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

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

Abstract

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

Keywords:

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

1. Introduction

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

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

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

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

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

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

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

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

2. Material and methods

2.1. Materials

Acetonitrile (ACN, LC-MS grade), methanol (MeOH, LC-MS grade), urea, thiourea, tris(hydroxymethyl)aminomethane (TRIS), trypsin (T0303, type IX-S,13,000–20,000 BAEE units/mg protein), dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma Aldrich (St. Louis, USA). Analytical grade hydrochloride, acetic acid and formic acid (FA) were obtained from POCH (Gliwice, Poland). Ultrapure water was prepared using a HLP_5 system (Hydrolab, Wiślina, Poland).

2.2. Preparation of meat products

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

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

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

to this procedure have been referred to as 'fried burgers' throughout the paper. Preparation and analysis of all individual raw and fried burgers listed in Table 1 were done in triplicate (n = 3). Table 1 summarises the compositions and the processing conditions of the meat products used in the study.

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 (Φ 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 (Φ 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 (Φ 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		

2.3. Sample preparation for HPLC-MS/MS analysis

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

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

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

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

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

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

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

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
					619.4	-11	-18	-28
Myoglobin	P02189	P2, HPGDFGADAQGAMSK	Pork	744.8	234.1	-24	-34	-23
					1254.5	-22	-26	-46
					692.3	-22	-28	-26
					1351.6	-20	-28	-48
Myoglobin	P02192	B1, VLGFHG	Beef	315.2	417.2	-11	-10	-19
					213.2	-11	-15	-23
					530.3	-11	-12	-26
					360.2	-10	-17	-17
Myoglobin	P02192	B2, HPSDFGADAQAAMSK	Beef	766.8	234.1	-24	-35	-10
					1298.6	-26	-26	-36
					706.4	-28	-31	-26
					1395.6	-20	-28	-40
G1 (global marker)	-	G1, LFDLR	*n/a*, global marker	332.2	550.3	-11	-13	-18
					403.2	-11	-12	-17
G2 (global marker)	-	G2, DIDDLELTLAK	*n/a*, global marker	623.3	1017.5	-10	-18	-21
					674.5	-12	-19	-31
					902.5	-11	-19	-17
					787.5	-10	-20	-19
G3 (global marker)	-	G3, HQGVMVGMGQK	*n/a*, global marker	586.3	906.5	-15	-22	-18
					750.4	-15	-24	-21
					619.3	-13	-25	-23
					849.5	-13	-23	-25

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

2.5. Data processing

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

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

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

where:

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

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

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

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

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

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

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

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

3. Results and discussion

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

3.1. Species-specific and global marker analysis

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

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

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

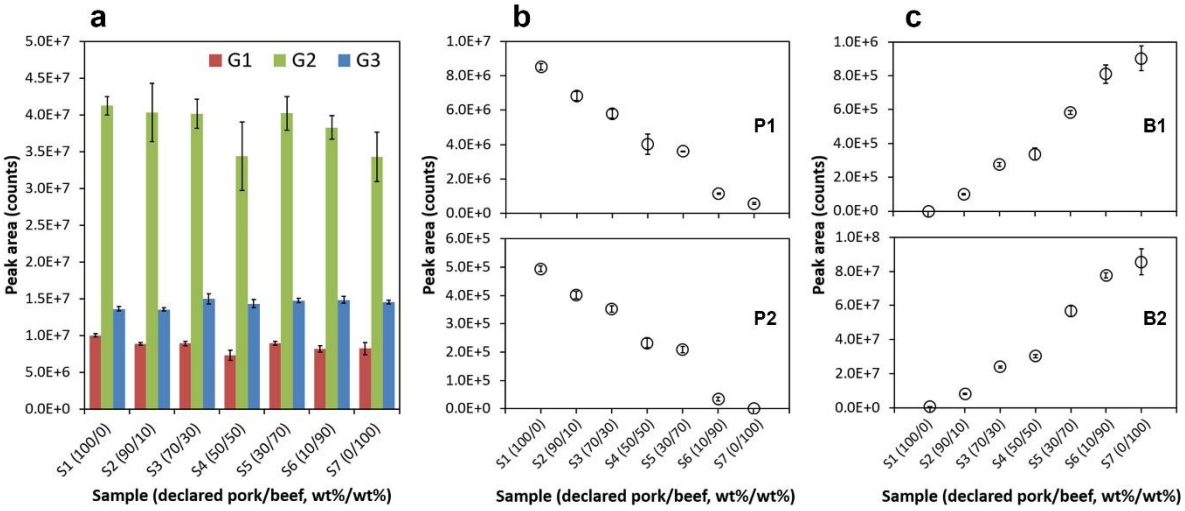

Fig. 1. MRM-MS analysis of sausages containing various pork/beef relative contents (samples S1-S7, Table 1). MS signals recorded for (a) global markers G1, G2 and G3, (b) pork-specific markers P1 and P2, and (c) beef-specific markers B1 and B2. Individual data points are shown as the mean ± SD (n = 3).

