



Analytical Methods

A sensitive HPLC-MS/MS screening method for the simultaneous detection of barley, maize, oats, rice, rye and wheat proteins in meat products



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ABSTRACT

The use of grain proteins in various types of meat products is common practice. A reliable detection of these food ingredients is required to control specifications and regarding food fraud and allergenic potential. Consequently, a sensitive HPLC-MS/MS method for the simultaneous detection of barley, maize, oats, rice, rye and wheat proteins in meat products was developed. After protein extraction and tryptic digestion, three to four selected marker peptides for each grain species were measured by HPLC-MS/MS. Emulsion-type sausages with grain-based protein concentrations in the range of 5–1000 mg/kg and blank values were produced. The limits of detection of the method were ≤ 5 or ≤ 10 mg grain protein/kg meat product for each grain species and no false-positive or -negative results were obtained. The detectability of the marker peptides only slightly decreased after storage and grilling of sausages, whereas the influence of the canning process was noticeably higher.

1. Introduction

The addition of foreign proteins to meat products (especially emulsion-type sausages) is a very common practice. In addition to soybean and milk proteins, wheat gluten is most frequently added (Lopez & Alegre, 2009). Wheat is the third most important cereal in terms of worldwide production with an annual production rate of 729 million tonnes; the two cereals with the highest production rate: maize (1038 million tonnes) and rice (741 million tonnes), and barley (144 million tonnes), oats (23 million tonnes) and rye (15 million tonnes) are conceivable non-meat proteins (FAO, 2014). Several attempts have been made to add wheat protein to meat products (Ulu, 2004), due to its advantageous functional properties, such as water- and fat-binding, texturizing properties and low cost (Lopez & Alegre, 2009). However, the use of the gel properties of wheat gluten is limited due to its poor solubility. Besides wheat, the grain species barley (Petersson, Godard, Eliasson, & Tornberg, 2014), oats (Petersson et al., 2014), rice (Liu, Wu, & Tan, 2010) and rye (Petersson et al., 2014) have also been used as foreign proteins in meat products. The reasons for the use of maize as a non-meat protein are primarily economical ones, whereas technological reasons are less important due to the low functionality and poor solubility of maize protein (Lopez & Alegre, 2009). However, the use of modified corn gluten in an emulsified meat model system has been reported in the literature (Homco-Ryan, Ryan, & Brewer, 2003).

In addition to the deliberate use of grain proteins as a protein

replacement in meat products, the grain proteins mentioned could also be unintentionally transferred into meat products via cross-contamination while processing, and contaminations of spice mixtures or other food additives and processing aids. In this context, even small amounts of grain proteins may be relevant to human health. Within the group of cereals containing gluten, proteins from wheat, rye, barley and, in rare cases, oats, are responsible for health problems such as celiac disease, non-celiac gluten sensitivity and wheat allergy (Schalk, Lexhaller, Koehler, & Scherf, 2017; Scherf, Koehler, & Wieser, 2016). Gluten is especially responsible for triggering these hypersensitive reactions, but other proteins, such as lipid-transfer proteins and amylase-trypsin inhibitors may also cause harmful effects (Fasano, Sapone, Zevallos, & Schuppan, 2015). Food containing no more than 20 mg/kg is defined as 'gluten-free' in the European Union (European Union, 2014), whereas 'gluten' means the corresponding protein fractions from wheat, rye, barley and oats. However, oats contain only low amounts of prolamin (avenin) and uncontaminated oats seem to be safe concerning the triggering of celiac disease in the vast majority of patients (European Food Safety Authority, 2014). Furthermore, a reference dose for allergenic food residues of 1 mg wheat protein exists (Taylor et al., 2014). The cereals rice and maize containing no gluten may also have the potential to induce food allergies (European Food Safety Authority, 2014), however these grain species are not included in the list of the 14 major food allergens, that must be declared as allergens in the European Union.

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The most common analytical methods to detect grain proteins (especially wheat) in food are currently polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISAs; [Codex Alimentarius Commission, 2006](#)). Such methods are of great importance for checking the food specifications and for the identification of food fraud and the detection of substances triggering allergies and food intolerances. Proficiency tests for the detection of wheat flour in sausages showed that PCR and ELISA methods were suitable for a qualitative detection of the presence of gluten in sausages in the concentration range of 11–34 mg/kg ([Scharf, Kasel, Wichmann, & Besler, 2013](#)). The disadvantages of PCR are that it is an indirect method and suffers from the problem of DNA contamination due to the amplification of a minor component ([Bönick, Huscchek, & Rawel, 2017](#)). The ELISA methods are, in most cases, only applicable for single allergen detection. Furthermore, the sandwich ELISA based on the R5 antibody recommended by the [Codex Alimentarius Commission \(2006\)](#) for the detection of gliadin, secalins and hordeins cannot differentiate between these prolamins and shows a strong influence of food processing ([Sajic et al., 2017](#)). Due to these disadvantages of PCR and ELISA methods, analytical methods based on high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) have been developed for the detection of allergens and other proteins in food. Trypsin or chymotrypsin and a combination of both proteases was used ([Manfredi, Mattarozzi, Giannetto, & Careri, 2015](#)) for the mass spectrometric detection of wheat, oat, barley and rye prolamins in rice flour and rice bread. Trypsin, not in combination with other proteases, was used by [Simonato, Mainente, Tolin, and Pasini \(2011\)](#), who detected residual proteins in gluten fined red wine. Furthermore, tryptic marker peptides were used for the detection of wheat proteins in grain products using lipid transfer proteins ([Heick, Fischer, & Popping, 2012](#)) or glutenins and alpha amylase inhibitors ([Colgrave et al., 2015](#)) as target proteins. Also tryptic marker peptides were used for the detection of barley contaminations in grain products ([Colgrave, Byrne, Blundell, & Howitt, 2016](#)), gluten from barley, oats, rye and wheat in grain products ([Lock, 2014](#)), and wheat and barley gluteins in various foods, but no meat products ([Liao et al., 2017](#)). No HPLC-MS/MS methods for the detection of grain proteins in meat products exist. Therefore, an HPLC-MS/MS-method for the detection of wheat proteins in meat products using tryptic marker peptides from gliadins was established in a previous study ([Jira, Hirschfeld, Speer, & Schwägele, 2015](#)).

The main objective of this study was to develop a novel sensitive analytical HPLC-MS/MS screening method for the simultaneous detection of proteins of the six main grain species: barley (*Hordeum vulgare*), maize (*Zea mays*), oats (*Avena sativa*), rice (*Oryza sativa*), rye (*Secale cereale*) and wheat (*Triticum aestivum*), in meat products with limits of detection (LODs) below 20 mg/kg using characteristic tryptic marker peptides. The influence of storage, heating and processing on the detectability of the grain marker peptides should also be investigated.

