FOODINTEGRITY

Ensuring the Integrity of the European food chain

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Title: Outcomes of ring trials to transfer methods between partners and MS platforms

Author(s): Jens Brockmeyer, Yvonne Gunning, Kate Kemsley, Ilona Kłosowska-Chomiczewska, Alexandra Klußmann, Adam Macierzanka, Laura Viet, Andrzej Wasik, Andrew Watson

Beneficiary(s): Quadram Institute Biosciences, Gdansk University of Technology, Analytical Food Chemistry Stuttgart

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1 Description of Deliverable

1.1 Overview of the deliverable

Outcomes of ring trials to transfer methods between partners and MS platforms. Work will include an exploration of porting the methods developed to alternate MS platforms; ease of transfer of standard operating procedures between partners; ring trial examination of commercial samples from supermarkets and from industrial collaborators.

1.1 Rationale and strategy of the deliverable

One important criteria for the successful uptake of any analytical testing method is the capacity to generate consistent results with different personnel, instrumentation and laboratories. This capacity can be assessed using a ring trial, in which different sites take measurements on the same blinded samples and compare results. This process has the additional benefits of establishing more realistic errors on the testing method, of clarifying issues involved in disseminating the test standard operating procedures (SOP) to other sites, and also in sharing and discussing best practice between those sites.

This deliverable covers a ring trial involving the three WP13 partners, Gdansk, Stuttgart and Norwich. Each partner created a sample set that was split into three parts, with two parts going to each of the other partners and one part measured in house. Measurements were performed blind, in that the sample composition was not known, though for the purpose of the testing all samples were from a restricted space of possibilities. It should be noted that all three threads of the ring trial involved quantitation, not simply species detection. Quantitation is more challenging that species detection. Because the testing involved quantitation, all samples contained controlled amounts of a labelled meat as well as an 'undeclared' meat, the task then being to see how close results were, both to those from other partners, and to the known composition of the sample.

Stuttgart provided raw meat samples comprising four different mixtures of horse and beef. The challenge was to use their method for detecting global markers to test for quantitation of one species mixed with

another. Global markers (c.f. deliverable D13.3) are species non-specific and can be used to flag the presence of species other than the declared species in the sample. The test therefore requires knowledge of a species-specific marker for the declared species, plus knowledge of a global marker that will reveal any one of a spectrum of candidate undeclared species: horse and beef mixtures provide a test bed for this approach. Stuttgart's SOP identified markers and set out the extraction protocols and detection protocols. The point of this test is to confirm that this wholly new *untargeted* method can be reproduced in other centres. Untargeted methods are key for detecting undeclared species for which no marker is known or for which a known marker is not included in the test SOP for operational reasons.

Norwich provided samples of cooked meat-based infant foods. One was created in-house using a commercially relevant recipe starting with raw ingredients, followed by a commercially relevant retort cooking process. This sample contained a mixture of horse and beef. Pure samples of uncooked horse and beef were also provided. In addition, Norwich circulated a sample comprising a mixture of supermarket infant food products combined to give a lamb with beef system. The supermarket-derived sample therefore is fully commercially relevant but lacks direct knowledge of the levels of meat species. The testing requires knowledge of species-specific markers for all species in the test (horse, beef, lamb) and these were identified in the circulated SOP, together with extraction and detection methods. The marker peptides are all derived from myoglobins of the different species. The point of this trial is to confirm that testing for adulteration in a complex food, meaning a food that is a mixture of ingredients and which has endured significant processing, can be reproduced at other centres. Complex foods are of particular concern in food adulteration testing since the multiple components may spawn erroneous results in some testing regimes, and processing may damage markers used in some testing.

Gdansk provided samples of six different 'as-sold' sausage products, all pork and beef mixtures and all created as bespoke products by a commercial partner in Poland. Testing involved species-specific markers for pork and beef based on myoglobins from the different species. These markers were identified in the circulated SOP, together with extraction and detection methods. The point of this trial is to confirm that testing of sausage products can be reproduced across multiple sites. Cured sausage products are of special interest to some retailers, who view them as potential sources of undeclared species. Secondly, sausage products are unique complex foods, possessing an emulsion structure and being subject to heat treatment and curing processes having the potential to disrupt markers or make markers difficult to extract.

The three SOPs circulated to partners for this ring trial are reproduced verbatim as appendices to this deliverable document.