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

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

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

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

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

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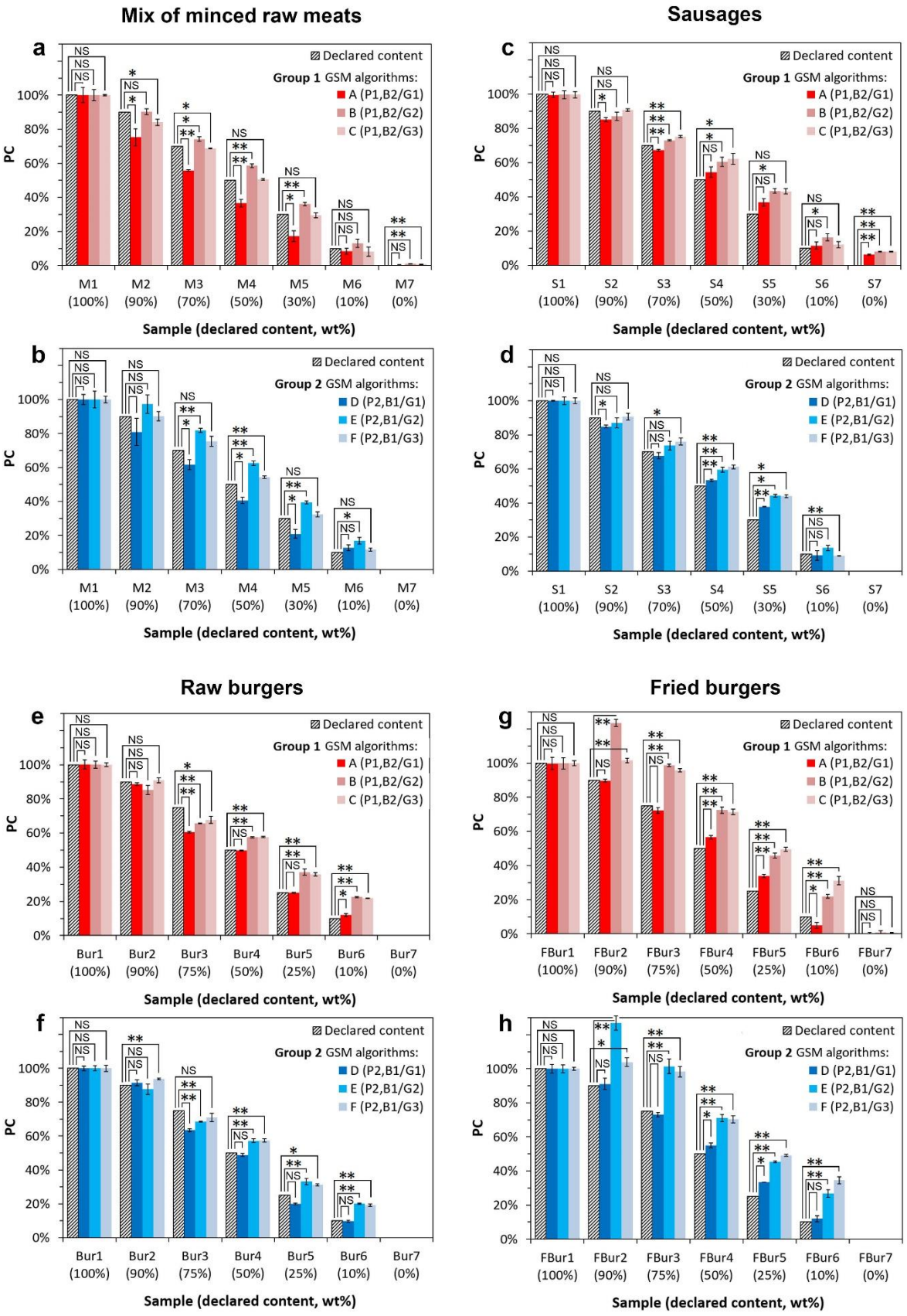

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Fig. 2. The relative pork contents (PC) calculated (equation 3) with the use of various GSM algorithms (Section 2.5., Table S2) for four groups of meat products: (a, b) mixes of raw, minced pork and beef, (c, d) sausages, (e, f) raw burgers, and (g,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