2. Materials and methods

2.1. Materials and reagents

Whole grain flours of wheat (*Triticum aestivum*) and rye (*Secale cereale*) were obtained from Dennree (Töpen, Germany). Whole grain flours of barley (*Hordeum vulgare*), oats (*Avena sativa*) and maize flour (*Zea mays*) were purchased from Adlerrmühle (Bahlingen am Kaiserstuhl, Germany), and rice flour (*Oryza sativa*) from Carptime (Mihla, Germany). The grain kernels of wheat (spring wheat, Quintus), barley (winter feed barley, Antonella) and rye (population rye, Matador) were purchased from Nordsaat Saatucht GmbH (Langenstein, Germany). Maize grains originated from a cornfield near Kulmbach (Germany). Rice grains were purchased from Transimpex (Lambsheim, Germany). Oat grains (Pergamon) and wheat grains (spring wheat, Thasos and winter wheat, Exquisit) were obtained from the test ground of the Julius Kühn-Institut in Groß Lüsewitz (Germany).

Maize protein was purchased from Common Baits (Rosenfeld, Germany), rice protein from Biohealth International GmbH (Münchberg, Germany) and wheat gluten from Pacovis food solutions GmbH (Sassenberg, Germany).

The solvents acetone, acetonitrile (ACN), and water were purchased from LGC Standards (Wesel, Germany) in Optigrade quality. Ethanol (absolute, p.A.) was obtained from Th. Geyer (Renningen, Deutschland), Tris(hydroxymethyl)-aminomethane (TRIS; $\geq 99.3\%$) was purchased from Carl Roth (Karlsruhe, Germany), trypsin (sequencing grade) from Promega (Madison, USA), and hydrochloric acid (HCl) and formic acid (p.A.) from Merck (Darmstadt, Germany). Iodoacetamide (IAA), DL-Dithiothreitol (DTT; $\geq 98\%$), and formic acid (for LC-MS) were bought from Sigma (St. Louis, USA). Dimethyl sulfoxide (DMSO; p.A.) was purchased from J. T. Baker (Center Valley, USA).

2.2. HPLC-MS/MS-identification of characteristic tryptic peptides for barley, maize, oats, rice, rye and wheat in flour, whole grain flour and grain protein isolates

The experimental workflow of this study is shown in Supplementary data Fig. S1.

2.2.1. Sample preparation

About 1 g flour or protein isolate was defatted. This material was extracted and digested with trypsin, as described later (see [Sections 2.7.1 to 2.7.3](#)) with a few minor changes:

In contrast to the conditions described for pressurized liquid extraction in [Section 2.7.1](#), the flour or protein isolate was placed on laboratory wipes, the temperature was set to 50 °C and a static time of 5 min was applied. Furthermore, after homogenization of the resulting dried powder in the ball mill MM 2000 from Retsch (Haan, Germany), 25 mg defatted and dehydrated material was used for the protein extraction performed with 1 mL buffer (TRIS-HCl [1M, pH 8.2]/ethanol, 50/50, v/v).

2.2.2. Liquid chromatography

Separation of peptides was performed with a Dionex UltiMate 3000 RS HPLC from Thermo Scientific (Waltham, USA). The column temperature was 40 °C and the injection volume was 2 μ L. The analytical column used was a Nucleosil 100-3 C18 HD (125 $\times$ 2.0 mm; particle size: 3 μ m) from Macherey-Nagel (Düren, Germany). The mobile phase consisted of solvent A: 0.1% formic acid and 3% ACN in water; and solvent B: 0.1% formic acid and 90% ACN in water. The LC run (total time: 52 min) started with 2% B for 3 min, followed by a gradient to 60% B in 30 min and another gradient to 100% B in 1 min. An isocratic step at 100% B continued for 10 min. At the end of the run, the column was allowed to equilibrate at 2% B for 7 min. The flow rate was 250 μ L/min.

2.2.3. High resolution tandem mass spectrometry

Peptide identification was carried out on a maxIS UHR-QToF system (Bruker Daltonik, Bremen, Germany) in the positive ESI mode (capillary voltage: 3500 V). The ESI interface setting parameters were 180 °C drying gas temperature and 4 bar ESI nebulizer gas (N_2) pressure. The mass range of the LC-MS/MS measurements (data-dependent MS/MS) was m/z 100–1600 with a spectra rate of 2 Hz. Selected precursors analyzed more than two times were actively excluded from analysis for 60 s. The collision energy of the quadrupole ranged between 25 and 50 V. Peak lists from the MS/MS data acquired were submitted to the online version of the MASCOT database search tool (www.matrixscience.com). The following parameters were set for the database search: The databases selected were SwissProt and NCBIprot. One missed trypsin cleavage was allowed. Peptide tolerance and MS/MS tolerance were set to 0.05 Da. The variable amino acid modification was carbamidomethyl. The resulting peptides originating from barley,

Table 1

Percentage compositions of the emulsion-types sausages of the different batches in the three production series produced with and without the addition of a flour mixture containing barley, maize, oats, rice, rye and wheat at different levels of grain-based protein.

	Production series 1				Production series 2				Production series 3							
Batch name ^a	1-0	1-A	1-C	1-E	2-0	2-A	2-B	2-C	2-D	2-E	2-F	3-0	3-A	3-C	3-E	
Protein (grain) [%] ^b	–	0.1	0.01	0.001	–	0.1	0.05	0.01	0.005	0.001	0.0005	–	0.1	0.01	0.001	
Pork [%]	53.45	50.07	53.09	53.41	54.10	50.73	52.42	53.77	53.94	54.07	54.09	54.03	50.66	53.69	53.99	
Fat [%]	24.75	23.18	24.58	24.73	23.48	22.02	22.75	23.33	23.41	23.46	23.47	23.11	21.67	22.97	23.09	
Ice [%]	19.80	18.54	19.66	19.78	20.42	19.14	19.78	20.29	20.35	20.40	20.41	20.39	19.12	20.26	20.37	
Curing salt [%]	1.78	1.67	1.77	1.78	1.80	1.69	1.74	1.79	1.79	1.80	1.80	1.83	1.72	1.82	1.83	
Ascorbate [%]	0.03	0.03	0.03	0.03	–	–	–	–	–	–	–	0.03	0.03	0.03	0.03	
Phosphate [%]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
Spice mixture [%]	–	–	–	–	–	–	–	–	–	–	–	0.41	0.38	0.41	0.41	
Flour mixture [%]	–	6.33	0.63	0.06	–	6.24	3.12	0.62	0.31	0.06	0.03	–	6.23	0.62	0.06	
Flour mixture	–	1	1	1	2	2	2	2	2	2	2	2	2	2	2	
Sausage in casing	–	–	–	–	x	x	x	x	x	x	x	–	–	–	–	
Home canned can	–	–	–	–	x	x	x	x	x	x	x	–	–	–	–	
Normal can (F-value)	x (3.9)	x (3.9)	x (3.9)	x (3.9)	–	–	–	–	–	–	–	x (4.1)	x (4.1)	x (4.1)	x (4.1)	
Can for use under tropical conditions (F-value)	–	–	–	–	x (13.7)	x (13.7)	x (13.7)	x (13.7)	x (13.7)	x (13.7)	x (13.7)	–	–	–	–	

^a Same letters indicate same concentration levels.

^b The protein concentration refers to each of the six types of grain.

maize, oats, rice, rye and wheat were additionally searched (BLAST) in the UniProt and the NCBI database and preferably unique peptides were preselected for the further method development on the QTrap 5500 (AB Sciex, Darmstadt, Germany) (see Section 2.7).