2 Achievement of the Deliverable

2.1 A method for locating global markers in animals and birds

Two global markers, present in all vertebrates, are denoted G2 and G3 (c.f. D13.3). 'B' and 'H' denote beef and horse respectively. Quantitation was performed by calculating ratios of the peak areas from species-specific markers to global markers. The analysis was performed using technical duplicates (post tryptic digestion). Values listed under H/G2 and H/G3 are ratios of horse to global marker peptides. Ratios declared B/G2 and B/G3 result from determination of beef levels which are then subtracted from 100% to give a horse meat percentage. The actual horse levels, known from the sample preparation, are highlighted in green.

	amount of horse meat [%]			
	B/G2	B/G3	H/G2	H/G3
Sample 9.1	9,0	8,2	10,7	8,5
Sample 9.2	7,3	5,5	10,2	8,2
mean	8,2	6,8	10,5	8,3
SD	0,8	1,3	0,3	0,2
Real amount:	9,7			
Error:	-1,5	-2,9	0,8	-1,4
	B/G2	B/G3	H/G2	H/G3
Sample 10.1	21,4	20,6	18,4	14,6
Sample 10.2	14,9	14,8	19,0	14,9
mean	18,1	17,7	18,7	14,8
SD	3,3	2,9	0,3	0,1
Real amount:	17,8			
Error:	0,3	-0,1	0,9	-3,0
	B/G2	B/G3	H/G2	H/G3
Sample 11.1	47,5	42,9	49,1	42,0
Sample 11.2	45,7	42,7	53,1	44,0
Mean	46,6	42,8	51,1	43,0
SD	0,9	0,1	2,0	1,0
Real amount:	45,5			
Error:	1,1	-2,7	5,6	-2,5
	B/G2	B/G3	H/G2	H/G3
Sample 12.1	84,0	70,3	49,2	71,9
Sample 12.2	84,4	70,3	47,6	71,4
Mean	84,2	70,3	48,4	71,6
SD	0,2	0,0	0,8	0,3
Real amount:	75,7			
Error:	8,5	-5,4	-27,3	-4,1

Table 2.1.1 Horse-beef system analysed by Stuttgart using a Sciex Triple TOF 6600 with Micro-LC M3 system. The true horse meat percentages were 9.7, 17.8, 45.5 and 75.7.

	amount of horse meat [%]			
	B/G2	B/G3	H/G2	H/G3
Sample 5	57,1	22,2	6,2	7,7
Real amount:	9,8			
Error:	47,3	12,4	-3,6	-2,1
	B/G2	B/G3	H/G2	H/G3
Sample 6	16,0	16,0	24,1	16,6
Real amount:	18,1			
Error:	-2,1	-2,1	6,0	-1,5
	B/G2	B/G3	H/G2	H/G3
Sample 7	82,9	0,8	20,9	83,2
Real amount:	48,1			
Error:	34,8	-47,3	-27,2	35,1
	B/G2	B/G3	H/G2	H/G3
Sample 8	86,4	85,3	68,9	51,2
Real amount:	81,5			
Error:	4,9	3,8	-12,6	-30,3

Table 2.1.2 Horse-beef system analysed by Norwich using a Sciex Q-Trap 4000 with Agilent 1200 LC system. The true horse meat percentages were 9.8, 18.1, 48.1 and 81.5.

	amount of horse meat [%]			
	B/G2	B/G3	H/G2	H/G3
Sample 1	11,4	0,2	8,3	9,5
Real amount:	10,1			
Error:	1.3	-9.9	-1.8	-0.6
	B/G2	B/G3	H/G2	H/G3
Sample 2	35,5	21,0	15,9	19,8
Real amount:	20,9			
Error:	14.6	0.1	-5.0	-1.1
	B/G2	B/G3	H/G2	H/G3
Sample 3	46,4	31,6	35,5	45,9
Real amount:	44,8			
Error:	1.6	-13.2	-9.3	1.1
	B/G2	B/G3	H/G2	H/G3
Sample 4	75,1	71,2	69,5	81,1
Real amount:	75,6			
Error:	-0.5	-4.4	-6.1	5.5

Table 2.1.3 Horse-beef system analysed by Gdansk using a Sciex Q-Trap 4000 with Agilent 1200 LC system. The true horse meat percentages were 10.1, 20.9, 44.8 and 75.6.

2.2 Quantitation of undeclared meat in processed infant food

Cooked, processed infant food samples comprising 5% horse in beef were distributed to Gdansk (triplicate samples C4 to C6), Stuttgart (triplicate samples C7 to C9) and Norwich (triplicate samples C1 to C3).

	Norwich	**Gdansk**	**Stuttgart**
	% horse in beef	% horse in beef	% horse in beef
Transition	average C1 - C3	average C4 - C6	average C7- C9
1268/1298	12.08	9.75	11.74
1366/1396	10.96	10.51	12.10
706	24.53	6.30	15.58
248/234	1.22	4.73	8.47
1097	13.38	not in method	12.59
Average all transitions	12.43	7.82	12.09

Figure 2.2.1 Horse in beef infant food system comprising 5% horse as analysed at the three sites. The percentage horse values as determined by transition peak areas are not corrected for the different levels of myoglobin in the two species, so horse levels are over-reported.

		Norwich	**Stuttgart**	**Gdansk**
y Value	**Transition**	**Ratio horse: beef**	**Ratio horse: beef**	**Ratio horse: beef**
4	374.700/564.300 Da	1.44	1.81	1.58
3	374.700/435.300 Da	1.54	1.86	1.50
1	374.700/175.100 Da	1.57	1.88	1.54
5	374.700/677.400 Da	1.57	1.78	1.67
	Average	1.53	1.83	1.57

Figure 2.2.2 Ratios of transition peak areas for the identical peptide ALELFR (precursor m/z 374.7 Da) measured for pure horse and pure beef at the three sites showing that, weight for weight, horse has a higher level of myoglobin than beef. The meats used in this measurement were from the same original selection used to create horse in beef mixtures.

