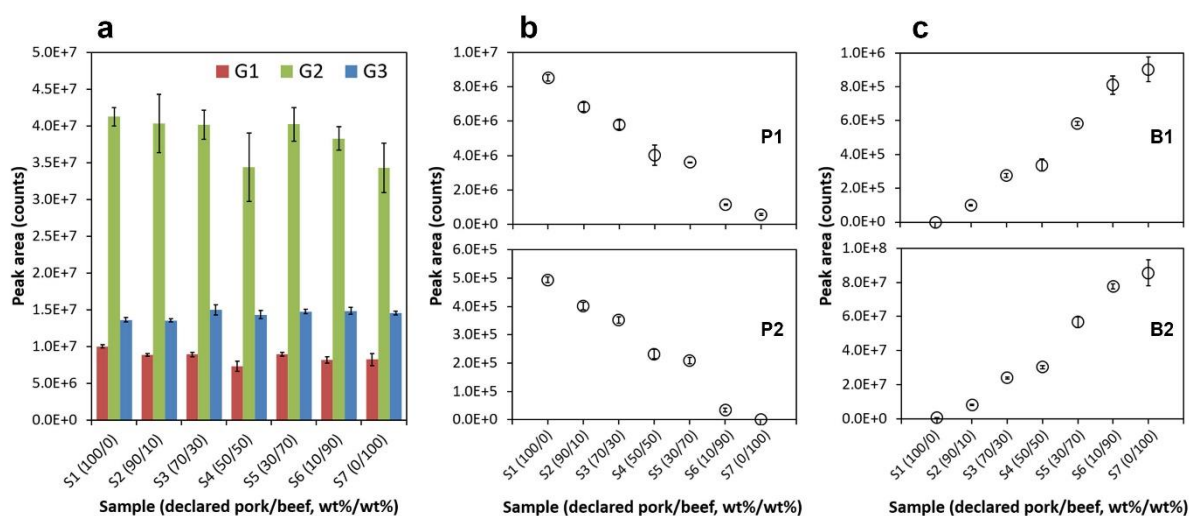
310 We have used a range of custom made processed meat products containing pork and beef (**P/B**) at  
311 various proportions as well as mixtures of the two raw meat species prepared in-house (Table 1). This  
312 made it possible to **gain** full control over the declared contents of individual meats in all the samples  
313 that have been analysed in this study. Hence, it was feasible to evaluate how the analysis of a  
314 combination of any particular global and species-specific peptide markers (GSM, Global and Specific  
315 Markers) in the samples reflected the true, declared pork and beef **relative** contents. The ultimate goal  
316 of this study was to identify GSM algorithm(s), which would allow for a reliable validation of the **relative**  
317 contents of individual meat species across the whole range of the meat products included in the study.

#### 318 **3.1. Species-specific and global marker analysis**

319 The presence of global markers (G1, G2 and G3, Table 2) in all the analysed meat products was  
320 confirmed by targeted proteomics in MRM mode. We have selected these three global markers as  
321 they have recently been identified as highly conserved amino acid sequence signatures in the core  
322 muscle proteome of 84 vertebrate species (including taxonomic classes *Aves*, *Pesceres*, *Mammalia*,  
323 *Amphibia*, and *Reptilia*) and 17 invertebrate species (*Mollusca* and *Arthropoda*) (Brümmer et al, 2022).  
324 The analyses performed for sausages containing various P/B proportions showed the MS signal was  
325 relatively constant for the global markers regardless of the ratio of the two meat species used in  
326 sausage manufacturing (Fig. 1a). This confirms a general suitability of the global markers for relative  
327 quantitation of P/B binary mixtures. However, some fluctuations in the MS signal generated by global  
328 markers may occur if a meat matrix is highly processed. In our study, this was observed mostly for the  
329 high-temperature processed P/B products (i.e., fried burgers; Fig. S2d-f).

330 Peptides specific for pork (P1 and P2) and beef (B1 and B2) derived from myoglobin (Mb) and their  
331 selection for this study was based on the information provided in previous reports (Watson et al.,  
332 2015) (Sentandreu & Sentandreu, 2014), where they were found to be more suitable over other Mb

333 peptides in terms of the MS signal quality, high discriminating power, etc. They were also successfully  
 334 used in our previous study for authentication of meat products containing pork or beef (Nalazek-  
 335 Rudnicka et al., 2019). In the present work, the MRM-MS analysis showed the MS signal obtained for  
 336 P1 and P2 declined proportionally to the decrease of pork content in the sausages (Fig. 1b). At the  
 337 same time the signal intensity recorded for beef markers (B1 and B2) increased with the increasing  
 338 content of this meat species in the sausages (Fig. 1c). Similar analyses were also performed for other  
 339 types of meat products (Table 1), and the results obtained for the species-specific markers and the  
 340 global markers were used for the relative quantitative determination of pork and beef contents in all  
 341 the meat products included in this study.



342  
 343 Fig. 1. MRM-MS analysis of sausages containing various pork/beef relative contents (samples S1-S7,  
 344 Table 1). MS signals recorded for (a) global markers G1, G2 and G3, (b) pork-specific markers P1 and  
 345 P2, and (c) beef-specific markers B1 and B2. Individual data points are shown as the mean ± SD (n =  
 346 3).

347  
 348 **3.2. Selection of GSM algorithms for quantitative validation of meat product composition**

349 Having completed the MRM-MS analysis of the specific and global markers in all 28 meat products  
 350 (Table 1), we applied six different GSM algorithms for calculating relative contents of pork and beef in  
 351 the products. This has produced 168 individual data points presented in Figs. 2 and 3. Each algorithm  
 352 took into account the MS signal recorded for one of the three global markers (G1, G2 or G3) in the  
 353 analysed sample. The algorithms were divided into two groups depending on whether MS signals  
 354 recorded for specific markers P1 and B2 (group 1) or P2 and B1 (group 2) were used in calculations  
 355 (see Section 2.5., Table S2). The efficiency of each GSM algorithm in reflecting declared contents of a  
 356 specific meat species in samples of analysed meat products have been evaluated after calculating *P*-  
 357 values and absolute errors (AEs) for the measured/calculated contents versus the declared contents  
 358 of a given meat species in all meat products included in this study (Table 1). The relative pork content  
 359 (PC) was always calculated (equations 3, Section 2.5.) using the analysed contents of both pork and

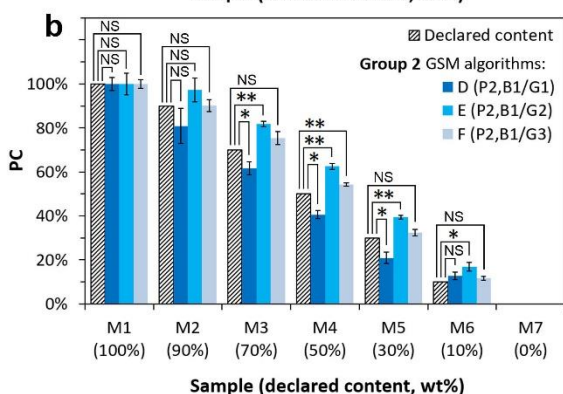
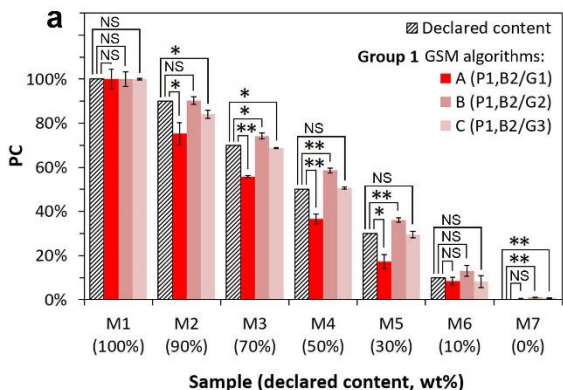
360 beef (CPM and CBM) for each meat product. Similar approach was made for calculating the relative  
361 beef content (BC; equations 4, Section 2.5.). The GSM algorithm(s), which returned, in a most  
362 consistent fashion, non-significant ( $P > 0.05$ ) differences between measured and declared PC and/or  
363 BC values have been considered most efficient for determining the relative contents of the two meat  
364 species. Algorithms have also been required to yield AE values  $\leq 10\%$  for both the raw and the  
365 thermally processed meat products.