2.3. Production of flour mixtures of barley, maize, oats, rice, rye and wheat

The six commercial grain flours mentioned (see Section 2.1) were mixed to obtain comparable protein concentrations of the different grain species for the production of flour mixture 1 (see Table 1; used for sausage production series 1). Therefore, the protein concentration of each type of grain flour was determined in accordance with the respective method in the German Food and Feed Code (§64 LFGB). An amount of 20 g of each type of grain were separately filled in a 40 mL grinding chamber and carefully homogenized (3 cycles of 30 s at 25000 rpm) with a Tube Mill control from IKA GmbH (Staufen, Germany) to obtain flour mixture 2 (see Table 1; used for sausage production series 2 and 3). The six fresh ground flours obtained were combined in such a manner to obtain approximately comparable protein concentrations for all six types of grain.

2.4. Production of emulsion-type sausages

Three series of emulsion-type sausages containing different protein concentrations of the six types of grain mentioned were produced. The formulations of the different batches of the emulsion-type sausages for the three production series are shown in Table 1.

The meat was minced in a meat grinder (Moulinex, Alencon, France). The sausages were produced in a 13 L bowl chopper (Müller Food Machines, Saarbrücken, Germany) and the maximum temperature of the sausage meat was 12 °C. The sausage meat was stuffed into 200 g tin-plate cans (type 99/36 mm; Dosen-Zentrale Züchner GmbH, Cologne, Germany) or casings (caliber: 60 mm; Nalo-Top, Kalle GmbH, Wiesbaden, Germany). The sausages were heated and, subsequently, cooled overnight and stored at 2 °C. In addition, a storage experiment was performed with sausages in casings from production series 2 at 2 °C for six weeks.

2.5. Performance of the grilling experiments

Grilling experiments of slices of emulsion-type sausages filled in casings (see Section 2.4; production series 2) were performed with a Silex S-162 contact grill (Hamburg, Germany) with Duranel plates. The upper and lower grill plate were preheated to 180 °C. After reaching the

plate temperatures, the meat slices (diameter: 6 cm; thickness: 1 cm) were wrapped in aluminum foil and grilled for 1.3 min. The core temperature (about 75 °C) was measured with a NiCr-Ni temperature sensor 922 from Testo (Lenzkirch, Germany). The grilled meat slices were cooled to room temperature (RT) and the samples were homogenized and used for analysis.

2.6. Synthesis of labelled marker peptides and preparation of peptide standard solutions

The labelled marker peptides IVPLA(d₄)IDTR (barley), FSLMNPVL (¹³C₆, ¹⁵N)SR (maize), ALPVDVLANA(d₄)YR (oats), LQA(d₄)FEPIR (rice), ILWGIPTLL(d₁₀)K (rye) and SVAVSQVA(d₄)R (wheat) (see Table 2) were synthesized at the 0.05 mmol scale with the Liberty Blue Microwave Peptide Synthesizer (CEM, Kamp-Lintfort, Germany) using solid-phase Fmoc (9-fluorenylmethoxycarbonyl) chemistry with free amines at the N-terminal ends and free carboxyls at the C-terminal ends. The peptides were purified by an ÄKTA pure chromatography system (GE Healthcare Life Sciences, Freiburg, Germany). The preparative column used was a Reprosil 100 C18 (250 × 16 mm; particle size: 5 µm) from Dr. Maisch GmbH (Ammerbuch, Germany). The peptides were characterized by HPLC-MS/MS and the purities (86 to > 99%) were confirmed by HPLC-UV (216 nm) (Dionex UltiMate 3000 RS HPLC with a Diode Array Detector from Thermo Fisher Scientific (Waltham, Massachusetts, USA). An amount of 1 mg of each peptide was dissolved in 5 mL of solvent A (0.1% formic acid and 3% ACN in water) for the preparation of the stock solutions (200 ng/µL). The concentrations of the peptides mentioned above used as internal peptide standard mixture (ISTD) were 0.3 ng/µL, each.

2.7. HPLC-MS/MS detection of barley, maize, oats, rice, rye and wheat in meat products

Different method parameters concerning protein extraction and tryptic digestion were optimized (see Section 3). The final conditions are reported in this paragraph.

2.7.1. Pressurized liquid extraction (PLE)

Approximately 2 g of the homogenized emulsion-type sausages were applied on filter paper circles (type 589, Schleicher und Schüll, Dassel, Germany) and transferred into 20 mL cells for defatting of the samples. The PLE was accomplished with a Speed Extractor E-916 obtained from Büchi (Flawil, Switzerland), and acetone was used as the solvent. Two static cycles were performed; the operating conditions of the extraction

Table 2

Parameters of the MRM method (MRM detection window 120 s; CE = Collision Energy; CXP = Cell Exit Potential; DP = Declustering Potential).