The commercial infant food trial samples comprised 10% of a beef-based supermarket product combined with 90% of a lamb-based supermarket product. According to the product label information both products contained the same proportion of meat. Triplicate samples D4 to D6 were sent to Gdansk, triplicate samples D7 to D9 went to Stuttgart, and triplicate samples D1 to D3 stayed in Norwich.

	Norwich	Gdansk	Stuttgart
	% beef in lamb	% beef in lamb	% beef in lamb
Transition	average D1 - D3	average D4 - D6	average D7- D9
1268/1298	10.05	0.00	9.84
1366/1396	7.99	0.00	0.00
706	16.65	0.00	8.92
248/234	4.70	0.00	46.66
1097	6.18	0.00	0.00
Average all transitions	9.11	0.00	13.08

Figure 2.2.3 Commercial product. Beef in lamb supermarket infant food system comprising 10% beef product and 90% lamb product. Results not corrected for the difference in myoglobin levels of beef and lamb.

2.3 Quantitation of meat species in processed sausage products

Six samples of sausage product comprising different levels of pork and horse, and prepared by a commerical partner, were analysed at the three sites.

	Norwich analysis	Gdansk Analysis	Stuttgart Analysis	Actual amounts
Sample No.	% pork in beef	% pork in beef	% pork in beef	% pork in beef
1	3.69	4.52	4.62	**30**
2	11.48	4.90	12.76	**50**
3	75.81	36.75	46.43	**90**
4	32.78	50.08	0	**70**
5	10.16	10.53	0	**10**
6	50.12	71.51	85.15	**90**

Figure 2.3.1 Samples of six pork plus beef sausage samples analysed by transition peak area ratios, based on myoglobin peptides HPSDFGADAQAAMSK, m/z =767 Da (beef) and HPGDFGADAQGAMSK, m/z = 745 Da, (pork) fragment y2 only. No correction has been made for the different levels of myoglobin in beef and pork, so pork will be under-reported.

3 Summary

The ring trial was largely successful, in the sense that site-specific effects were mostly absent. In other words, if the data from each site for a given test were to be combined into a single table, it is not immediately clear which number was generated by which site. For the most part, marker peptides were identified by all three partners, and the overall quantitation pattern agreed between all three partners. However, there were some site-related issues: for reasons that are yet to be investigated Gdansk was unable to detect beef in a beef-in-lamb commercial product, and Stuttgart was unable to detect pork in two of the pork-in-beef sausage products. The Norwich group's results on the universal marker trial were substandard due to an unexplained peak masquerading as a horse peptide.

The ring trial did highlight some unexpected variabilities in the methodology, as distinct from site-dependent variability. In some cases, quantitation values are significantly beyond the implied distribution and may be legitimate outliers. Improved statistics from more samples, combined with manual inspection of the data would help to clarify their status. Variation in the reported levels across each trial, if they cannot be reduced by further protocol refinement or improved statistics, imply a more realistic and robust confidence level than might be given by a single lab, even if that confidence level is unhelpfully broad.

The sharing of protocols between sites revealed the desirability of having a contact expert to address any queries and provide clarifications. There is a case for site visits by the creator of the original protocol to the site attempting to implement that protocol in instances were problems emerge, or at least the sharing of actual data for debugging analysis. The protocol sharing also revealed the need for a data recording template to be distributed as part of the protocol. This template needs to contain embedded code that gives identical and unambiguous handing of the input values. Where possible a common format for the protocols themselves is desirable.

In the future, Gdansk will refine the sausage methodology using more tightly controlled materials, and perhaps look to other proteins and markers that show less intra-animal variation than myoglobin. This might control some of the uncertainties revealed by the trial. The trial demonstrated that testing in complex foods such as infant foods and cured meat products is reproducible across sites and we anticipate that this type of testing will gradually be implemented by centres equipped for routine testing. The global marker approach likewise is shown to be transferable: Stuttgart is improving the method to enhance its robustness, and Norwich is creating a student project with the object of implementing a commercial testing service, for example testing that a vegetarian food product is correctly labelled.

4 Appendices

4.1 Appendix 1: A method for locating global markers in animals and birds

Materials

Chemicals

Acetic acid	96 %, Grüssing, Filsum, Germany

Aceton	Chromasolv for HPLC > 99.8 %, Sigma Aldrich, St. Louis, MO, USA
Acetonitrile (ACN)	LC-MS grade, Fisher Scientific, Hampton, NH, USA
Dithiothreitol (DTT)	> 99 %, p.a., Roth, Karlsruhe, Germany
Formic acid	99 %, Grüssing, Filsum, Germany
Hydrochloric acid	37 %, Grüssing, Filsum, Germany
Iodoacetamide (IAA)	> 99 %, Sigma Aldrich, St. Louis, MO, USA
Methanol	LC-MS grade, Fisher Scientific, Hampton, NH, USA
Thiourea	> 99 %, p.a., Roth, Karlsruhe, Germany
Tris(hydroxymethyl)aminomethane (TRIS)	> 99.9 %, Roth, Karlsruhe, Germany
Trypsin from porcine pancreas	> 90 % (HPLC), tryptic activity: > 6000 U/g, Serva Electrophoresis, Heidelberg, Germany
Urea	> 99.5 %, p.a., Roth, Karlsruhe, Germany