366 Fig. 2a,b shows the PC results obtained for the mixtures of minced raw pork and beef at various  
367 proportions (M1–M7, Table 1). In most of the P/B mixes analysed (i.e., in 4-6 out of 7), only the group  
368 1 algorithm C and the group 2 algorithms D and F returned PC values that were not significantly  
369 different from the true, declared contents. The same was also confirmed for BC results produced for  
370 the P/B mixes (Fig. 3a,b). The AE analysis showed the PC and BC values calculated with the use of  
371 these three algorithms did not differ by more than 10% from the declared PCs for all seven M1-M7  
372 products (Tables 3 and S3). Similar accuracy has been shown in a recent study where a metal oxide  
373 semiconductor based E-Nose technique, supported with machine learning, was applied for detecting  
374 adulteration of minced beef with pork (0–60%) (Huang & Gu, 2022). The authors recorded a maximum  
375 AE of approx. 10%. However, in another similar study on beef adulteration (Zhao, Feng, Chen, & Jia,  
376 2019), the application of a visible near-infrared (Vis-NIR) hyperspectral imaging and least squares  
377 support vector machine (LS-SVM) model returned AE values up to approx. 16%.

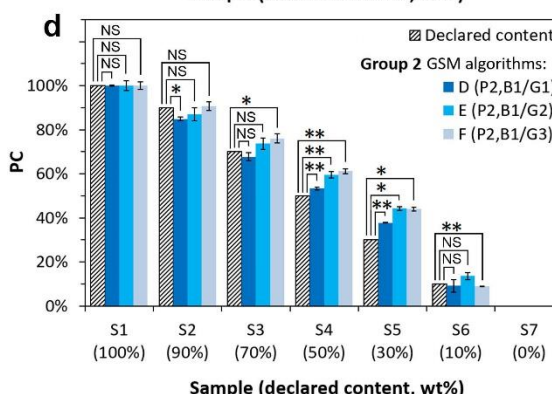
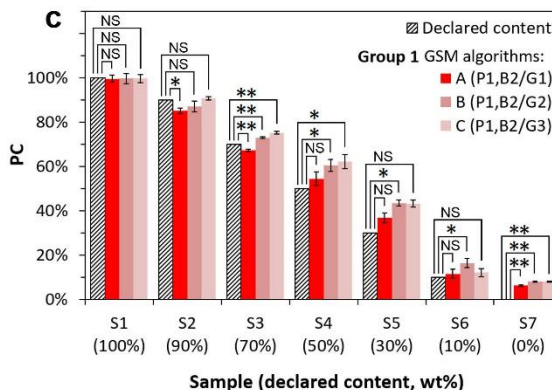
378 The PC and BC results obtained for the mixtures of raw pork and beef (Figs. 2a,b and 3a,b) were  
379 subsequently compared against the data obtained for sausages that had been produced with the  
380 same P/B ratios. They were manufactured by a meat processing company in a multi-step procedure  
381 that involved food processing conventionally used in preparation of such meat products for  
382 commercial purposes (Section 2.2). Thus, the sausages represented real meat products that were  
383 processed to a higher extent than the mixes of raw pork and beef. Despite this more complex  
384 processing, the AE values calculated for the sausages with GSM algorithms were found to be  $\leq 10\%$   
385 for most of the individual sausage types and the algorithms used (Tables 3 and S3). One of the few  
386 exceptions was sample S5, for which the AE of 13.2–14.2% was obtained after the PC and BC values  
387 had been calculated using algorithms B, C, E or F. Low ( $\leq 10\%$ ) AE values were most consistently  
388 shown for the use of algorithms A and D. The two algorithms were also amongst those that yielded  
389 non-significant differences between the calculated PC or BC values and the declared pork or beef  
390 ratios, for at least four out of seven different types of sausage (Figs. 2c,d and 3c,d). However, all the  
391 group 1 GSM algorithms, including algorithm A, showed that sample S7 (100% beef) contained 6–8%  
392 pork (Fig. 2c). This result is difficult to account for as all the other GSM algorithms – i.e., the group 2  
393 algorithms - did not confirm any contamination with pork in S7 (Fig. 2d), which, otherwise, would be an  
394 obvious suspicion here. It is worth noting that the same three algorithms (i.e., the group 1 algorithms  
395 A, B and C) also showed the presence of pork in sample M7 (100% beef, Fig. 2a), although it was  
396 much smaller (PC  $< 1\%$ ) than for sample S7. In this case too, the group 2 algorithms did not confirm  
397 the M7 sample contained any pork (Fig. 2b). All the above might suggest a small level of nonspecific  
398 signal could be recorded with the use of group 1 algorithms.



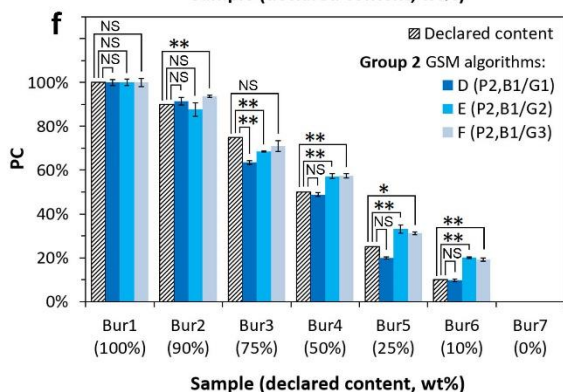
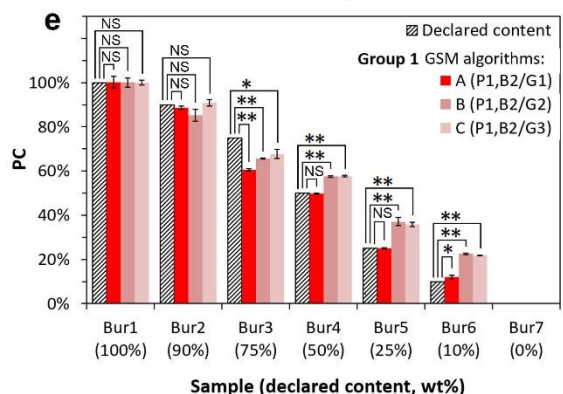
Mix of minced raw meats