Marker peptide	RT [min]	m/z (charge state)	Product Ions (Mass Transition 1, 2, and 3)	CE [V]	CXP [V]	DP [V]
Barley1 IETPGPPYLAK	6.0	593.3 (+2)	842.5 (y8), 421.7 (y8 ²⁺), 243.1 (b2)	24/28/30	35/20/15	70/70/70
Barley2 IVPLAIDTR ^{a,b}	7.2	499.3 (+2)	785.5 (y7), 575.3 (y5), 276.2 (y2)	20/26/36	35/30/15	70/70/70
Barley3 IVPLA(d ₄)IDTR	7.2	501.3 (+2)	789.5 (y7), 579.3 (y5), 276.2 (y2)	20/26/36	35/30/15	70/70/70
Barley3 TLPTMC*SVNVPLYR ^a	10.3	825.9 (+2)	761.4 (y6), 718.9 (y12 ²⁺), 548.3 (y4)	44/32/32	37/33/25	60/60/60
Barley4 ILPFGIDTR ^c	10.6	516.3 (+2)	805.4 (y7), 403.2 (y7 ²⁺), 276.2 (y2)	20/18/34	35/20/15	70/70/70
Maize1 LEDLPGC*PR	4.8	528.8 (+2)	586.3 (y5), 358.2 (b3), 243.1 (b2)	22/26/24	30/20/15	50/50/50
Maize2 TNPC*GVSAIIPYY	8.8	755.4 (+2)	971.5 (b10), 629.3 (b6), 539.3 (y4)	20/18/28	45/30/25	50/50/50
Maize3 FSLMNPVLSR	11.5	582.3 (+2)	816.4 (y7), 685.4 (y6), 571.4 (y5)	29/29/29	35/30/25	70/70/70
Maize3 FSLMNPVL(¹³ C ₆ , ¹⁵ N)SR	11.5	585.8 (+2)	823.4 (y7), 692.4 (y6), 578.4 (y5)	29/29/29	35/30/25	70/70/70
Maize4 QQPIVGGAIIF	12.0	515.3 (+2)	751.4 (b8), 680.4 (b7), 279.2 (y2)	18/22/16	35/33/15	60/60/60
Oat1 C*TGVSIVIR	4.6	446.2 (+2)	731.4 (y7), 630.4 (y6), 474.3 (y4)	23/22/24	35/30/25	70/70/70
Oat2 ALPVDVLANAYR ^{d,e}	11.7	651.4 (+2)	921.5 (y8), 707.4 (y6), 559.3 (y10 ²⁺)	32/34/24	40/35/25	50/50/50
Oat2 ALPVDVLANA(d ₄)YR	11.7	653.4 (+2)	925.5 (y8), 711.4 (y6), 561.3 (y10 ²⁺)	32/34/24	40/35/25	50/50/50
Oat3 DFPITWPKW	15.6	595.3 (+2)	927.5 (y7), 464.3 (y7 ²⁺), 430.2 (y3)	22/22/36	40/23/23	60/60/60
Rice1 LQAFEPPIR	6.5	487.3 (+2)	732.4 (y6), 385.3 (y3), 242.1 (b2)	20/30/20	35/23/15	60/60/60
Rice1 LQA(d ₄)FEPIR	6.5	489.3 (+2)	736.4 (y6), 385.3 (y3), 242.1 (b2)	20/30/20	35/23/15	60/60/60
Rice2 ELGAPDVGHMSEVFR	7.9	580.9 (+3)	865.4 (y7), 685.8 (y12 ²⁺), 457.6 (y12 ³⁺)	30/20/20	40/32/20	60/60/60
Rice3 VIQPGLLVPR ^c	8.5	610.4 (+2)	879.5 (y8), 341.2 (b3), 272.2 (y2)	27/24/26	40/20/15	50/50/50
Rice4 VIEPQGLLVPR ^c	9.1	610.9 (+2)	879.5 (y8), 654.4 (y6), 440.3 (y8 ²⁺)	30/35/26	40/35/22	60/60/60
Rye1 TPFASTVAGIGGQ	7.8	603.3 (+2)	945.5 (b10), 431.2 (y5), 261.1 (y3)	16/20/18	45/22/15	60/60/60
Rye2 SLVLQNLPTMC*NVYVPR ^a	13.3	668.7 (+3)	747.4 (y6), 618.8 (y10 ²⁺), 534.3 (y4)	29/19/25	35/30/25	70/70/70
Rye3 ILWGIPTLLK	16.5	577.4 (+2)	927.6 (y8), 571.4 (y5), 199.2 (a2)	24/33/26	40/25/10	70/70/70
Rye3 ILWGIPTLL(d ₁₀)K	16.5	582.4 (+2)	937.6 (y8), 581.4 (y5), 199.2 (a2)	24/33/26	40/25/10	70/70/70
Wheat1 SVAVSQVAR ^a	7.2	458.8 (+2)	730.4 (y7), 560.3 (y5), 258.1 (b3)	21/22/21	35/27/15	70/70/70
Wheat1 SVAVSQVA(d ₄)R	7.2	460.8 (+2)	734.4 (y7), 564.3 (y5), 258.1 (b3)	21/22/21	35/27/15	70/70/70
Wheat2 VFLQQQC*SPVAMPQSLAR ^f	9.0	687.4 (+3)	873.5 (y8), 802.4 (y7), 671.4 (y6)	30/27/30	40/35/30	50/50/50
Wheat3 APFASIVAGIGGQ ^{c,f,g,h}	12.8	594.3 (+2)	927.5 (b10), 686.4 (b7), 502.3 (y6)	17/17/19	40/30/25	60/60/60
Wheat4 IFWGIPALLK ^{a,f}	17.5	579.4 (+2)	897.6 (y8), 711.5 (y7), 541.4 (y5)	21/26/32	40/33/25	70/70/70

* = Carbamidomethyl; ISTD in Italics.

^a Lock, 2014.^b Colgrave et al., 2014.^c Liao et al., 2017.^d Dawson et al., 2018.^e Chang et al., 2011.^f Uvackova et al., 2013.^g Jira et al., 2015.^h Fallahbagheri et al., 2017.

were 30 °C, 50 bar, static time 15 min and purge time 10 min. The defatted and dehydrated meat product was removed from the filter papers and, firstly, dried at RT in the air for at least 1 h and, subsequently, overnight in a desiccator.

2.7.2. Protein extraction

The defatted and dehydrated meat product was homogenized with the ball mill for 1 min at maximum amplitude and 50 mg was filled into 1.5-mL microtubes (polypropylene, LoBind, Eppendorf, Germany). After the addition of 0.5 mL buffer (TRIS-HCl [1 M, pH 8.2]/ethanol, 50/50, v/v) the samples were tightly closed, premixed, shaken for 2 h at 90 °C and 1400 rpm and then cooled to RT. The extract was centrifuged for 10 min at 12000 rpm. An amount of 0.5 mL TRIS-HCl (1 M, pH 8.2) and 10 µL ISTD were added to a 100 µL sample of the protein extract.

2.7.3. Tryptic digestion

The diluted protein extract (see Section 2.7.2) was reduced with 10 µL DTT solution (200 mM) for 30 min in a thermomixer (60 °C, 1400 rpm) and then alkylated by the addition of 5 µL IAA solution (1 M; 30 min at RT in the dark). Afterwards, 20 µL Trypsin solution (0.1 µg/mL in 50 mM acetic acid) was added and incubated at 37 °C for 18 h. The digestion was stopped by the addition of 20 µL concentrated formic acid.

2.7.4. Solid phase extraction (SPE)

The digested samples were loaded on a Strata-X SPE column

(30 mg/1 mL) from Phenomenex (Aschaffenburg, Germany), which was conditioned previously with 1 mL ACN and 1 mL water. The samples were washed with 1 mL water. Elution was performed with 500 µL of 80% ACN in water. The eluate was collected in 1.5-mL microtubes (see Section 2.7.2), prefilled with 5 µL DMSO and carefully concentrated to a volume of 5 µL by a SpeedVac (RVC 2-18 CD plus, Christ, Osterode am Harz, Germany). The concentrated eluate was dissolved in 50 µL of solvent A and transferred to a 1-mL tapered glass vial.

2.7.5. Liquid chromatography

Separation of peptides was performed, as described in Section 2.2.2. The LC run (total time: 33 min) started with 5% B, followed by a gradient to 17% B in 1 min, a further gradient to 23% B in 7 min, followed by gradients to 30% B in 3 min and to 34% B in 6 min, and another gradient step to 100% B in 3 min. An isocratic step at 100% B continued for 6 min. At the end of the run, the column was allowed to equilibrate at 5% B for 6 min. The flow rate was 250 µL/min.

2.7.6. Tandem mass spectrometry (MS/MS)

Peptide detection was carried out on an AB Sciex QTrap 5500 (Darmstadt, Germany) in positive ESI mode using the following parameters: Source temperature: 430 °C, ion spray voltage: 5.5 kV, curtain gas flow: 35 and an entrance potential of 10 V. Details of the multiple reaction monitoring (MRM) method are shown in Table 2.