Solutions and buffers (all solutions and buffers are prepared using ultrapure water)

Extraction

Extraction Buffer	6 M urea 1 M thiourea 50 mM TRIS pH 8.0 (adjusted with HCl)

Digestion

TRIS Stocksolution (used for the following digestion buffers and solutions)	400 mM TRIS pH 7.8 (adjusted with 6M HCl)

Digestion buffer	6 M urea 100 mM TRIS
Reducing agent	200 mM DTT 100 mM TRIS
Alkylating agent	200 mM IAA 100 mM TRIS
Trypsin solution	200 ng/µL trypsin 100 mM TRIS

Desalting

Conditioning solution	methanol LC Grade
Equilibration and washing solution	1 % (v/v) formic acid
Elution solution	90 % (v/v) methanol 1 % (v/v) formic acid
HPLC starting condition	3 % (v/v) acetonitrile 0.1 %(v/v) formic acid

HPLC

Mobile phase A	0.1 % formic acid in H_2O
Mobile phase B	0.1 % formic acid in ACN

<u>Instrumentation</u>

Micro LC	M3 Micro LC System, AB Sciex, Framingham, MA, USA
Autosampler	CTC PAL-1, CTC Analytics, Zwingen, Switzerland
Column	YMC, Triart C18, 150 mm x 0.3 mm, 3 µm, YMC CO., Kyoto, Japan
Mass spectrometer	Triple-TOF 6600 System, Framingham, MA, USA
Software for the mass spectrometer	Eksigent, AB Sciex, Framingham, MA, USA Analyst 1.7, AB Sciex, Framingham, MA, USA

Software for the analysis	Peak View 2.2, AB Sciex, Framingham, MA, USA MultiQuant MD 3.0.1, AB Sciex, Framingham, MA, USA Skyline 3.7, MacCoss Lab at the University of Washington, WA, USA

1. Methods

Extraction

1. Add 10 mL of extraction buffer per 1 g of sample
2. Homogenize for 2 min at 9600 rpm using the Ultra Turrax
3. Centrifuge for 1 h at 4°C with 10000 g
4. Transfer supernatant into 15 mL centrifuge tubes

Digestion

1. Transfer 50 µL of the extract to a 1.5 mL reaction tube
2. Add 2.5 µL of reducing agent
3. Incubate for 1 h at room temperature
4. Add 10 µL of alkylating agent
5. Incubate in the dark for 1 h
6. Add 10 µL of reducing agent
7. Incubate for 1 h at room temperature
8. Add 387.5 µL of water
9. Add 50 µL of freshly prepared trypsin solution
10. Incubate over night at 37°C
5. Add 2.5 µL acetic acid to inactivate the trypsin

Desalting

1. Desalt using Phenomenex Strata-X 33µ polymeric reversed-phase cartridges filled with 30 mg/ml RP material.
2. For conditioning and equilibration of the cartridge use 1 mL methanol and 1 mL washing solution, respectively.
3. Load the sample onto the cartridge
4. Wash using 1 mL of washing solution
5. Elute the peptides with 1 mL of elution solution into a 1.5 mL reaction tube

Preparation for LC/MS

1. Remove the solvent under vacuum at 40°C using the centrifugal evaporator (approx. 2 hours)
2. Redissolve in 50 µL "HPLC starting condition" and transfer to an autosampler vial for LC/MS analysis

LC/MS-Analysis

Table 1: General LC-parameters.

Parameters	Settings
Flowrate	7 µl/min
Injection volume	7 µL
Autosampler temperature	10 °C

Table 2: Gradient profile.

Time (min)	%A	%B
0	97	3
12	65	35
12.5	5	95
14.5	5	95
15	97	3
17	97	3

Table 3: General MS-parameters.

Parameter	Settings
Source	
Source type	Duo spray ion source
Source temperature [°C]	300
Experiment Parameters	
Curtain gas (CUR)	30
Ion source gas 1 (GS1)	25
Ion source gas 2 (GS2)	15
Ion spray voltage floating (ISVF)	5500
Temperature (TEM)	300

Mass range parameters	
Collision energy (CE) [V]	Depending on the precursor
Collision energy spread (CES) [V]	5
Declustering potential (DP) [V]	80
IDIx	0
IDUx	5
Ion release Delay (IRD) [ms]	30
IRDx	15 000
Ion release width (IRW) [ms]	15
IRWx	10 000
IWIx	0
IWUx	5
XA1	Depending on the precursor
Start mass [m/z]	150
End mass [m/z]	1 200
RF Transmission	
Mass	140
Time [%]	100

Table 4: Peptide specific MS-parameters.