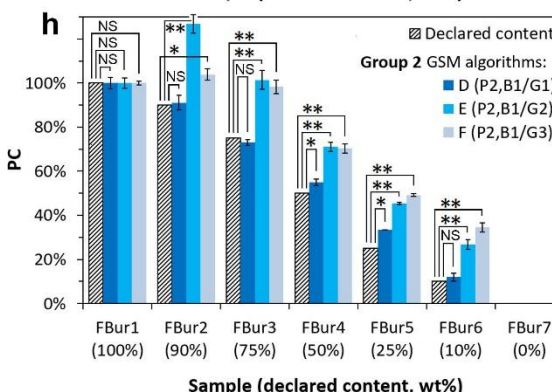
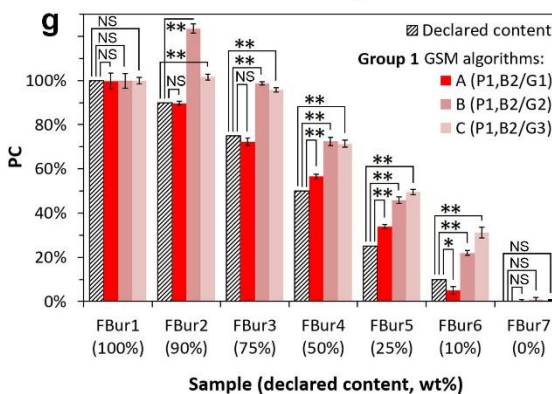
Sausages



Raw burgers



Fried burgers



401 Fig. 2. **The relative** pork contents (PC) calculated (equation 3) with the use of various GSM algorithms  
402 (Section 2.5., Table S2) for **four groups** of meat products: (a, b) mixes of raw, minced pork and beef, (c,  
403 d) sausages, **(e, f) raw burgers, and (g, h) fried burgers. All sets of the meat products were** made with  
404 various pork/beef proportions. Calculated PC values are plotted against the true, declared PC in the  
405 meat products. Individual results are shown as the mean  $\pm$  SD ( $n = 3$ ). \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; NS, not  
406 significant ( $P > 0.05$ ). The exact  $P$ -values have been given in Supplementary **Table S4.**

407

408 In the next step, we analysed results obtained for raw and fried burgers (Bur1–Bur7 and FBur1–FBur7,  
409 respectively; Table 1). The preparation of raw burgers required separate grinding of pork and beef,  
410 mixing at different P/B proportions (with addition of salt and pepper), and freezing the formed burgers  
411 to store before analysis. This relatively mild processing might have been the reason why the pork- and  
412 beef-specific peptides could be quite easily extracted from a meat matrix, and reflected well the true,  
413 declared contents of pork and beef (Figs. 2e,f and 3e,f). The PC and BC values finally obtained  
414 suggested the extraction of global markers was also largely unhindered (Fig. S2a-c). Tables 3 and S3  
415 show that the AE values of  $>10\%$  between the measured/calculated PC or BC and the **respective**  
416 **declared relative contents** were only evident in **several** cases; most notably for samples Bur5 and Bur6,  
417 both determined with algorithms B and C.

418 Frying burgers in oil heated to  $190\text{ }^{\circ}\text{C}$  resulted in some GSM algorithms losing their efficiency in  
419 validating the declared **relative contents of pork and beef** in burgers. This was especially true for the  
420 algorithms that utilised the MS data obtained for global markers G2 (i.e., algorithms B and E) and G3  
421 (i.e., algorithms C and F). Tables 3 and S3 show that the use of global marker G3 for calculating PC  
422 and BC can result in AE values being as large as 24–25%, whereas for G2 they were even higher; up  
423 to approx. 37%. The  $P$ -values obtained for the four GSM algorithms were predominantly  $\leq 0.01$  (Figs.  
424 2g,h and 3g,h), confirming the differences between the measured and the declared PC or BC were very  
425 significant. This might have been caused by different levels of hindrance in extracting G2 and G3  
426 peptides from a heat-denatured matrix of burgers, depending on the P/B content. Fig. S2e,f shows the  
427 MS signals recorded for the two global marker peptides was reduced roughly 4-fold between the  
428 samples containing 100% pork (FBur1) and 100% beef (FBur7). This is in contrast to relatively constant  
429 levels of MS signals obtained for the G2 and G3 peptides in the mildly processed raw burgers (Fig.  
430 S2b,c) or even in the sausages (Fig. 1a), the manufacturing of which involved cold smoking for 4h.

431 The MS signal recorded in the fried burgers for global marker G1 was also reduced, but only by 50%  
432 between the pure-pork and the pure-beef samples (Fig. S2d). This guaranteed the algorithms based on  
433 G1 (i.e., algorithms A and D) were still useful in validating PC and BC. In contrast to algorithms B, C, E  
434 and F, the PC and BC results obtained with algorithms A and D showed a non-significant discrepancy  
435 ( $P > 0.05$ ) for the **measured/calculated values** relative to the declared contents, for most of the fried  
436 burger samples (i.e., for 4–5 out of 7 burgers with various P/B proportions; Figs. 2g,h and 3g,h). All the  
437 AE values calculated for the use of algorithms A and D were found to be well below the 10% limit (Tables  
438 3 and S3).

439 **The detection limit (DL) calculated for the use of algorithm A in the analysis of fried burgers was 4.8%**  
440 **for PC and 2.1% for BC, whereas for algorithm D it was 5.1% and 6.2%, respectively. These values**



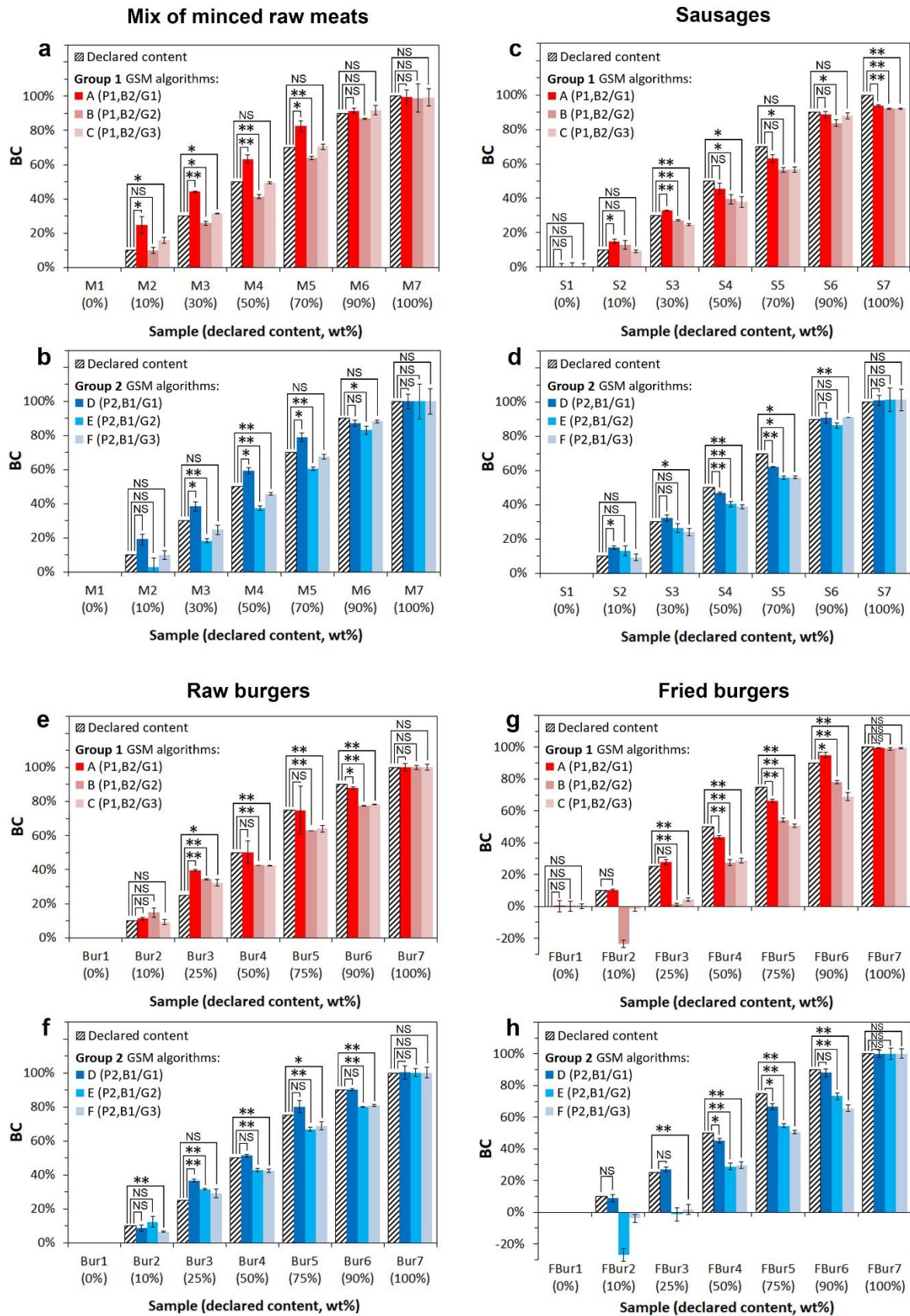
441 seem to be satisfactory when compared with the results presented for a number of different methods  
 442 that analysed specific protein/peptide markers for meat authentication purposes; where DL values have  
 443 been reported to fall in the range of 0.5–7.5% (Prandi et al., 2017) (Pan et al., 2018) (Montowska &  
 444 Fornal, 2017) (Feng et al., 2021) (Sezer et al., 2021) (Jiang et al., 2021).