3. Results and Discussion

3.1. Determination of suitable marker peptides for barley, maize, oats, rice, rye and wheat in plant materials

Peak lists were submitted to the online version of the MASCOT database search tool (www.matrixscience.com) based on the HPLC-MS/MS measurements (data-dependent MS/MS) of the tryptic digests of the defatted plant materials using the maXis UHR-QToF system. As a result, large numbers of peptides derived from proteins of barley, maize, oats, rice, rye and wheat were obtained. Within these groups of peptides, potential marker peptides (8 to 18 amino acids) for each type of grain were preselected (barley: 36 peptides, maize: 51 peptides, oats: 24 peptides, rice: 63 peptides, rye: 13 peptides, wheat: 36 peptides). Particularly the criteria abundance, extractability and digestibility of the corresponding target proteins (Johnson et al., 2011) were considered for the preselection of the marker peptides, in addition to uniqueness. Moreover, different target proteins for each grain species should be used as far as possible to minimize the general risk of adverse analytical effects due to different technological treatments of the samples and grain ingredients, respectively. Three to four peptides for barley, maize, oats, rice, rye and wheat from each of these preselected peptides were finally chosen as marker peptides due to their advantageous detectability in spiked meat products. The results of a BLAST search to verify the uniqueness of the marker peptides selected using NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) are shown in Supplementary data Table S1. Similar results were obtained by using UniProt (<http://www.uniprot.org/blast/>). Some of these marker peptides had already been described in the literature (Chang, Alli, Konishi, & Ziomek, 2011; Colgrave, Goswami, Blundell, Howitt, & Tanner, 2014; Dawson et al., 2018; Fallahbaghery, Zou, Byrne, Howitt, & Colgrave, 2017; Jira et al., 2015; Liao et al., 2017; Lock, 2014; Uvackova, Skultety, Bekesova, McClain, & Hajdúch, 2013).

Cereal flours of the individual grain species were analyzed separately due to the homologies according to the BLAST search (see Supplementary data Table S1) observed for barley1 (rye), barley3 (wheat), maize1 (rice) and all rye marker peptides (wheat). Thereby, only the homology of barley1 with rye was experimentally confirmed. The analysis of rye peptides in cereal grains of different commercial varieties of wheat (N = 18) showed that rye1 was only detected in one commercial wheat line. The marker peptides rye2, rye3 and barley3 were not detected in any of these samples (see Supplementary data Table S2). The marker peptide barley1 was detected in all cereal grain samples of commercial rye varieties whereas the marker peptide maize1 was not detected in any of the samples of commercial rice varieties (analyzed varieties are listed in Supplementary data Table S3). In these samples of commercial cultivars, all corresponding marker peptides were detectable. The variations of the marker peptide levels for commercial cultivars were as follows: 19–22% for barley (N = 6), 23–46% for maize (N = 7), 11–49% for oats (N = 9), 20–45% for rice (N = 9), 12–15% for rye (N = 7), and 23–28% for wheat (N = 16) (see Supplementary data Table S3).

In addition, the uniqueness of the marker peptides selected was checked by analyzing nine groups containing several common possible ingredients of meat products and twelve different commercial spice mixtures (see Supplementary data Table S4), as well as meat products without the addition of any type of grain (blank values) to prevent false positive results. Since none of the peptides mentioned above were detected in the ingredients analyzed or in sausages without added grain protein, the selected marker peptides were considered as appropriate for the detection of barley, maize, oats, rice, rye and wheat in meat products. In order to check, if rice4 is not only a deamidation product of rice3, spiking experiments were performed: At first, the addition of the synthesized peptide rice3 to emulsion-type sausages (control samples) showed that no rice4 was formed during the sample preparation. In a second experiment, the ratios of the peak areas of rice3 and rice4 in

sausages containing rice protein and control samples spiked with rice protein afterwards were compared. It was shown that a formation of rice4 from rice3 during the processing of meat products (e.g. by the enzyme tissue transglutaminase; Colgrave et al., 2014) is rather unlikely. As a consequence, rice3 and rice4 are two different marker peptides.

3.2. Optimization of the conditions of protein extraction and tryptic digestion in meat products with added cereal flours

The analytical method for the detection of barley, maize, oats, rice, rye and wheat was optimized according to the conditions of protein extraction, tryptic digestion and SPE. The improvement of the conditions mentioned was performed by the investigation of meat products with added cereal flours, however, only the results analyzing samples with the highest concentration of grain protein (batch 1-A; see Table 1) are shown. Comparable results were obtained by testing samples with lower concentrations.

Since the extraction efficiency of the various proteins from the six different types of grain was low applying the commonly used TRIS-HCl buffer (Heick, Fischer, & Popping, 2011; Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017), in a first step, several attempts were made to optimize the extraction buffer. As it is known from the literature (Díaz-Amigo & Popping, 2013) that alcohol is required for the extraction of gluten, the extraction with TRIS-HCl was compared, amongst others, with TRIS-HCl/ethanol (1:1) and TRIS-HCl/methanol (1:1), with a special focus on the extraction of the proteins of the cereals maize and rice containing no gluten. The effects of the extraction buffers on the peak areas of the two least sensitive marker peptides for each of the six different types of grain, which are the limiting factors for the detection, were considered (Supplementary data Fig. S2). Only minor differences between the extraction buffers tested were observed regarding the marker peptides wheat1, wheat4 and rye3. However, noticeably higher peak areas compared to the other extraction buffers were obtained for all other marker peptides for TRIS-HCl/ethanol (1:1). Therefore, this extraction system was used for the further investigations.

These findings comply with the literature, because it is known that gluten is a water-insoluble complex of proteins (Sollid & Jabri, 2013). The different components of gluten can be subdivided into the alcohol-soluble prolamins and the other fraction of glutelins, which are not soluble in alcohol. Several extraction solutions containing alcohol are used for the detection of gluten in food by ELISA methods (Bruins Slot, Bremer, van der Fels-Klerx, & Hamer, 2015). In contrast to that, a comparison of different sample extraction solutions for the mass spectrometric detection of wheat proteins in different types of food (Heick, et al., 2012) showed that an extraction with TRIS-HCl was more suitable than the use of 70% ethanol. However, in that study, a buffer system consisting of a mixture of TRIS-HCl and alcohol was not tested. Moreover, there is also evidence that glutelins are not only soluble in alkali or acid solutions, but also in a combination of an aqueous alcohol solution and a TRIS-HCl buffer (Lock, 2014; Schalk et al., 2017). The suitability of an extraction solution containing ethanol/TRIS-HCl (1:1; 1M, pH 8.2) for the mass spectrometric detection of wheat proteins in meat products was shown in a further study (Jira et al., 2015).