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

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

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

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

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

Table 3. Absolute error (AE) values showing the difference between the relative pork content (PC) calculated (equation 3) with the use of different SGM algorithms (A – F; see Section 2.5.) and the true 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	**0%**	**0%**	**0%**	S1	**0.4%**	**0.3%**	**0.3%**
M2	14.7%	**0.2%**	**5.9%**	S2	**5.0%**	**3.0%**	**0.7%**
M3	14.2%	**4.2%**	**1.4%**	S3	**2.8%**	**2.9%**	**5.2%**
M4	13.3%	**8.6%**	**0.6%**	S4	**4.4%**	10.5%	12.2%
M5	12.7%	**6.1%**	**0.5%**	S5	**6.8%**	13.5%	13.2%
M6	**1.5%**	**3.1%**	**1.9%**	S6	**1.4%**	**6.4%**	**2.1%**
M7	**0.4%**	**1%**	**0.8%**	S7	**6.2%**	**7.9%**	**8.0%**
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Mix of minced raw meats	D	E	F	Sausages	D	E	F
M1	**0%**	**0%**	**0%**	S1	**0.001%**	**0.04%**	**0.02%**
M2	**9.1%**	**7.2%**	**0.1%**	S2	**5.1%**	**2.9%**	**0.8%**
M3	**8.4%**	11.8%	**5.4%**	S3	**2.3%**	**3.7%**	**6.1%**
M4	**9.4%**	12.5%	**4.3%**	S4	**3.3%**	**9.6%**	11.2%
M5	**9.1%**	**9.5%**	**2.4%**	S5	**7.7%**	14.2%	14.0%
M6	**2.7%**	**6.8%**	**1.7%**	S6	**0.8%**	**3.6%**	**1.1%**
M7	**0%**	**0%**	**0%**	S7	**0%**	**0%**	**0%**
	Group 1 GSM algorithms				Group 1 GSM algorithms		
Raw burgers	A	B	C	Fried burgers	A	B	C
Bur1	**0.08%**	**0.05%**	**0.02%**	FBur1	**0.3%**	**0.2%**	**0.2%**
Bur2	**1.3%**	**4.7%**	**0.8%**	FBur2	**0.2%**	33.5%	11.5%
Bur3	14.5%	**9.3%**	**7.3%**	FBur3	**2.8%**	23.8%	20.7%
Bur4	**0.2%**	**7.5%**	**7.7%**	FBur4	**6.5%**	22.5%	21.4%
Bur5	**0.1%**	12.2%	10.8%	FBur5	**8.8%**	20.8%	24.6%
Bur6	**2.0%**	12.5%	11.8%	FBur6	**5.0%**	12.0%	21.1%
Bur7	**0%**	**0%**	**0%**	FBur7	**0.5%**	**1.0%**	**0.6%**
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Raw burgers	D	E	F	Fried burgers	D	E	F
Bur1	**0.03%**	**0.02%**	**0.03%**	FBur1	**0.02%**	**0.01%**	**0.01%**
Bur2	**1.4%**	**2.3%**	**3.6%**	FBur2	**1.1%**	36.7%	13.9%
Bur3	11.5%	**6.5%**	**4.1%**	FBur3	**1.9%**	26.4%	23.2%
Bur4	**1.2%**	**7.3%**	**7.5%**	FBur4	**5.0%**	21.1%	20.3%
Bur5	**5.2%**	**8.1%**	**6.2%**	FBur5	**8.4%**	20.4%	24.2%
Bur6	**0.3%**	10.1%	**9.2%**	FBur6	**2.0%**	16.8%	24.5%
Bur7	**0%**	**0%**	**0%**	FBur7	**0%**	**0%**	**0%**

Values shown in bold print indicate AE ≤10%.