Peptide	**m/z of the precursor**	**CE [V]**	**XA1**	**m/z of fragments**
DIDDLEITLAK (G2)	623.3	31.3	199.733	1017.5 (y9)
				674.5 (y6)
				902.5 (y8)
				787.5 (y7)
HQGVMVGMGQK (G3)	586.3	30	193.806	906.5 (y9)
				750.4 (y7)
				619.3 (y6)
				849.5 (y8)
LVNELTEFAK (B)	582.5	29.8	193.171	708.4 (y6)
				569.1 (y5)
				837.1 (y7)
LVNDLTGQR (H)	508.3	27.2	181.325	803.4 (y7)
				574.3 (y5)
				689.4 (y6)

Analysis

Samples consist of varying amounts of beef and horse meat. Two samples containing 100 % horse meat or 100 % beef are provided for testing the method.

Since the analysis in our lab was carried out using a TripleTOF 6600 a mean CE value for all transitions of one peptide was used. For MRM measurements CE values should be optimised for each transition. This can be done using the 100 % horse and 100 % beef sample.

To estimate the amount of beef and horse meat in each of the samples the ratio of the peak areas of the specific marker (B for beef and H for horse) and one of the global markers (G2 or G3) must be calculated. For each peptide the transition with the highest intensity should be identified in the 100 % samples and used to calculate the ratios in all of the samples.

Example:

The ratio B/G2 for the 100 % beef sample is 3.4. If the calculated ratio B/G2 for one of the unknown samples is 1.8 this corresponds to **1.8/3.4*100 %=53 %** beef in the sample.

4.2 Appendix 2: Protocol – undeclared meat in processed infant food

Extraction and Analysis Protocol for Infant Food

Chemicals Required

Potassium chloride
Potassium monophosphate
Potassium hydroxide
Urea
Acetonitrile
Ammonium bicarbonate
DMSO
Trypsin (TPCK treated from bovine pancreas)
Methanol
Concentrated acetic acid

Solvents/Buffers/Reagents

0.15M KCl, 0.15M Phosphate buffer at pH 6.5
25mM Ammonium bicarbonate
1mg/ml Trypsin in 25mM ammonium bicarbonate
1% Formic Acid (FA)
5%MeOH/1% FA
Acetonitrile/H_2O (90:10; 0.1% FA)

Extraction

1. Weigh 120g of the infant food puree (as supplied) into a plastic beaker
2. Add 50ml of extraction buffer (0.15M KCl, 0.15M phosphate buffer at pH 6.5)
3. Extract by blending in the Ultra Turrax for 5 min
4. Transfer 2ml of the extract into a 2ml centrifuge tube
5. Centrifuge for 5 min at 4°C with 17000g
6. Remove 200µl aliquots of the supernatant into 2ml centrifuge tubes and use the centrifugal evaporator to remove the buffer (50°C, approx. 1 hour)

Digestion

1. Redissolve the dried residue in 1ml of 25mM ammonium bicarbonate solution. Mix well.
2. Heat the sample in the hot block at 95°C for 30 minutes.
3. Cool to room temperature
4. Add urea (0.5M = 30mg)
5. Carry out tryptic digest: Add 20ul of trypsin solution. Mix sample by gentle vortex and carry out digestion overnight at 37°C

Desalting

1. Dilute the sample 1:2 with water.
2. Desalt using Phenomenex Strata-X 33µ polymeric reversed-phase cartridges filled with 30 mg/ml RP material.
3. Wash cartridge and activate using 1ml of MeOH followed by equilibration with 1ml of 1% formic acid.
4. Load the sample onto cartridge and wash with 1ml of 5% MeOH/1% formic acid.
5. Elute the peptides with 1ml of acetonitrile/H_2O (90:10; 0.1% FA).
6. Collect eluate in Eppendorf reaction tubes prefilled with 5 µl DMSO.

Preparation for LC/MS

1. Remove the solvent under vacuum at 40°C using the centrifugal evaporator (approx. 2 hours).
2. Redissolve in 250µl acetonitrile/H_2O (3:97; 0.1% FA) and transfer to autosampler vials for LC/MS analysis.

LC/MS Analysis

HPLC-MS/MS: Agilent 1200 LC system coupled to an AB Sciex Q-Trap 4000 triple quadrupole mass spectrometer
Column: Phenomenex XB C-18 reversed-phase (RP) column (100 × 2.1 mm, 2.6 µm)
Column temperature: 40°C
Flow rate: 300 µL/min
ESI source: positive ion mode
Curtain gas pressure: 25 psi
Source temperature: 550°C
Desolvation gas: 50 psi
Sheath gas: 20 psi
Gradient profile: binary gradient from 97% A (water + 0.1% formic acid) and 3% B (acetonitrile + 0.1% formic acid) to 28.4% B over 22 min, increasing to 100% B at 23 min and held for 5 min. Column re-equilibration was for a further 6 min.
Injection volume: 10 µL
The peptides eluted from the column can be detected using a scheduled dynamic monitoring mode with a scan time of 2 s and a nominal retention time widow of ±50 s. The peptides and their fragments for the method are listed below:

m/z	Fragments	R_t *(min)*	Species	Sequence
375	(175; 435; 564; 677)	15.8	BPLH	ALELFR
697	(147; 260; 487; 430)	21.9	BPL	HGNTVLTALGGILK
797	(1009; 1080; 815; 952)	14.1	BPL	VEADVAGHGQEVLIR
637	(716; 1012; 147; 911)	12.6	BHL	LFTGHPETLEK
326	(204; 76; 408; 351)	14.2	HP	ELGFQG
471	(580; 509; 711; 310)	8.7	BL	NDMAAQYK
690	(886; 985; 1242; 773)	21.1	H	HGTVVLTALGGILK
752	(1269; 706; 248; 1366)	12.0	H	HPGDFGADAQGAMTK
804	(1009; 815; 952; 500)	14.9	H	VEADIAGHGQEVLIR
928	(953; 1081; 1394; 1523)	8.4	H	GHHEAELKPLAQSHATK
456	(716; 490; 147; 619)	5.2	P	GHPETLEK
745	(234; 1255; 692; 1352)	11.9	P	HPGDFGADAQGAMSK
914	(953; 1367; 1054; 1496)	9.8	P	GHHEAELTPLAQSHATK
311	(76; 204; 408; 351)	16.2	L	VLGFQG
760	(234; 1285; 692; 1083)	11.8	L	HPSDFGADAQGAMSK
315	(417; 213; 530; 360)	13.4	B	VLGFHG
767	(234; 1299; 706; 1396)	13.2	B	HPSDFGADAQAAMSK

Identify the different meat species in the samples from the presence of the species peptides listed above. (We use the criteria for confirmation of the presence of at least 4 peptides for the species, including the species-specific peptides, with 3 transitions per peptide present).
To estimate the relative amounts of myoglobin for the meat species in the mixture we need to compare the peak areas of the common peptide (375) in the single meat samples. This will give us an approximate ratio of the levels of myoglobin.
To calculate the quantities of each species in the mixture we need to combine the above myoglobin ratio calculation with a comparison of the measured levels of a selected CPCP (corresponding protein, corresponding peptide).

A CPCP and y value should be chosen from the list below:

Species	CPCP	y values	Fragments
Beef and lamb	B(767) & L(760)	2, 11, 13 and 14	234, 1097/1083, 1299/1285, 1396/1382
Beef and horse	B(767) & H(752)	7, 11, 13 and 14	706, 1097, 1299/1269, 1396/1366
	B(697) & H(690)	5 & 9	487, 886
Beef and pork	B(767) & P(745)	2, 7, 11, 13 & 14	234, 706/692, 1097/1083, 1299/1255, 1396/1352
Lamb and horse	L(760) & H(752)	11, 13 & 14	1083/1097, 1285/1269, 1382/1366
	L(697) & H(690)	1, 2, 5 & 9	147, 260, 487, 886
Lamb and pork	L(760) & P(745)	2 & 13	234, 1285/1255
Horse and pork	H(752) & P(745)	7, 13 & 14	706/692, 1269/1255, 1366/1352
	H(690) & P(697)	1, 2, 5 & 9	147, 260, 487, 886

Determine the peak areas for a particular transition for the chosen CPCP and calculate the ratio of species 1 in species 2 using the equation below:
Ratio of peak areas = peak area (species 1)/(peak area (species 1) + peak area (species 2))

4.3 Appendix 3: Protocol – meat species in processed sausage products

Materials

1.1. Materials

Reagent	Description	Producer/supplier
Acetonitrile	LC-MS grade	Sigma-Aldrich
Urea		Sigma-Aldrich
Trypsin	T0303, type IX-S, 13,000-20,000 BAEE units/mg protein	Sigma-Aldrich
Ammonium hydrogen carbonate		Sigma-Aldrich
Dimethyl sulfoxide (DMSO)		Sigma-Aldrich
Potassium chloride	Analytical grade	
Potassium hydroxide	Analytical grade	
Potassium hydrogen phosphate	Analytical grade	
Formic acid	Analytical grade	
Ultrapure water		

1.2. Selected solutions

- Extraction buffer (0.3 M potassium chloride, 0.3 M potassium hydrogen phosphate, pH 6.5)
- Ammonium hydrogen carbonate solution (30 mM in ultrapure water)
- Trypsin solution (1 mg/mL in the 30mM ammonium hydrogen carbonate solution)

2. Instrumentation and settings (Gdansk)

The HPLC-MS/MS analyses of the marker peptides (Table 2) were performed using an Agilent 1200 LC system (Santa Clara, USA) equipped with a binary pump, an online degasser, an autosampler and a thermostated column compartment equipped with a switching valve. The HPLC system was coupled with a Q-Trap 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, USA). Kinetex XB C-18 reversed-phase (RP) column (100 × 2.1 mm, 2.6 µm, Phenomenex) was used for RP-HPLC separation of the peptides. In order to acquire data and control the instrument, Analyst software ver. 1.5.2 (AB Sciex, CA, USA) was used.