After the choice of the extraction buffer, different extraction temperatures were tested analyzing samples of batch 1-A (see Table 1). Extraction temperatures of 50, 70, and 90 °C were tested in triple determinations (see Fig. 1). Increasing the extraction temperatures resulted in higher peak areas of all the least sensitive marker peptides mentioned of the six types of grain analyzed. The only exception was marker peptide rye3, which showed the lowest peak area at the highest temperature. However, this decrease of peak area was less important, since the differences between the three temperatures were only minor and, otherwise, the standard deviations were relatively high for this peptide. Moreover, rye3 showed a relatively high peak area compared to the other marker peptides (see Fig. 1). An increase of the extraction

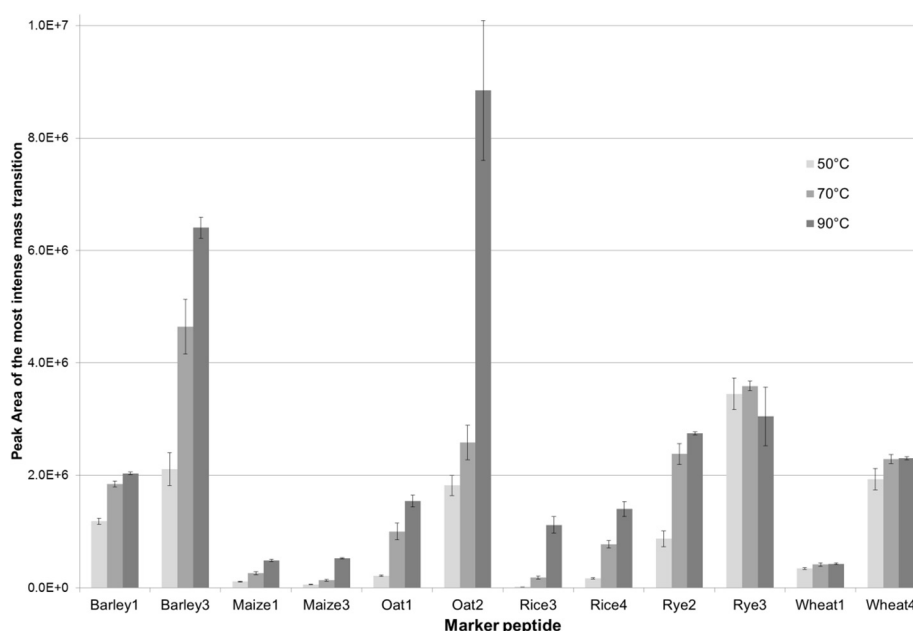


Fig. 1. Peak areas with standard deviations (N = 3) of the two least sensitive marker peptides for barley, maize, oats, rice, rye and wheat applying different extraction temperatures (50, 70 and 90 °C) in emulsion-type sausages with 0.1% grain protein (batch 1-A; QTrap 5500).

temperature from 70 to 90 °C for wheat1 and wheat4 resulted in only very small increases of the peak areas. The main reason for the decision to select 90 °C as the extraction temperature was the fact that the marker peptides with the lowest peak areas (maize1, maize3, oat1, rice3 and rice4) reached much higher values using this temperature: Raising the temperature from 70 to 90 °C, the peak areas of these marker peptides increased from 55% (oat1) to 501% (rice3). Temperatures higher than 90 °C proved to be disadvantageous, because sample losses were observed due to the high vapor pressure of the ethanol proportion in the extraction buffer.

Different conditions of tryptic digestion (especially various concentrations of trypsin, DTT and IAA as well as different digestion times) and SPE were tested (data not shown). The optimal parameters are described in Section 2.7.3.

3.3. Detection of marker peptides for barley, maize, oats, rice, rye and wheat in emulsion-type sausages

Three to four marker peptides were determined for each grain species (see Table 2) for the unambiguous detection of grain proteins in meat products, even in low concentrations (mg/kg range). Seven of these marker peptides are known to be potentially modified: Five peptides (barley3, maize3, rice2, rye2 and wheat2) contain methionine (susceptible to oxidation), maize4 contains N-terminal glutamine (formation of pyroglutamate) and oat1 contains N-terminal cysteine (formation of pyrolysine). In order to check the proportions of the peak areas of the modified peptides compared to the peak areas of the corresponding unmodified peptides, sausages containing 0.01%, 0.05% and 0.1% grain protein (N = 23) were analyzed. The proportions of the modified peptides (mean value ± standard deviation) were as follows (unmodified peptide = 100%): barley3(M-ox) (9 ± 2%), maize3(M-ox) (15 ± 4%), maize4(pyro-Q) (16 ± 3%), rice2(M-ox) (14 ± 4%), rye2(M-ox) (11 ± 1%) and wheat2(M-ox) (6 ± 1%), whereas the modified peptide oat1(pyro-C) was not detected. The relatively small proportions of the modified peptides compared to the unmodified form (all of them < 20%) and the low standard deviations (all of them ≤ 4 percentage points) confirm the suitability of the selected grain marker peptides susceptible to modifications, especially since different conditions of processing were included in the mentioned experiments.

Three preferably intensive mass transitions were necessary for each

marker peptide to provide a reliable detection, each of which must have a signal-to-noise ratio (S/N) equal to or greater than 3, according to the commonly used definition of the LOD. The ratios between the peak areas of the three selected MRM transitions can be used as an additional meaningful quality feature for the reliable detection of a marker peptide. These ratios should be fairly constant (deviation ≤ 20%) for different concentration levels, using the values for the highest concentration of grain protein as a reference (criterion 1), and show no high standard deviation (RSD < 20%) by measuring several samples within the same concentration level (criterion 2). The ratios of the mass transitions (always in relation to the most intensive mass transition) and the corresponding relative standard deviations (RSDs) were calculated for the meat products analyzed containing different concentrations of grain protein (in total N = 78; see Table 3).

A review of criterion 1 showed no deviations higher than 20% at the concentration level of 0.05% grain protein. At the concentration levels of 0.01 and 0.005%, deviations higher than 20% were observed for maize1 (b2/y5), rice2 (y7/y12²⁺) and wheat1 (b3/y7), and at 0.005% only for wheat1 (y5/y7) (grey marking of the left numerical series in Table 3). In addition to the mass transitions of peptides mentioned above, barley4 (y7/y2 and y7²⁺/y2), maize1 (b3/y5) and rice3 (y2/b3) also showed a higher deviation than 20% at a concentration level of 0.001% (10 mg/kg) grain protein of each type of cereal. This also applied to rye2 (y6/y4), with a marginal higher deviation than 20%, however the deviation at 5 mg/kg rye protein was acceptable.

Regarding criterion 2, the RSDs of the ratios of all marker peptides were below 10% in emulsion-type sausages containing 0.1 and 0.05% protein of each of the six types of grain. For concentrations of 0.01 and 0.005%, three ratios of the MRM transitions (maize1 (b2/y5), rice2 (y7/y12²⁺), only at the concentration level of 0.01%, and wheat1 (b3/y7)) showed higher RSDs than 10%, which were still well below 20% and, therefore, considered acceptable, with the exception of wheat1 (b3/y7) at the concentration level of 0.005% (grey marking of the right numerical series in Table 3). The ratios of the mass transitions still showed acceptable RSDs (below 20%) even in sausages with 0.001% protein of each of the six types of grain, with the exception of maize1 (both ratios), rice2 (y7/y12²⁺), rice3 (y2/b3) and wheat1 (b3/y7).

Consequently, three marker peptides for each of the six types of grain analyzed, with the exception of rice (two marker peptides), fulfilled both criteria for samples with 0.001% (10 mg/kg) grain protein of

Table 3

Ratios of the peak areas of the mass transitions and corresponding RSDs (in brackets) of the different marker peptides for barley, maize, oats, rice, rye and wheat at varying concentrations of grain protein in emulsion-type sausages (grey marking of the left numerical series = ratio differs by more than 20% from the ratio observed for the highest concentration of grain protein; grey marking of the right numerical series = standard deviation $\geq 20\%$; marker peptides in bold allow the detection at a level of 10 mg/kg or even lower).