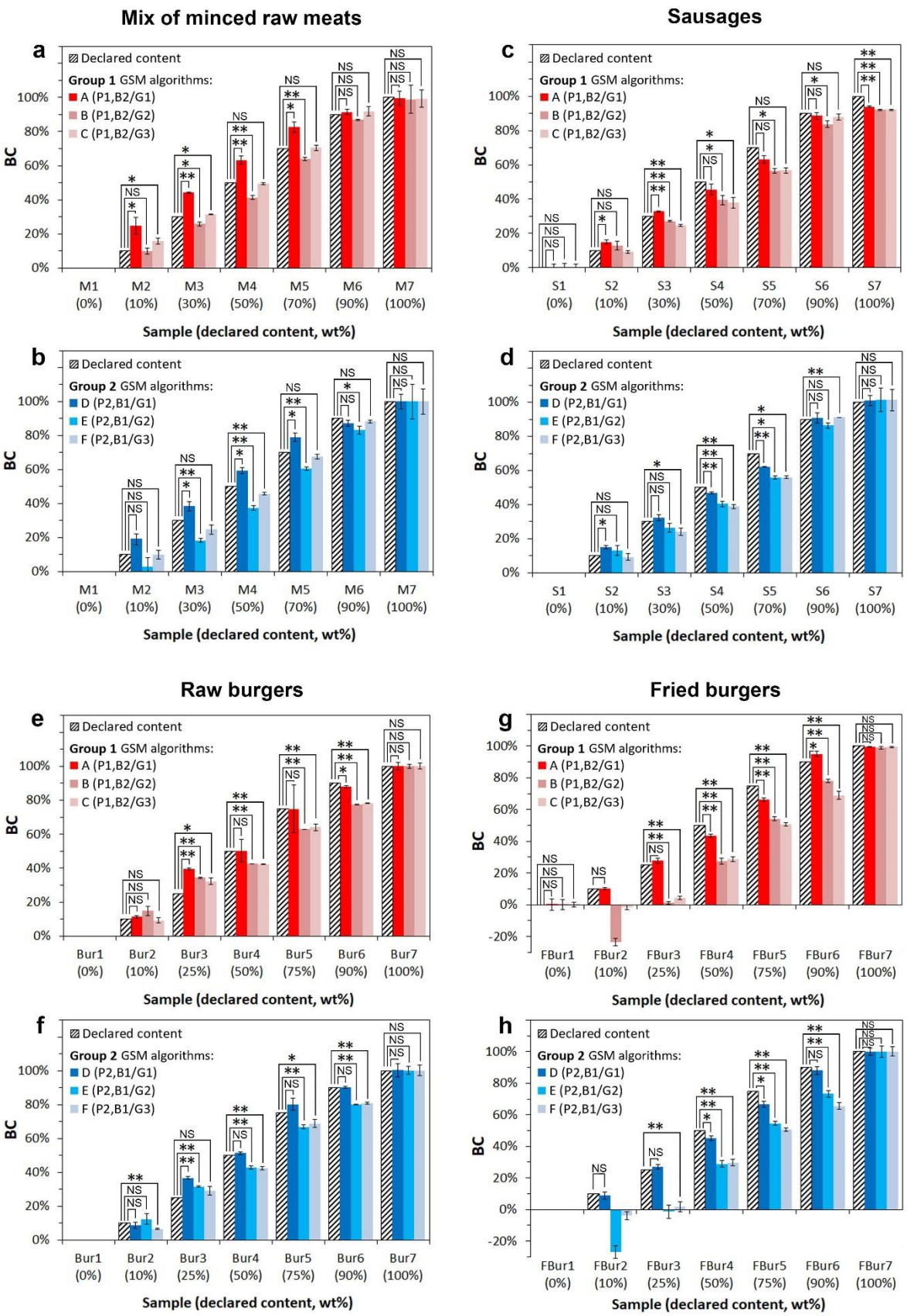

Fig. 3. The relative beef contents (BC) calculated (equation 4) with the use of various GSM algorithms (Section 2.5., Table S2) for four groups of meat products: (a, b) mixes of raw, minced pork and beef, (c, d) sausages, (e, f) raw burgers, and (g, h) fried burgers. All sets of the meat products were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

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

4. Conclusions

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

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

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Supplementary Material

Additional characterisation of the methods used and supporting data.

Literature

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586

Figure captions

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

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

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

Supplementary Material

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

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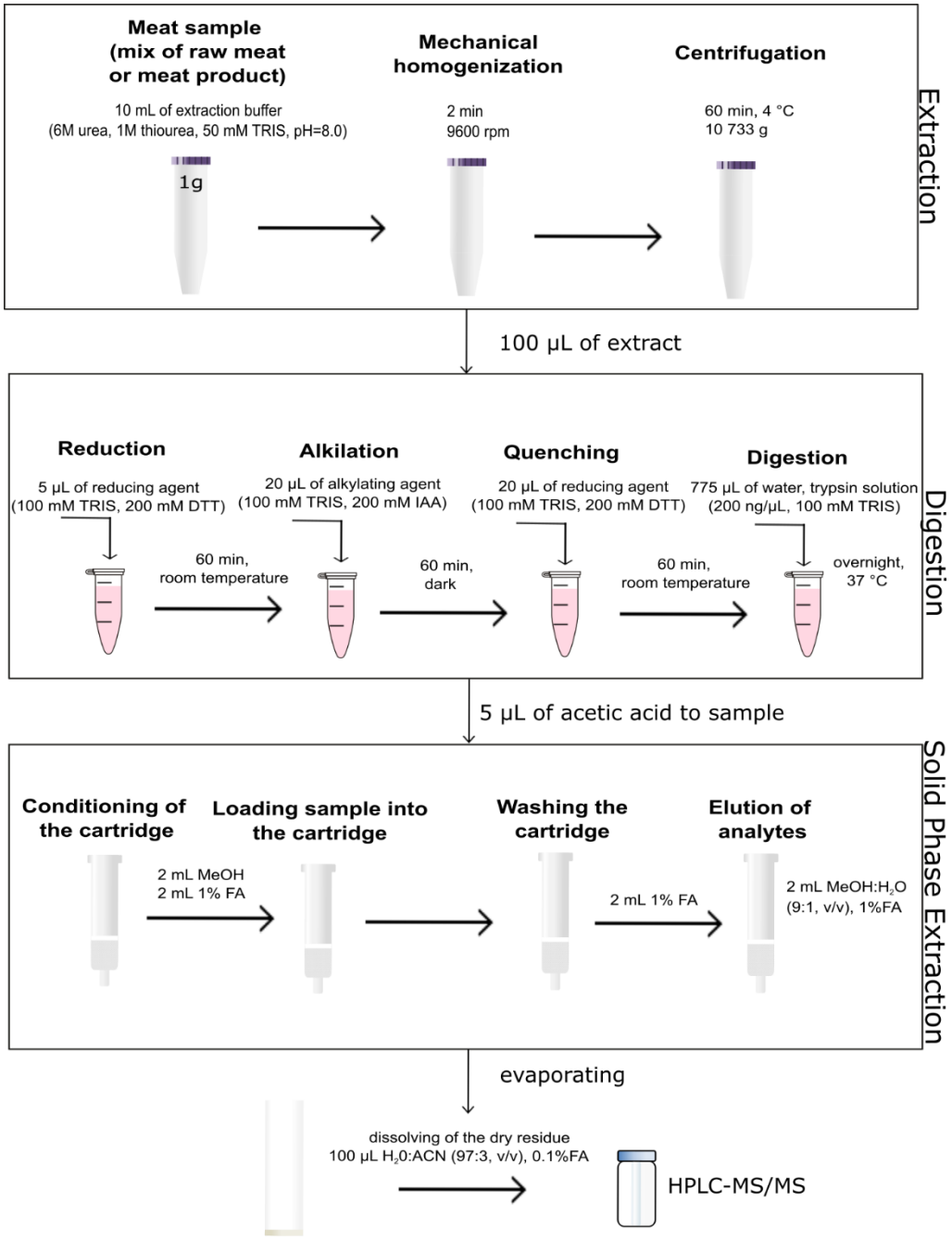