445 Table 3. Absolute error (AE) values showing the difference between the relative pork content (PC)  
 446 calculated (equation 3) with the use of different SGM algorithms (A – F; see Section 2.5.) and the true  
 447 PC declared in the analysed meat products

Meat product/Sample	Group 1 GSM algorithms			Meat product/Sample	Group 1 GSM algorithms		
Mix of minced raw meats	A	B	C	Sausages	A	B	C
M1	<b>0%</b>	<b>0%</b>	<b>0%</b>	S1	<b>0.4%</b>	<b>0.3%</b>	<b>0.3%</b>
M2	14.7%	<b>0.2%</b>	<b>5.9%</b>	S2	<b>5.0%</b>	<b>3.0%</b>	<b>0.7%</b>
M3	14.2%	<b>4.2%</b>	<b>1.4%</b>	S3	<b>2.8%</b>	<b>2.9%</b>	<b>5.2%</b>
M4	13.3%	<b>8.6%</b>	<b>0.6%</b>	S4	<b>4.4%</b>	10.5%	12.2%
M5	12.7%	<b>6.1%</b>	<b>0.5%</b>	S5	<b>6.8%</b>	13.5%	13.2%
M6	<b>1.5%</b>	<b>3.1%</b>	<b>1.9%</b>	S6	<b>1.4%</b>	<b>6.4%</b>	<b>2.1%</b>
M7	<b>0.4%</b>	<b>1%</b>	<b>0.8%</b>	S7	<b>6.2%</b>	<b>7.9%</b>	<b>8.0%</b>
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Mix of minced raw meats	D	E	F	Sausages	D	E	F
M1	<b>0%</b>	<b>0%</b>	<b>0%</b>	S1	<b>0.001%</b>	<b>0.04%</b>	<b>0.02%</b>
M2	<b>9.1%</b>	<b>7.2%</b>	<b>0.1%</b>	S2	<b>5.1%</b>	<b>2.9%</b>	<b>0.8%</b>
M3	<b>8.4%</b>	11.8%	<b>5.4%</b>	S3	<b>2.3%</b>	<b>3.7%</b>	<b>6.1%</b>
M4	<b>9.4%</b>	12.5%	<b>4.3%</b>	S4	<b>3.3%</b>	<b>9.6%</b>	11.2%
M5	<b>9.1%</b>	<b>9.5%</b>	<b>2.4%</b>	S5	<b>7.7%</b>	14.2%	14.0%
M6	<b>2.7%</b>	<b>6.8%</b>	<b>1.7%</b>	S6	<b>0.8%</b>	<b>3.6%</b>	<b>1.1%</b>
M7	<b>0%</b>	<b>0%</b>	<b>0%</b>	S7	<b>0%</b>	<b>0%</b>	<b>0%</b>
	Group 1 GSM algorithms				Group 1 GSM algorithms		
Raw burgers	A	B	C	Fried burgers	A	B	C
Bur1	<b>0.08%</b>	<b>0.05%</b>	<b>0.02%</b>	FBur1	<b>0.3%</b>	<b>0.2%</b>	<b>0.2%</b>
Bur2	<b>1.3%</b>	<b>4.7%</b>	<b>0.8%</b>	FBur2	<b>0.2%</b>	33.5%	11.5%
Bur3	14.5%	<b>9.3%</b>	<b>7.3%</b>	FBur3	<b>2.8%</b>	23.8%	20.7%
Bur4	<b>0.2%</b>	<b>7.5%</b>	<b>7.7%</b>	FBur4	<b>6.5%</b>	22.5%	21.4%
Bur5	<b>0.1%</b>	12.2%	10.8%	FBur5	<b>8.8%</b>	20.8%	24.6%
Bur6	<b>2.0%</b>	12.5%	11.8%	FBur6	<b>5.0%</b>	12.0%	21.1%
Bur7	<b>0%</b>	<b>0%</b>	<b>0%</b>	FBur7	<b>0.5%</b>	<b>1.0%</b>	<b>0.6%</b>
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Raw burgers	D	E	F	Fried burgers	D	E	F
Bur1	<b>0.03%</b>	<b>0.02%</b>	<b>0.03%</b>	FBur1	<b>0.02%</b>	<b>0.01%</b>	<b>0.01%</b>
Bur2	<b>1.4%</b>	<b>2.3%</b>	<b>3.6%</b>	FBur2	<b>1.1%</b>	36.7%	13.9%
Bur3	11.5%	<b>6.5%</b>	<b>4.1%</b>	FBur3	<b>1.9%</b>	26.4%	23.2%
Bur4	<b>1.2%</b>	<b>7.3%</b>	<b>7.5%</b>	FBur4	<b>5.0%</b>	21.1%	20.3%
Bur5	<b>5.2%</b>	<b>8.1%</b>	<b>6.2%</b>	FBur5	<b>8.4%</b>	20.4%	24.2%
Bur6	<b>0.3%</b>	10.1%	<b>9.2%</b>	FBur6	<b>2.0%</b>	16.8%	24.5%
Bur7	<b>0%</b>	<b>0%</b>	<b>0%</b>	FBur7	<b>0%</b>	<b>0%</b>	<b>0%</b>

448 Values shown in bold print indicate AE ≤10%.

449  
 450  
 451



452

453 Fig. 3. The relative beef contents (BC) calculated (equation 4) with the use of various GSM algorithms  
 454 (Section 2.5., Table S2) for four groups of meat products: (a, b) mixes of raw, minced pork and beef, (c,  
 455 (d) sausages, (e, f) raw burgers, and (g, h) fried burgers. All sets of the meat products were made with

456 various pork/beef proportions. Calculated BC values are plotted against the true, declared BC in the  
457 meat products. Individual results are shown as the mean  $\pm$  SD ( $n = 3$ ). \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; NS, not  
458 significant ( $P > 0.05$ ). The exact  $P$ -values have been given in Supplementary Table S5.