Marker Peptide (ratio of mass transition)	Concentration of grain protein (number of samples)					
	0.0005% (7)	0.001% (19)	0.005% (7)	0.01% (19)	0.05% (7)	0.1% (19)
Barley1 (y8/b2)	0.56 (55%)	0.34 (13%)	0.35 (5%)	0.38 (3%)	0.40 (3%)	0.39 (3%)
(y8 ²⁺ /b2)	0.70 (52%)	0.38 (17%)	0.36 (4%)	0.38 (4%)	0.37 (3%)	0.37 (2%)
Barley2 (y5/y2)	0.36 (3%)	0.34 (7%)	0.36 (4%)	0.35 (3%)	0.37 (3%)	0.37 (6%)
(y7/y2)	0.33 (5%)	0.32 (6%)	0.34 (4%)	0.33 (4%)	0.36 (6%)	0.35 (7%)
Barley3 (y4/y12 ²⁺)	0.39 (7%)	0.38 (3%)	0.39 (3%)	0.38 (2%)	0.39 (3%)	0.38 (2%)
(y6/y12 ²⁺)	0.12 (6%)	0.11 (6%)	0.11 (6%)	0.11 (5%)	0.11 (7%)	0.11 (5%)
Barley4* (y7/y2)	---	0.27 (5%)	---	0.38 (2%)	---	0.40 (1%)
(y7 ²⁺ /y2)	---	1.38 (4%)	---	1.97 (2%)	---	2.07 (1%)
Maize1 (b2/y5)	4.16 (88%)	2.20 (20%)	0.91 (13%)	0.78 (17%)	0.56 (7%)	0.55 (5%)
(b3/y5)	2.26 (94%)	1.41 (21%)	0.97 (8%)	0.95 (6%)	0.84 (2%)	0.83 (2%)
Maize2 (b10/y4)	0.27 (15%)	0.26 (13%)	0.26 (9%)	0.25 (6%)	0.26 (8%)	0.24 (7%)
(b6/y4)	0.24 (46%)	0.26 (19%)	0.26 (8%)	0.26 (4%)	0.26 (5%)	0.26 (4%)
Maize3 (y6/y5)	0.63 (19%)	0.62 (14%)	0.68 (5%)	0.68 (2%)	0.70 (1%)	0.69 (2%)
(y7/y5)	0.45 (21%)	0.44 (13%)	0.42 (3%)	0.41 (5%)	0.41 (5%)	0.40 (5%)
Maize4 (b7/b8)	0.22 (26%)	0.22 (14%)	0.20 (6%)	0.20 (4%)	0.19 (3%)	0.19 (3%)
(y2/b8)	0.33 (5%)	0.38 (14%)	0.45 (4%)	0.46 (2%)	0.47 (3%)	0.47 (2%)
Oat1 (y4/y6)	0.58 (12%)	0.67 (8%)	0.72 (2%)	0.73 (2%)	0.73 (1%)	0.73 (1%)
(y7/y6)	0.20 (9%)	0.21 (15%)	0.18 (6%)	0.18 (3%)	0.18 (2%)	0.18 (1%)
Oat2 (y6/y10 ²⁺)	0.11 (8%)	0.12 (6%)	0.12 (1%)	0.12 (2%)	0.12 (2%)	0.12 (1%)
(y8/y10 ²⁺)	0.13 (9%)	0.12 (5%)	0.14 (4%)	0.13 (4%)	0.13 (5%)	0.13 (4%)
Oat3 (y3/y7 ²⁺)	0.32 (15%)	0.33 (7%)	0.30 (2%)	0.30 (1%)	0.30 (1%)	0.30 (1%)
(y7/y7 ²⁺)	0.13 (6%)	0.13 (4%)	0.13 (2%)	0.13 (2%)	0.13 (2%)	0.12 (1%)
Rice1 (y3/b2)	0.39 (9%)	0.35 (5%)	0.34 (4%)	0.33 (4%)	0.34 (3%)	0.32 (4%)
(y6/b2)	0.72 (7%)	0.69 (6%)	0.70 (4%)	0.68 (4%)	0.70 (4%)	0.68 (4%)
Rice2 (y12 ³⁺ /y12 ²⁺)	0.37 (4%)	0.40 (10%)	0.37 (5%)	0.37 (3%)	0.37 (4%)	0.36 (3%)
(y7/y12 ²⁺)	0.45 (55%)	0.72 (68%)	0.32 (7%)	0.32 (15%)	0.27 (8%)	0.26 (5%)
Rice3 (y2/b3)	0.05 (245%)	0.32 (41%)	0.44 (2%)	0.44 (5%)	0.45 (3%)	0.44 (2%)
(y8/b3)	0.62 (8%)	0.70 (10%)	0.75 (5%)	0.75 (4%)	0.76 (5%)	0.75 (3%)
Rice4 (y6/y8 ²⁺)	0.34 (17%)	0.29 (14%)	0.26 (5%)	0.26 (3%)	0.26 (2%)	0.27 (1%)
(y8/y8 ²⁺)	0.46 (19%)	0.49 (7%)	0.47 (3%)	0.47 (3%)	0.47 (3%)	0.47 (2%)
Rye1 (b10/y3)	0.19 (5%)	0.19 (9%)	0.19 (4%)	0.19 (3%)	0.19 (2%)	0.19 (2%)
(y5/y3)	0.21 (14%)	0.20 (12%)	0.18 (4%)	0.17 (2%)	0.18 (2%)	0.18 (2%)
Rye2 (y10 ²⁺ /y4)	0.68 (12%)	0.70 (7%)	0.70 (1%)	0.70 (1%)	0.70 (2%)	0.70 (1%)
(y6/y4)	0.18 (16%)	0.20 (13%)	0.18 (4%)	0.18 (3%)	0.17 (1%)	0.16 (2%)
Rye3 (y5/a2)	0.33 (13%)	0.36 (10%)	0.33 (7%)	0.34 (5%)	0.32 (5%)	0.33 (3%)
(y8/a2)	0.29 (10%)	0.31 (7%)	0.33 (2%)	0.34 (3%)	0.34 (2%)	0.34 (2%)
Wheat1 (b3/y7)	1.65 (41%)	1.96 (29%)	1.25 (39%)	1.24 (14%)	0.96 (6%)	0.95 (4%)
(y5/y7)	0.19 (53%)	0.30 (17%)	0.53 (7%)	0.69 (5%)	0.80 (2%)	0.83 (1%)
Wheat2 (y7/y6)	0.19 (8%)	0.18 (8%)	0.18 (2%)	0.18 (2%)	0.18 (1%)	0.18 (2%)
(y8/y6)	0.25 (5%)	0.26 (5%)	0.28 (2%)	0.27 (2%)	0.27 (1%)	0.27 (1%)
Wheat3 (b10/y6)	1.08 (7%)	0.90 (7%)	0.83 (2%)	0.80 (2%)	0.81 (2%)	0.80 (2%)
(b7/y6)	0.60 (3%)	0.59 (4%)	0.59 (1%)	0.58 (2%)	0.59 (1%)	0.58 (1%)
Wheat4 (y5/y8)	0.95 (5%)	0.94 (5%)	0.91 (3%)	0.92 (2%)	0.91 (3%)	0.92 (2%)
(y7/y8)	0.73 (5%)	0.75 (3%)	0.74 (3%)	0.74 (2%)	0.73 (2%)	0.74 (1%)

* Barley4 was only detectable in sausages of production series 1 (N=9, each)

each type of cereal. This means that these marker peptides (Table 3, marked in bold) allow the detection of the six types of grain analyzed at a protein level of 10 mg/kg each in emulsion-type sausages, some of them even at a level of 5 mg/kg. The marker peptide barley4 was only detectable in samples containing the barley flour used to produce sausages from series 1. This shows that marker peptides may be dependent on grain variety and, consequently, the need to use several marker peptides for each grain species.