Fig.S1. Procedure of sample preparation required for HPLC-MS/MS analysis of peptide markers.

Table S1. Separation conditions of peptide markers

Column	Kinetex XB C-18 (100 x 2.1 mm, 2.6 µm)
Flow rate (mL/min)	0.3
Temperature of thermostat (ºC)	40 ºC
Injection volume	1µL
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 →22 min, 3-30 % B 22→28 min, 30-70 % B 28→29 min, 70-100 % B 29→31 min, 100 % B 31→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
A	P1,B2/G1	**Group 1** (P1 and B2)
B	P1,B2/G2	
C	P1,B2/G3	
D	P2,B1/G1	**Group 2** (P2 and B1)
E	P2,B1/G2	
F	P2,B1/G3	

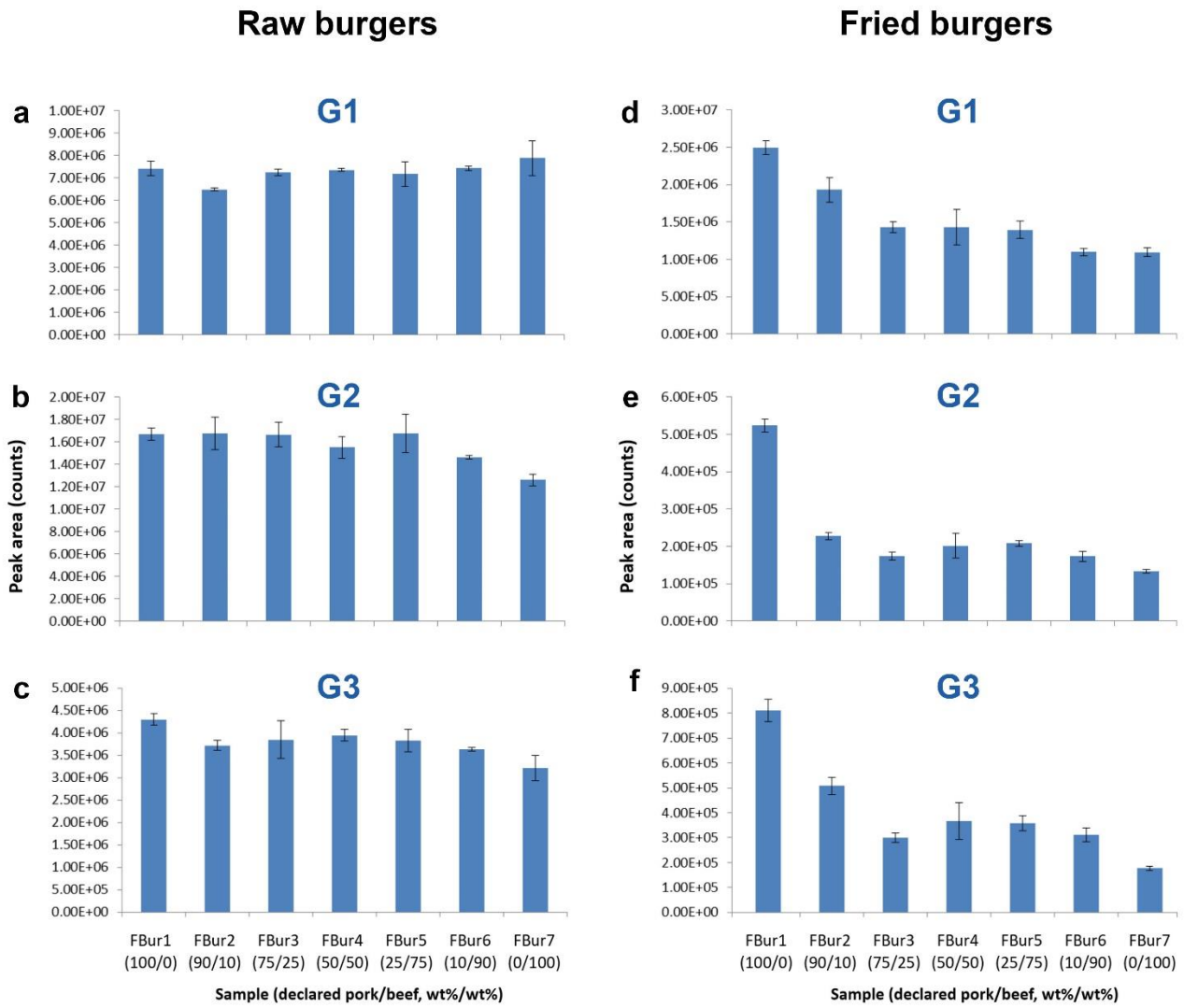

Fig. S2. MRM-MS analysis of global markers G1, G2 and G3 in (a-c) raw burgers and (d-f) fried burgers, made with various pork/beef proportions. The declared proportions are true proportions (samples Bur1–Bur7 and FBur1–FBur7, Table 1). Individual MS-signal data points are shown as the mean ± SD (n = 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	**0%**	**0%**	**0%**	S1	**0.4%**	**0.3%**	**0.3%**
M2	14.7%	**0.2%**	**5.9%**	S2	**5.0%**	**3.0%**	**0.7%**
M3	14.2%	**4.2%**	**1.4%**	S3	**2.8%**	**2.9%**	**5.2%**
M4	13.3%	**8.6%**	**0.6%**	S4	**4.4%**	10.5%	12.2%
M5	12.7%	**6.1%**	**0.5%**	S5	**6.8%**	13.5%	13.2%
M6	**1.5%**	**3.1%**	**1.9%**	S6	**1.4%**	**6.4%**	**2.1%**
M7	**0.4%**	**1.0%**	**0.8%**	S7	**6.2%**	**7.9%**	**7.9%**
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Mix of minced raw meats	D	E	F	Sausages	D	E	F
M1	**0%**	**0%**	**0%**	S1	**0.0%**	**0.0%**	**0.0%**