459

#### 460 **4. Conclusions**

461 From the above characterisation of the data presented in Figs. 2 and 3, it is clear that the analysis of  
462 the global marker G1, the pork-specific marker P2 and the beef-specific marker B1, followed by the  
463 combined use of their MS signals in calculating PC and BC (algorithm D) offered the most reliable  
464 validation of the relative P/B composition across the whole range of raw and thermally processed meat  
465 products. Thus, the hypothesis of this study has been confirmed. The mean detection limit of the  
466 method utilising algorithm D was in the range of 5–6% for PC and BC across all the meat products  
467 analysed in this study. Nevertheless, further studies are required to confirm the efficiency of the  
468 algorithm in analysing different, commercial meat products containing highly processed pork and beef.  
469 However, the present work on the four different sets of meat products suggests an absolute error of  
470 determining the relative pork and beef contents in any such mixed meat products should not exceed  
471 10%.

472 This novel work demonstrates that a combined MS analysis of global and species-specific peptide  
473 markers allows for a quantitative validation of relative meat contents in food products made of more  
474 than one meat species. The method may, therefore, serve as a useful tool for the authentication of  
475 meat product composition.

476

#### 477 **Acknowledgements**

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482 for publication.

483

#### 484 **Supplementary Material**

485 Additional characterisation of the methods used and supporting data.

486

487

488 **Literature**

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586

587 **Figure captions**

588

589 Fig. 1. MRM-MS analysis of sausages containing various pork/beef **relative** contents (samples S1-S7,  
590 Table 1). MS signals recorded for (a) global markers G1, G2 and G3, (b) pork-specific markers P1 and  
591 P2, and (c) beef-specific markers B1 and B2. Individual data points are shown as the mean  $\pm$  SD (n =  
592 3).

593 Fig. 2. **The relative** pork contents (PC) calculated (equation 3) with the use of various GSM algorithms  
594 (Section 2.5., Table S2) for **four groups** of meat products: (a, b) mixes of raw, minced pork and beef, (c,  
595 d) sausages, **(e, f) raw burgers, and (g, h) fried burgers. All sets of the meat products were** made with  
596 various pork/beef proportions. Calculated PC values are plotted against the true, declared **PC** in the  
597 meat products. Individual results are shown as the mean  $\pm$  SD (n = 3). \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; NS, not  
598 significant ( $P > 0.05$ ). The exact  $P$ -values have been given in Supplementary **Table S4**.

599 **Fig. 3. The relative beef contents (BC) calculated (equation 4) with the use of various GSM algorithms**  
600 **(Section 2.5., Table S2) for four groups of meat products: (a, b) mixes of raw, minced pork and beef, (c,**  
601 **d) sausages, (e, f) raw burgers, and (g, h) fried burgers. All sets of the meat products were made with**  
602 **various pork/beef proportions. Calculated BC values are plotted against the true, declared BC in the**  
603 **meat products. Individual results are shown as the mean  $\pm$  SD (n = 3). \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; NS, not**  
604 **significant ( $P > 0.05$ ). The exact  $P$ -values have been given in Supplementary Table S5.**



**Relative quantification of pork and beef in meat products using global and species-specific peptide markers for the authentication of meat composition**

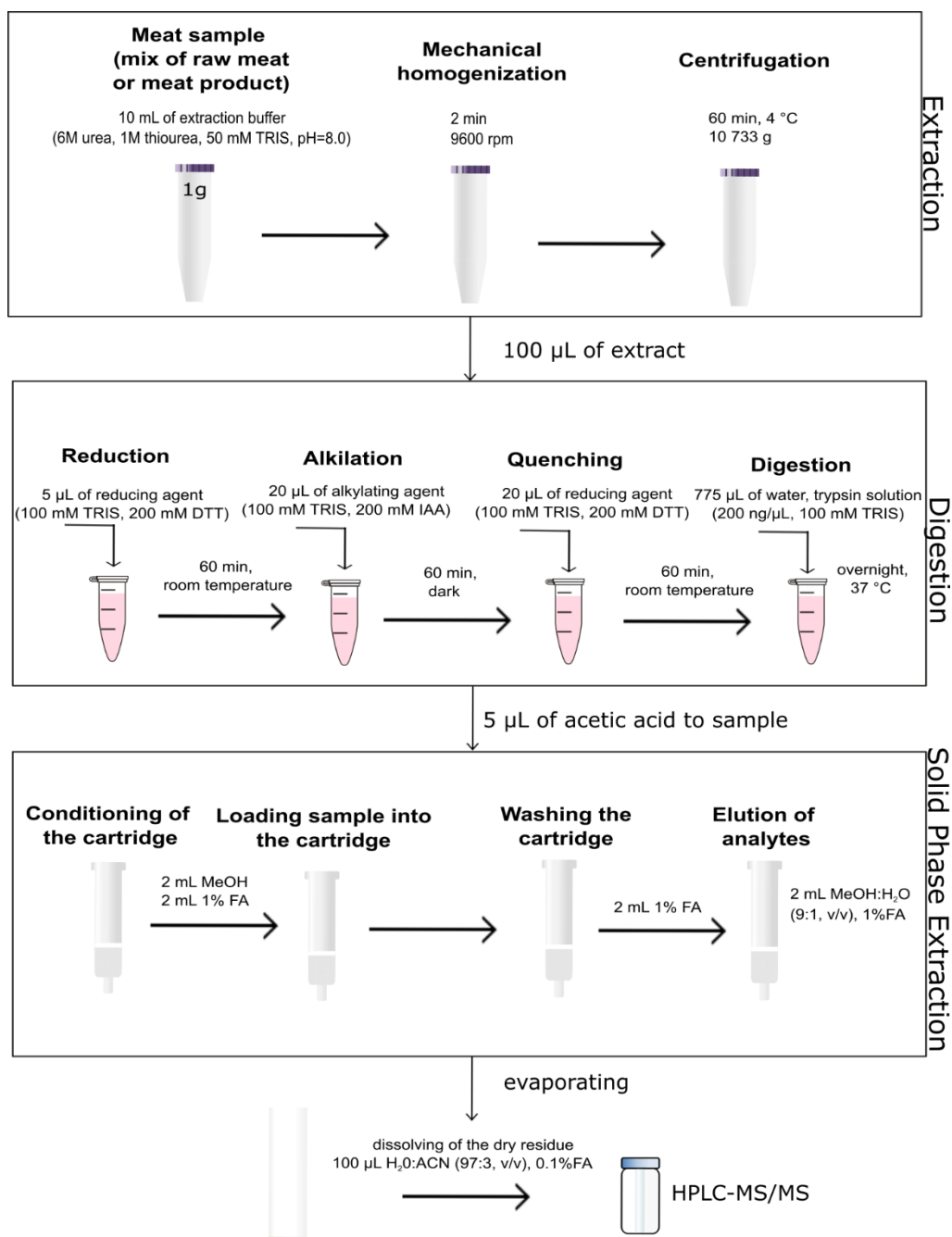
Katarzyna Nalazek-Rudnicka<sup>a</sup>, Ilona Kłosowska-Chomiczewska<sup>b</sup>, Jens Brockmeyer<sup>c</sup>, Andrzej Wasik<sup>a</sup>, Adam Macierzanka<sup>b,\*</sup>

<sup>a</sup> Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza 11/12, 80-233 Gdańsk, Poland

<sup>b</sup> Department of Colloid and Lipid Science, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza 11/12, 80-233 Gdańsk, Poland

<sup>c</sup> Department Food Chemistry, Institute for Biochemistry and Technical Biochemistry, University of Stuttgart, Allmandring 5B, 70569 Stuttgart, Germany

\*Corresponding author. E-mail address: adam.macierzanka@pg.edu.pl (A. Macierzanka).