The column temperature was maintained at 40°C throughout the separation process. The ESI source was operated in the positive ion mode with the following conditions: the curtain gas pressure at 25 psi, the temperature of source set to 550°C, the ion spray voltage at 5000 V, the nebulizer gas and the heater gas at 50 psi and 20 psi, respectively.

3. Meat samples preparation

3.1. Day one – preparation and digestion of the samples

Processed meat products were stored at -80°C prior to analysis. On the day of analysis, samples of meat products were defrosted at room temperature. Accurately weighed samples (1 g) were placed in plastic 50-mL centrifuge tubes and 10 mL of the extraction buffer was added. The samples were homogenized with an Ultra-Turrax homogenizer for 5 min (18 000 rpm). In the next step, the homogenizates were sonicated for

30 min in an ultrasound bath and finally centrifuged at room temperature for 40 min at 10733 g. Clear or almost clear supernatants were collected, transferred to plastic, 50-mL centrifuge tubes and vortexed. Subsequently, the extracts were centrifuged at room temperature for 2 min at 1315 g in order to suppress the foam that could have formed during the previous step, and finally 1.5-mL aliquots of extracts were transferred to 12-mL test tubes and mixed with 2.4 mL of 30 mM ammonium hydrogen carbonate. In the next step, the extracts were heated for 30 min at 90°C in order to denature the proteins present in solution. The extracts were then cooled to the room temperature, and 105 mg of urea was added to each test tube in order to accelerate the following enzymatic digestion of proteins. Subsequently, 1.5 mL of trypsin solution was added. The samples were immediately vortexed and incubated for 4 h at 37°C. Since the extraction and digestion procedure was time-consuming (around 10 h in total for 21 samples), the following steps were performed on the next day. Until then, the digested extracts were stored frozen (-20°C).

3.1. Day two – sample purification and enrichment

After bringing the samples back to room temperature, the extracts were cleaned-up and enriched using Strata-X 33 µm polymeric reversed-phase SPE cartridges (Phenomenex, Macclesfield, UK). The cartridges were activated with 2 mL of MeOH followed by 4 mL of aqueous 1% (v/v) FA; afterwards the extracts were loaded into the cartridges. The empty extract vials were additionally washed out with 500 µL of 1% (v/v) FA, and the washings were loaded into the cartridges to provide a quantitative transfer of the samples. The cartridges were then washed with 4 mL of 1% (v/v) FA. Finally, the peptides were eluted with 4 mL of ACN:water mixture (1:1 v/v; containing 0.1% v/v FA) into 12-mL glass test tubes containing 10 µL DMSO each. The elution was done in two runs, 2 mL each. The cartridges were left to soak for 10 minutes after the first batch of eluate in order to provide complete elution of the peptides. Subsequently, the solvents in the purified extracts were completely evaporated under the stream of nitrogen at 45°C. Prior to chromatographic analysis, the extracts were reconstituted with 200 µL of ACN:H_2O mixture (3:97 v/v; containing 0.1% v/v FA), vortexed for 30 s, centrifuged for 3 min at 2470 g and transferred to autosampler vials equipped with 250 µL inserts. The sample preparation procedure has been summarized in Figure 1.