M2	**9.1%**	**7.2%**	**0.1%**	S2	**5.1%**	**2.9%**	**0.8%**
M3	**8.4%**	11.8%	**5.4%**	S3	**2.3%**	**3.7%**	**6.1%**
M4	**9.4%**	12.5%	**4.3%**	S4	**3.3%**	**9.6%**	11.2%
M5	**9.1%**	**9.5%**	**2.4%**	S5	**7.7%**	14.2%	14.0%
M6	**2.7%**	**6.8%**	**1.7%**	S6	**0.8%**	**3.6%**	**1.1%**
M7	**0%**	**0%**	**0%**	S7	**0.9%**	**1.3%**	**1.3%**
	Group 1 GSM algorithms				Group 1 GSM algorithms		
Raw burgers	A	B	C	Fried burgers	A	B	C
Bur1	**0.0%**	**0.0%**	**0.0%**	FBur1	**0.3%**	**0.2%**	**0.2%**
Bur2	**1.3%**	**4.7%**	**0.8%**	FBur2	**0.2%**	33.5%	11.5%
Bur3	14.5%	**9.3%**	**7.3%**	FBur3	**2.8%**	23.8%	20.7%
Bur4	**0.2%**	**7.5%**	**7.7%**	FBur4	**6.5%**	22.5%	21.4%
Bur5	**0.1%**	12.2%	10.8%	FBur5	**8.8%**	20.8%	24.6%
Bur6	**2.0%**	12.5%	11.8%	FBur6	**5.0%**	12.0%	21.1%
Bur7	**0.1%**	**0.1%**	**0.1%**	FBur7	**0.5%**	**1.0%**	**0.6%**
	Group 2 GSM algorithms				Group 2 GSM algorithms		
Raw burgers	D	E	F	Fried burgers	D	E	F
Bur1	**0.0%**	**0.0%**	**0.0%**	FBur1	**0.0%**	**0.0%**	**0.0%**
Bur2	**1.4%**	**2.3%**	**3.6%**	FBur2	**1.1%**	36.7%	13.9%
Bur3	11.5%	**6.5%**	**4.1%**	FBur3	**1.9%**	26.4%	23.2%
Bur4	**1.2%**	**7.3%**	**7.5%**	FBur4	**5.0%**	21.1%	20.3%
Bur5	**5.2%**	**8.1%**	**6.2%**	FBur5	**8.4%**	20.4%	24.2%
Bur6	**0.3%**	10.1%	**9.2%**	FBur6	**2.0%**	16.8%	24.5%
Bur7	**0.2%**	**0.1%**	**0.2%**	FBur7	**0.02%**	**0.02%**	**0.006%**

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*≤0.05 indicates the GSM result differed significantly from the declared pork content.