**Fig.S1.** Procedure of sample preparation required for HPLC-MS/MS analysis of peptide markers.

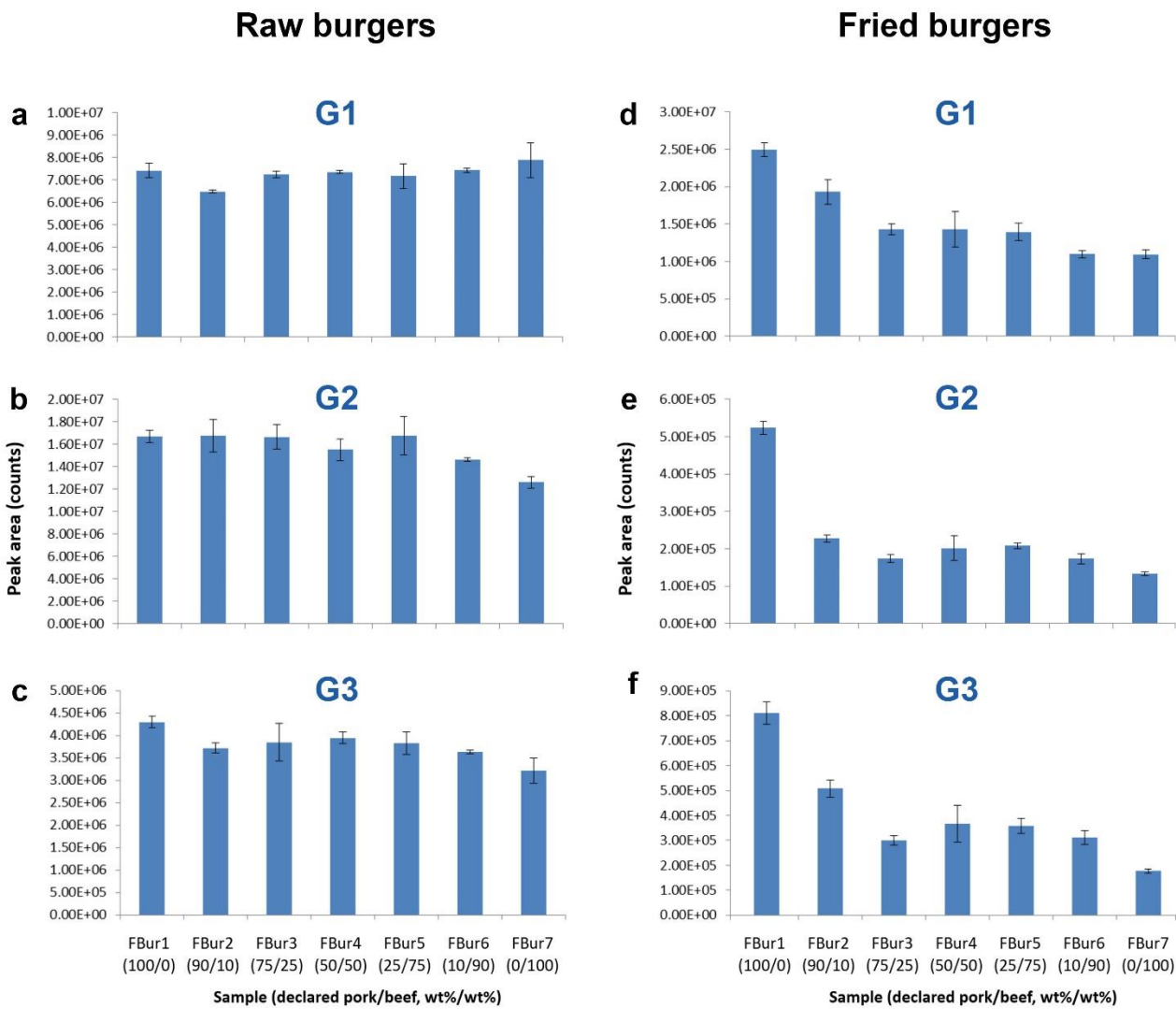
**Table S1.** Separation conditions of peptide markers

Column	Kinetex XB C-18 (100 x 2.1 mm, 2.6 $\mu$ m)
Flow rate (mL/min)	0.3
Temperature of thermostat ( $^{\circ}$ C)	40 $^{\circ}$ C
Injection volume	1 $\mu$ L
Analysis time	38 min
Mobile phase	A: water containing 0.1 % (v/v) FA B: ACN containing 0.1 % (v/v) FA
Gradient elution	0 $\rightarrow$ 22 min, 3-30 % B 22 $\rightarrow$ 28 min, 30-70 % B 28 $\rightarrow$ 29 min, 70-100 % B 29 $\rightarrow$ 31 min, 100 % B 31 $\rightarrow$ 38 min, 3 % B

**Table S2.** Global and Specific Markers (GSM) algorithms created for assessing quantitatively the relative composition of meat products

GSM algorithms (species-specific/global peptide markers)		Group of peptide markers
<b>A</b>	P1,B2/G1	<b>Group 1</b> (P1 and B2)
<b>B</b>	P1,B2/G2	
<b>C</b>	P1,B2/G3	
<b>D</b>	P2,B1/G1	<b>Group 2</b> (P2 and B1)
<b>E</b>	P2,B1/G2	
<b>F</b>	P2,B1/G3	





**Fig. S2.** MRM-MS analysis of global markers G1, G2 and G3 in (a-c) raw burgers and (d-f) fried burgers, made with various pork/beef proportions. The declared proportions are true proportions (samples Bur1–Bur7 and FBur1–FBur7, Table 1). Individual MS-signal data points are shown as the mean  $\pm$  SD ( $n = 3$ ).

**Table S3.** Absolute error (AE) values showing the difference between the relative beef content (BC) calculated (equation 4) with the use of different SGM algorithms (A – F; see Section 2.5. in the main text) and the true BC declared in the analysed meat products