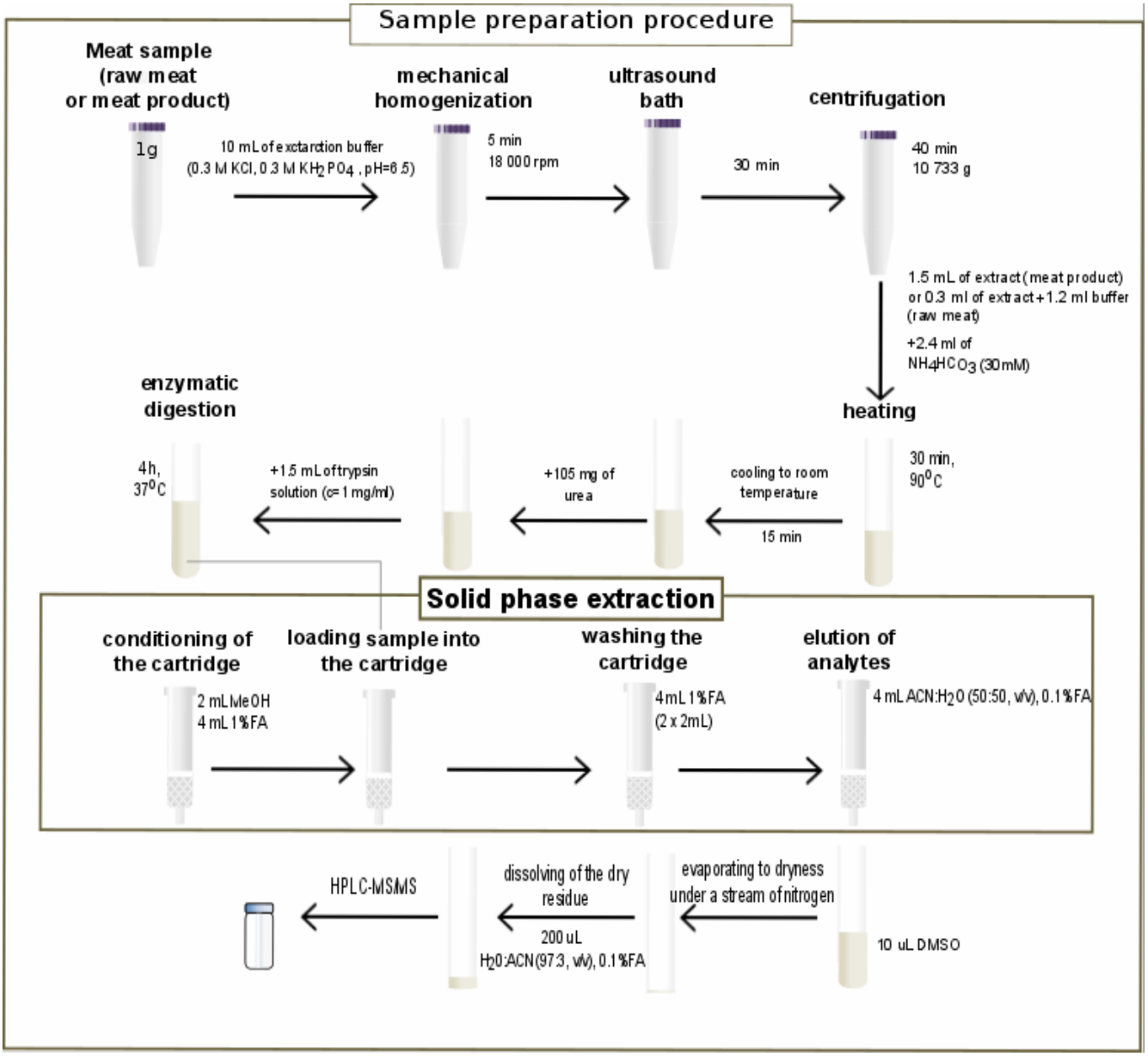

Fig. 1. Sample preparation procedure required for the HPLC-MS/MS analysis of marker peptides.

4. HPLC-MS/MS analysis of samples

4.1. Marker peptides

The HPLC-MS/MS analyses of the marker peptides (Table 2) were performed. Each peptide was monitored by four most intense MRM transitions. In the case of pork and beef samples, the intensities of three subsequent MRM transitions were very low (e.g. primary to secondary, tertiary and quaternary transitions for pork were around 100:4:4:4). Therefore, four MRM transitions were observed to confirm the identity of the markers peptides. When analyzing chicken meat, two MRM transitions were sufficiently intense to confirm the identity of markers peptides specific for this kind of meat. The source and MS parameters are shown in Table 2.

Table 2. MRM transition parameters for detection of marker peptides in meat samples

Meat	Protein	Uniprot ID	Marker peptide symbol and amino acid sequence	Parent ion (m/z)	Fragments (m/z)	Declustering potential (V)	Collision energy (V)
pork	myoglobin	P02189	P1 GHPETLEK	455.7	716.4 490.3 147.1 619.3	64.3	21.7
pork	myoglobin	P02189	P2 HPGDFGADAQGAMSK	744.8	234.1 1254.5 692.3 1351.6	97.7	47.8
beef	myoglobin	P02192	B1 VLGFHG	315.2	417.2 213.2 530.3 360.2	54.0	14.0
beef	myoglobin	P02192	B2 HPSDFGADAQAAMSK	766.8	234.1 1298.6 706.4 1395.6	86.0	39.0

4.2. Mobile phase

The mobile phase consisted of water containing 0.1% (v/v) FA (component A) and acetonitrile containing 0.1% (v/v) FA (component B).

4.3. Elution programme

The following gradient elution programme was used: a linear increase from 3% of B to 28.4% of B during 22 min, next, a linear increase from 28.4% of B to 90% of B during 1 min, followed by 90% of B maintained for 10 min. The last step was the conditioning of the column for 12 min with 3% of B. The flow rate of mobile phase was 300 µL/min, and the injection volume was 10 µL.

5. Template for reporting results

Please report results based on the following template provided as an Excel sheet:

Authentication of meat samples - blind tests	
Laboratory:	Enter laboratory name here
Date:	Enter date

Please enter peak areas in counts

Meat sample		P1 GHPETLEK	P2 HPGDFGADAQGAMSK	B1 VLGFHG	B2 HPSDFGADAQAAMSK
1	Assay 1				
	Assay 2				
	Assay 3				
	Mean				
	SD				
2	Assay 1				
	Assay 2				
	Assay 3				
	Mean				
	SD				
3	Assay 1				
	Assay 2				
	Assay 3				
	Mean				
	SD				
4	Assay 1				
	Assay 2				
	Assay 3				
	Mean				
	SD				
5	Assay 1				
	Assay 2				
	Assay 3				
	Mean				
	SD				
6	Assay 1				
	Assay 2				
	Assay 3				
	Mean				
	SD				