Mix of minced raw meats						Sausages					
GSM algorithms, Group 1			GSM algorithms, Group 2			GSM algorithms, Group 1			GSM algorithms, Group 2		
M1	algorithm A	1.0000	M1	algorithm D	1.0000	S1	algorithm A	0.7075	S1	algorithm D	0.9957
M2		**0.0366**	M2		0.1853	S2		**0.0193**	S2		**0.0108**
M3		**0.0003**	M3		**0.0372**	S3		**0.0096**	S3		0.1604
M4		**0.0094**	M4		**0.0117**	S4		0.1332	S4		**0.0097**
M5		**0.0201**	M5		**0.0251**	S5		0.1405	S5		**0.0076**
M6		0.2543	M6		0.1207	S6		0.3510	S6		0.6531
M7		0.1461	M7		x	S7		**0.0011**	S7		x
M1	algorithm B	1.0000	M1	algorithm E	1.0000	S1	algorithm B	0.8641	S1	algorithm E	0.9768
M2		0.8321	M2		0.1434	S2		0.1723	S2		0.2239
M3		**0.0279**	M3		**0.0037**	S3		**0.0052**	S3		0.1282
M4		**0.0049**	M4		**0.0044**	S4		**0.0225**	S4		**0.0077**
M5		**0.0099**	M5		**0.0028**	S5		**0.0427**	S5		**0.0291**
M6		0.1569	M6		**0.0264**	S6		**0.0317**	S6		0.0588
M7		**0.0020**	M7		x	S7		**0.0004**	S7		x
M1	algorithm C	1.0000	M1	algorithm F	1.0000	S1	algorithm C	0.8117	S1	algorithm F	0.9874
M2		**0.0290**	M2		0.9487	S2		0.2281	S2		0.5505
M3		**0.0108**	M3		0.0826	S3		**0.0047**	S3		**0.0397**
M4		0.2346	M4		**0.0076**	S4		**0.0224**	S4		**0.0032**
M5		0.6403	M5		0.0947	S5		0.0532	S5		**0.0273**
M6		0.3404	M6		0.0758	S6		0.1818	S6		**0.0018**
M7		**0.0070**	M7		x	S7		**0.0002**	S7		x

Raw burgers						Fried burgers					
GSM algorithms, Group 1			GSM algorithms, Group 2			GSM algorithms, Group 1			GSM algorithms, Group 2		
Bur1	algorithm A	0.9652	Bur1	algorithm D	0.9734	FBur1	algorithm A	0.8967	FBur1	algorithm D	0.9926
Bur2		0.0805	Bur2		0.2997	FBur2		0.6384	FBur2		0.6381
Bur3		**0.0004**	Bur3		**0.0015**	FBur3		0.1012	FBur3		0.1370
Bur4		0.1147	Bur4		0.1315	FBur4		**0.0084**	FBur4		**0.0289**
Bur5		0.9306	Bur5		0.1255	FBur5		**0.0039**	FBur5		**0.0149**
Bur6		**0.0464**	Bur6		0.5417	FBur6		**0.0339**	FBur6		0.3070
Bur7		x	Bur7		x	FBur7		0.1672	FBur7		x
Bur1	algorithm B	0.9728	Bur1	algorithm E	0.9843	FBur1	algorithm B	0.9244	FBur1	algorithm E	0.9972
Bur2		0.0940	Bur2		0.3242	FBur2		**0.0014**	FBur2		**0.0039**
Bur3		**0.0004**	Bur3		**0.0007**	FBur3		**0.0004**	FBur3		**0.0088**
Bur4		**0.0010**	Bur4		**0.0072**	FBur4		**0.0022**	FBur4		**0.0033**
Bur5		**0.0046**	Bur5		**0.0092**	FBur5		**0.0010**	FBur5		**0.0011**
Bur6		**0.0003**	Bur6		**0.0003**	FBur6		**0.0031**	FBur6		**0.0057**
Bur7		x	Bur7		x	FBur7		0.2015	FBur7		x
Bur1	algorithm C	0.9763	Bur1	algorithm F	0.9803	FBur1	algorithm C	0.8312	FBur1	algorithm F	0.9923
Bur2		0.4329	Bur2		**0.0067**	FBur2		**0.0052**	FBur2		**0.0106**
Bur3		**0.0243**	Bur3		0.1032	FBur3		**0.0007**	FBur3		**0.0055**
Bur4		**0.0004**	Bur4		**0.0058**	FBur4		**0.0017**	FBur4		**0.0035**
Bur5		**0.0054**	Bur5		0.0496	FBur5		**0.0006**	FBur5		**0.0007**
Bur6		**0.0001**	Bur6		**0.0020**	FBur6		**0.0045**	FBur6		**0.0024**
Bur7		x	Bur7		x	FBur7		0.0584	FBur7		x

Numbers shown in bold indicate *P ≤ 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*≤0.05 indicates the GSM result differed significantly from the declared beef content.