Meat product/Sample	Group 1 GSM algorithms			Meat product/Sample	Group 1 GSM algorithms		
Mix of minced raw meats	A	B	C	Sausages	A	B	C
M1	<b>0%</b>	<b>0%</b>	<b>0%</b>	S1	<b>0.4%</b>	<b>0.3%</b>	<b>0.3%</b>
M2	14.7%	<b>0.2%</b>	<b>5.9%</b>	S2	<b>5.0%</b>	<b>3.0%</b>	<b>0.7%</b>
M3	14.2%	<b>4.2%</b>	<b>1.4%</b>	S3	<b>2.8%</b>	<b>2.9%</b>	<b>5.2%</b>
M4	13.3%	<b>8.6%</b>	<b>0.6%</b>	S4	<b>4.4%</b>	10.5%	12.2%
M5	12.7%	<b>6.1%</b>	<b>0.5%</b>	S5	<b>6.8%</b>	13.5%	13.2%
M6	<b>1.5%</b>	<b>3.1%</b>	<b>1.9%</b>	S6	<b>1.4%</b>	<b>6.4%</b>	<b>2.1%</b>
M7	<b>0.4%</b>	<b>1.0%</b>	<b>0.8%</b>	S7	<b>6.2%</b>	<b>7.9%</b>	<b>7.9%</b>
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Mix of minced raw meats	D	E	F	Sausages	D	E	F
M1	<b>0%</b>	<b>0%</b>	<b>0%</b>	S1	<b>0.0%</b>	<b>0.0%</b>	<b>0.0%</b>
M2	<b>9.1%</b>	<b>7.2%</b>	<b>0.1%</b>	S2	<b>5.1%</b>	<b>2.9%</b>	<b>0.8%</b>
M3	<b>8.4%</b>	11.8%	<b>5.4%</b>	S3	<b>2.3%</b>	<b>3.7%</b>	<b>6.1%</b>
M4	<b>9.4%</b>	12.5%	<b>4.3%</b>	S4	<b>3.3%</b>	<b>9.6%</b>	11.2%
M5	<b>9.1%</b>	<b>9.5%</b>	<b>2.4%</b>	S5	<b>7.7%</b>	14.2%	14.0%
M6	<b>2.7%</b>	<b>6.8%</b>	<b>1.7%</b>	S6	<b>0.8%</b>	<b>3.6%</b>	<b>1.1%</b>
M7	<b>0%</b>	<b>0%</b>	<b>0%</b>	S7	<b>0.9%</b>	<b>1.3%</b>	<b>1.3%</b>
	Group 1 GSM algorithms				Group 1 GSM algorithms		
Raw burgers	A	B	C	Fried burgers	A	B	C
Bur1	<b>0.0%</b>	<b>0.0%</b>	<b>0.0%</b>	FBur1	<b>0.3%</b>	<b>0.2%</b>	<b>0.2%</b>
Bur2	<b>1.3%</b>	<b>4.7%</b>	<b>0.8%</b>	FBur2	<b>0.2%</b>	33.5%	11.5%
Bur3	14.5%	<b>9.3%</b>	<b>7.3%</b>	FBur3	<b>2.8%</b>	23.8%	20.7%
Bur4	<b>0.2%</b>	<b>7.5%</b>	<b>7.7%</b>	FBur4	<b>6.5%</b>	22.5%	21.4%
Bur5	<b>0.1%</b>	12.2%	10.8%	FBur5	<b>8.8%</b>	20.8%	24.6%
Bur6	<b>2.0%</b>	12.5%	11.8%	FBur6	<b>5.0%</b>	12.0%	21.1%
Bur7	<b>0.1%</b>	<b>0.1%</b>	<b>0.1%</b>	FBur7	<b>0.5%</b>	<b>1.0%</b>	<b>0.6%</b>
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Raw burgers	D	E	F	Fried burgers	D	E	F
Bur1	<b>0.0%</b>	<b>0.0%</b>	<b>0.0%</b>	FBur1	<b>0.0%</b>	<b>0.0%</b>	<b>0.0%</b>
Bur2	<b>1.4%</b>	<b>2.3%</b>	<b>3.6%</b>	FBur2	<b>1.1%</b>	36.7%	13.9%
Bur3	11.5%	<b>6.5%</b>	<b>4.1%</b>	FBur3	<b>1.9%</b>	26.4%	23.2%
Bur4	<b>1.2%</b>	<b>7.3%</b>	<b>7.5%</b>	FBur4	<b>5.0%</b>	21.1%	20.3%
Bur5	<b>5.2%</b>	<b>8.1%</b>	<b>6.2%</b>	FBur5	<b>8.4%</b>	20.4%	24.2%
Bur6	<b>0.3%</b>	10.1%	<b>9.2%</b>	FBur6	<b>2.0%</b>	16.8%	24.5%
Bur7	<b>0.2%</b>	<b>0.1%</b>	<b>0.2%</b>	FBur7	<b>0.02%</b>	<b>0.02%</b>	<b>0.006%</b>

Values shown in bold print indicate AE ≤10%.

**Table S4.** *P*-values showing the level of statistical significance for the comparison between the mean relative pork contents, measured/calculated for meat products with GSM algorithms (Table S2), and the declared contents – the true relative contents of pork in meat products (see Table 1 for detailed characterisation of the meat products).  $P \leq 0.05$  indicates the GSM result differed significantly from the declared pork content.

Mix of minced raw meats				Sausages				Raw burgers				Fried burgers			
GSM algorithms, Group 1		GSM algorithms, Group 2		GSM algorithms, Group 1		GSM algorithms, Group 2		GSM algorithms, Group 1		GSM algorithms, Group 2		GSM algorithms, Group 1		GSM algorithms, Group 2	
M1	1.0000	M1	1.0000	S1	0.7075	S1	0.9957	Bur1	0.9652	Bur1	0.9734	FBur1	0.8967	FBur1	0.9926
M2	<b>0.0366</b>	M2	0.1853	S2	<b>0.0193</b>	S2	<b>0.0108</b>	Bur2	0.0805	Bur2	0.2997	FBur2	0.6384	FBur2	0.6381
M3	<b>0.0003</b>	M3	<b>0.0372</b>	S3	<b>0.0096</b>	S3	0.1604	Bur3	<b>0.0004</b>	Bur3	<b>0.0015</b>	FBur3	0.1012	FBur3	0.1370
M4	<b>0.0094</b>	M4	<b>0.0117</b>	S4	0.1332	S4	<b>0.0097</b>	Bur4	0.1147	Bur4	0.1315	FBur4	<b>0.0084</b>	FBur4	<b>0.0289</b>
M5	<b>0.0201</b>	M5	<b>0.0251</b>	S5	0.1405	S5	<b>0.0076</b>	Bur5	0.9306	Bur5	0.1255	FBur5	<b>0.0039</b>	FBur5	<b>0.0149</b>
M6	0.2543	M6	0.1207	S6	0.3510	S6	0.6531	Bur6	<b>0.0464</b>	Bur6	0.5417	FBur6	<b>0.0339</b>	FBur6	0.3070
M7	0.1461	M7	x	S7	<b>0.0011</b>	S7	x	Bur7	x	Bur7	x	FBur7	0.1672	FBur7	x
M1	1.0000	M1	1.0000	S1	0.8641	S1	0.9768	Bur1	0.9728	Bur1	0.9843	FBur1	0.9244	FBur1	0.9972
M2	0.8321	M2	0.1434	S2	0.1723	S2	0.2239	Bur2	0.0940	Bur2	0.3242	FBur2	<b>0.0014</b>	FBur2	<b>0.0039</b>
M3	<b>0.0279</b>	M3	<b>0.0037</b>	S3	<b>0.0052</b>	S3	0.1282	Bur3	<b>0.0004</b>	Bur3	<b>0.0007</b>	FBur3	<b>0.0004</b>	FBur3	<b>0.0088</b>
M4	<b>0.0049</b>	M4	<b>0.0044</b>	S4	<b>0.0225</b>	S4	<b>0.0077</b>	Bur4	<b>0.0010</b>	Bur4	<b>0.0072</b>	FBur4	<b>0.0022</b>	FBur4	<b>0.0033</b>
M5	<b>0.0099</b>	M5	<b>0.0028</b>	S5	<b>0.0427</b>	S5	<b>0.0291</b>	Bur5	<b>0.0046</b>	Bur5	<b>0.0092</b>	FBur5	<b>0.0010</b>	FBur5	<b>0.0011</b>
M6	0.1569	M6	<b>0.0264</b>	S6	<b>0.0317</b>	S6	0.0588	Bur6	<b>0.0003</b>	Bur6	<b>0.0003</b>	FBur6	<b>0.0031</b>	FBur6	<b>0.0057</b>
M7	<b>0.0020</b>	M7	x	S7	<b>0.0004</b>	S7	x	Bur7	x	Bur7	x	FBur7	0.2015	FBur7	x
M1	1.0000	M1	1.0000	S1	0.8117	S1	0.9874	Bur1	0.9763	Bur1	0.9803	FBur1	0.8312	FBur1	0.9923
M2	<b>0.0290</b>	M2	0.9487	S2	0.2281	S2	0.5505	Bur2	0.4329	Bur2	<b>0.0067</b>	FBur2	<b>0.0052</b>	FBur2	<b>0.0106</b>
M3	<b>0.0108</b>	M3	0.0826	S3	<b>0.0047</b>	S3	<b>0.0397</b>	Bur3	<b>0.0243</b>	Bur3	0.1032	FBur3	<b>0.0007</b>	FBur3	<b>0.0055</b>
M4	0.2346	M4	<b>0.0076</b>	S4	<b>0.0224</b>	S4	<b>0.0032</b>	Bur4	<b>0.0004</b>	Bur4	<b>0.0058</b>	FBur4	<b>0.0017</b>	FBur4	<b>0.0035</b>
M5	0.6403	M5	0.0947	S5	0.0532	S5	<b>0.0273</b>	Bur5	<b>0.0054</b>	Bur5	0.0496	FBur5	<b>0.0006</b>	FBur5	<b>0.0007</b>
M6	0.3404	M6	0.0758	S6	0.1818	S6	<b>0.0018</b>	Bur6	<b>0.0001</b>	Bur6	<b>0.0020</b>	FBur6	<b>0.0045</b>	FBur6	<b>0.0024</b>
M7	<b>0.0070</b>	M7	x	S7	<b>0.0002</b>	S7	x	Bur7	x	Bur7	x	FBur7	0.0584	FBur7	x

Numbers shown in bold indicate  $P \leq 0.05$ ,  
x, the pork specific peptide markers (P1, P2) not detected in sample declared as 100% beef.

**Table S5.** *P*-values showing the level of statistical significance for the comparison between the mean relative beef contents, measured/calculated for meat products with GSM algorithms (Table S2), and the declared contents – the true relative contents of beef in meat products (see Table 1 for detailed characterisation of the meat products).  $P \leq 0.05$  indicates the GSM result differed significantly from the declared beef content.

Mix of minced raw meats		Sausages		Raw burgers		Fried burgers									
GSM algorithms, Group 1		GSM algorithms, Group 2		GSM algorithms, Group 1		GSM algorithms, Group 1		GSM algorithms, Group 2							
M1	x	M1	x	S1	0.7066	S1	x	Bur1	x	Bur1	x	FBur1	0.9091	FBur1	x
M2	<b>0.0366</b>	M2	0.1853	S2	<b>0.0193</b>	S2	<b>0.0108</b>	Bur2	0.0806	Bur2	0.2997	FBur2	0.6386	FBur2	0.6395
M3	<b>0.0003</b>	M3	<b>0.0372</b>	S3	<b>0.0096</b>	S3	0.1609	Bur3	<b>0.0004</b>	Bur3	<b>0.0015</b>	FBur3	0.1011	FBur3	0.1335
M4	<b>0.0094</b>	M4	<b>0.0117</b>	S4	0.1332	S4	<b>0.0096</b>	Bur4	0.1121	Bur4	0.1315	FBur4	<b>0.0084</b>	FBur4	<b>0.0279</b>
M5	<b>0.0201</b>	M5	<b>0.0251</b>	S5	0.1404	S5	<b>0.0078</b>	Bur5	0.9318	Bur5	0.1255	FBur5	<b>0.0039</b>	FBur5	<b>0.0146</b>
M6	0.2543	M6	0.1207	S6	0.3504	S6	0.6529	Bur6	<b>0.0465</b>	Bur6	0.5415	FBur6	<b>0.0339</b>	FBur6	0.3123
M7	<b>0.0314</b>	M7	1.0000	S7	<b>0.0011</b>	S7	0.6391	Bur7	0.9233	Bur7	0.9229	FBur7	0.1666	FBur7	0.9915
M1	x	M1	x	S1	0.8639	S1	x	Bur1	x	Bur1	x	FBur1	0.9375	FBur1	x
M2	0.8354	M2	0.1434	S2	0.1723	S2	0.2247	Bur2	0.0936	Bur2	0.3242	FBur2	y	FBur2	y
M3	<b>0.0278</b>	M3	<b>0.0037</b>	S3	<b>0.0053</b>	S3	0.1279	Bur3	<b>0.0004</b>	Bur3	<b>0.0007</b>	FBur3	<b>0.0004</b>	FBur3	y
M4	<b>0.0049</b>	M4	<b>0.0044</b>	S4	<b>0.0224</b>	S4	<b>0.0077</b>	Bur4	<b>0.0010</b>	Bur4	<b>0.0072</b>	FBur4	<b>0.0022</b>	FBur4	<b>0.0033</b>
M5	<b>0.0099</b>	M5	<b>0.0028</b>	S5	<b>0.0427</b>	S5	<b>0.0291</b>	Bur5	<b>0.0045</b>	Bur5	<b>0.0093</b>	FBur5	<b>0.0010</b>	FBur5	<b>0.0011</b>
M6	0.1569	M6	<b>0.0399</b>	S6	<b>0.0316</b>	S6	0.0585	Bur6	<b>0.0003</b>	Bur6	<b>0.0003</b>	FBur6	<b>0.0031</b>	FBur6	<b>0.0059</b>
M7	<b>0.0021</b>	M7	1.0000	S7	<b>0.0004</b>	S7	0.7710	Bur7	0.9691	Bur7	0.9786	FBur7	0.2017	FBur7	0.9947
M1	x	M1	x	S1	0.8117	S1	x	Bur1	x	Bur1	x	FBur1	0.8591	FBur1	x
M2	<b>0.0290</b>	M2	0.9487	S2	0.2299	S2	0.5504	Bur2	0.4317	Bur2	<b>0.0067</b>	FBur2	y	FBur2	y
M3	<b>0.0111</b>	M3	0.0826	S3	<b>0.0047</b>	S3	<b>0.0396</b>	Bur3	<b>0.0244</b>	Bur3	0.1032	FBur3	<b>0.0007</b>	FBur3	<b>0.0057</b>
M4	0.2353	M4	<b>0.0076</b>	S4	<b>0.0223</b>	S4	<b>0.0032</b>	Bur4	<b>0.0004</b>	Bur4	<b>0.0058</b>	FBur4	<b>0.0017</b>	FBur4	<b>0.0036</b>
M5	0.6374	M5	0.0947	S5	0.0532	S5	<b>0.0273</b>	Bur5	<b>0.0054</b>	Bur5	0.0496	FBur5	<b>0.0006</b>	FBur5	<b>0.0006</b>
M6	0.3404	M6	0.0758	S6	0.1824	S6	<b>0.0016</b>	Bur6	<b>0.0001</b>	Bur6	<b>0.0020</b>	FBur6	<b>0.0045</b>	FBur6	<b>0.0024</b>
M7	<b>0.0064</b>	M7	1.0000	S7	<b>0.0004</b>	S7	0.7559	Bur7	0.9405	Bur7	0.9325	FBur7	0.0577	FBur7	0.9974

Numbers shown in bold indicate  $P \leq 0.05$ ,

x, the beef specific peptide markers (B1, B2) not detected in sample declared as 100% pork; y, negative value