Mix of minced raw meats						Sausages					
GSM algorithms, Group 1			GSM algorithms, Group 2			GSM algorithms, Group 1			GSM algorithms, Group 2		
M1	algorithm A	x	M1	algorithm D	x	S1	algorithm A	0.7066	S1	algorithm D	x
M2		**0.0366**	M2		0.1853	S2		**0.0193**	S2		**0.0108**
M3		**0.0003**	M3		**0.0372**	S3		**0.0096**	S3		0.1609
M4		**0.0094**	M4		**0.0117**	S4		0.1332	S4		**0.0096**
M5		**0.0201**	M5		**0.0251**	S5		0.1404	S5		**0.0078**
M6		0.2543	M6		0.1207	S6		0.3504	S6		0.6529
M7		**0.0314**	M7		1.0000	S7		**0.0011**	S7		0.6391
M1	algorithm B	x	M1	algorithm E	x	S1	algorithm B	0.8639	S1	algorithm E	x
M2		0.8354	M2		0.1434	S2		0.1723	S2		0.2247
M3		**0.0278**	M3		**0.0037**	S3		**0.0053**	S3		0.1279
M4		**0.0049**	M4		**0.0044**	S4		**0.0224**	S4		**0.0077**
M5		**0.0099**	M5		**0.0028**	S5		**0.0427**	S5		**0.0291**
M6		0.1569	M6		**0.0399**	S6		**0.0316**	S6		0.0585
M7		**0.0021**	M7		1.0000	S7		**0.0004**	S7		0.7710
M1	algorithm C	x	M1	algorithm F	x	S1	algorithm C	0.8117	S1	algorithm F	x
M2		**0.0290**	M2		0.9487	S2		0.2299	S2		0.5504
M3		**0.0111**	M3		0.0826	S3		**0.0047**	S3		**0.0396**
M4		0.2353	M4		**0.0076**	S4		**0.0223**	S4		**0.0032**
M5		0.6374	M5		0.0947	S5		0.0532	S5		**0.0273**
M6		0.3404	M6		0.0758	S6		0.1824	S6		**0.0016**
M7		**0.0064**	M7		1.0000	S7		**0.0004**	S7		0.7559

Raw burgers						Fried burgers					
GSM algorithms, Group 1			GSM algorithms, Group 2			GSM algorithms, Group 1			GSM algorithms, Group 2		
Bur1	algorithm A	x	Bur1	algorithm D	x	FBur1	algorithm A	0.9091	FBur1	algorithm D	x
Bur2		0.0806	Bur2		0.2997	FBur2		0.6386	FBur2		0.6395
Bur3		**0.0004**	Bur3		**0.0015**	FBur3		0.1011	FBur3		0.1335
Bur4		0.1121	Bur4		0.1315	FBur4		**0.0084**	FBur4		**0.0279**
Bur5		0.9318	Bur5		0.1255	FBur5		**0.0039**	FBur5		**0.0146**
Bur6		**0.0465**	Bur6		0.5415	FBur6		**0.0339**	FBur6		0.3123
Bur7		0.9233	Bur7		0.9229	FBur7		0.1666	FBur7		0.9915
Bur1	algorithm B	x	Bur1	algorithm E	x	FBur1	algorithm B	0.9375	FBur1	algorithm E	x
Bur2		0.0936	Bur2		0.3242	FBur2		y	FBur2		y
Bur3		**0.0004**	Bur3		**0.0007**	FBur3		**0.0004**	FBur3		y
Bur4		**0.0010**	Bur4		**0.0072**	FBur4		**0.0022**	FBur4		**0.0033**
Bur5		**0.0045**	Bur5		**0.0093**	FBur5		**0.0010**	FBur5		**0.0011**
Bur6		**0.0003**	Bur6		**0.0003**	FBur6		**0.0031**	FBur6		**0.0059**
Bur7		0.9691	Bur7		0.9786	FBur7		0.2017	FBur7		0.9947
Bur1	algorithm C	x	Bur1	algorithm F	x	FBur1	algorithm C	0.8591	FBur1	algorithm F	x
Bur2		0.4317	Bur2		**0.0067**	FBur2		y	FBur2		y
Bur3		**0.0244**	Bur3		0.1032	FBur3		**0.0007**	FBur3		**0.0057**
Bur4		**0.0004**	Bur4		**0.0058**	FBur4		**0.0017**	FBur4		**0.0036**
Bur5		**0.0054**	Bur5		0.0496	FBur5		**0.0006**	FBur5		**0.0006**
Bur6		**0.0001**	Bur6		**0.0020**	FBur6		**0.0045**	FBur6		**0.0024**
Bur7		0.9405	Bur7		0.9325	FBur7		0.0577	FBur7		0.9974

Numbers shown in bold indicate *P ≤ 0.05,*
x, the beef specific peptide markers (B1, B2) not detected in sample declared as 100% pork; y, negative value