Emulsion-type sausages from production series 1 were defatted to determine the repeatability of the analytical method developed.

Separate sample preparations were performed starting with the defatted materials (0.1, 0.01 and 0.001% grain protein of each type of cereal) for nine samples of each concentration level. Thereby, three samples of each concentration level were prepared and measured simultaneously. This procedure was performed three times in three different weeks. The resulting RSDs (N = 9) for the 22 marker peptides ranged between 3 and 13% (mean: 7%) for samples with 0.1% grain protein, between 3 and 18% (mean: 7%) for samples with 0.01% grain protein and between 4 and 19% (mean: 8%) for sausages with 0.001% grain protein. Based on the measurements of emulsion-type sausages

from production series 1 (nine measurements for each of the four concentrations; see Table 1), the correlations between the mean peak areas of the marker peptides (most intense mass transition, each) and the concentrations of grain proteins in the emulsion-type sausages were calculated. The coefficients of determination (R^2) ranged between 0.978 (rice1) and 0.997 (barley2).

It was possible to check the performance of each individual sample preparation (after protein extraction) and HPLC-MS/MS measurement with the help of selected isotope-labelled internal peptide standards (see Table 2), which were added to the samples after protein extraction (see Section 2.7.2). The RSDs of the peak areas of the internal standards ($N = 97$) ranged between 9 (ISTD for rice) and 16% (ISTD for rye), showing a sufficient and constant sensitivity of the HPLC-MS/MS measurements during the entire investigation period.

3.4. Influence of the storage, grilling and canning process of emulsion-type sausages on the detectability of the grain marker peptides

Emulsion-type sausages in casings from production series 2 were stored for six weeks at 2 °C to check the influence of storage on the detectability of the grain marker peptides. It was shown that the peak areas of the marker peptides in samples before and after storage showed only minor differences (see Supplementary data Fig. S3). The mean increases and decreases of the peak areas were in the range of 20% (ratio between 0.8 and 1.2). An extreme value was observed in the case of the marker peptide oat1, showing a decrease of the peak area after storage of about 40%. On the other hand, increases of the peak areas after storage were observed up to 40% for barley3, rice2 and wheat1. The maximum increases were even higher than 50% for barley2, maize2 and maize4.

In a second experiment, the sausages were grilled (after removal of the casing) to investigate the influence of this heat treatment on the detectability of the different marker peptides of the six types of grain analyzed (see Fig. 2). The grilling of the emulsion-type sausages resulted in an increase of the peak areas of all marker peptides for oats (an average of 11–74%) and rice (an average of 33–133%). The peak areas in grilled samples for two rice marker peptides (rice1 and rice3) were more than twice as high compared to unheated samples. Only minor differences between the peak areas in untreated and grilled samples were observed for the marker peptides of barley (an average of −13 to +9%), maize (an average of −6 to +14%), rye (an average of −4 to +3%) and wheat (an average of −9 to +17%). This shows that grilling

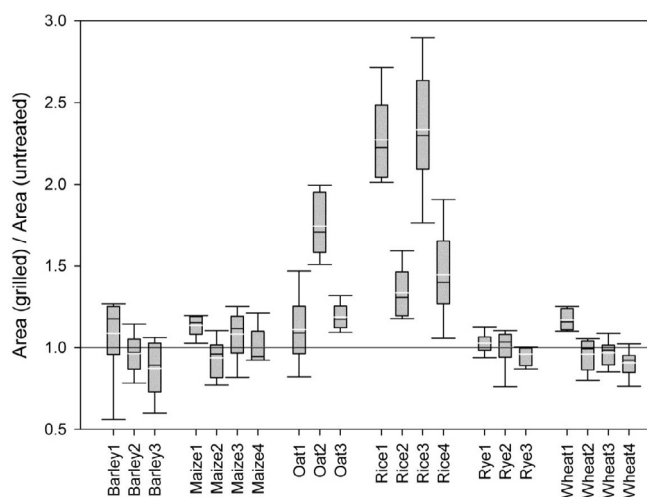


Fig. 2. Box Plots of the ratios of the marker peptide peak areas (most intense mass transition, each) of barley, maize, oats, rice, rye and wheat in grilled and untreated emulsion-type sausages ($N = 6$, six concentrations; black line in box: median; white line in box: mean; see Table 1, production series 2).

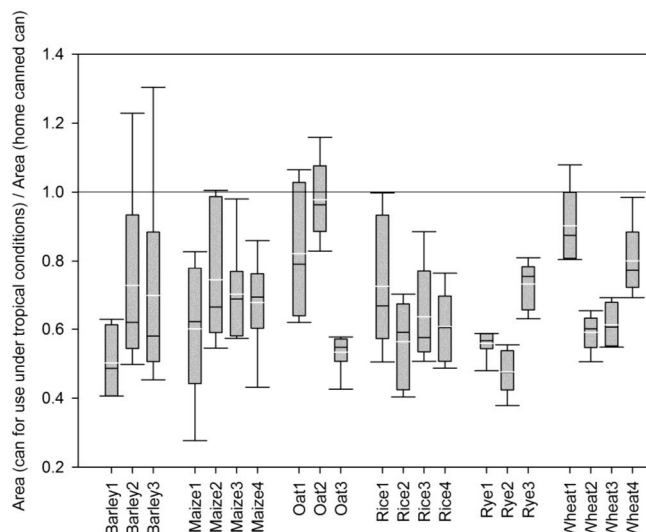


Fig. 3. Box Plots of the ratios of the marker peptide peak areas (most intense mass transition, each) of barley, maize, oats, rice, rye and wheat in cans for use under tropical conditions and in home canned products of emulsion-type sausages ($N = 6$, six concentrations; for barley1: $N = 4$, four concentrations; see Table 1, production series 2).

did not affect the detectability of the marker peptides adversely, but rather improved it. Increases in peak areas of grain marker peptides after grilling are possibly caused by a better extractability of the target proteins due to protein denaturation and/or a more efficient protease cleavage due to protein unfolding.

Cans for use under tropical conditions showed lower peak areas for all marker peptides compared to home-canned products (Fig. 3). The highest mean decrease was observed for the marker peptide rye2 (52%) and the lowest mean decrease was observed for oat2 (2%). Considering all marker peptides of a grain species, the highest mean decrease was observed for rye (41%) and the lowest for oats (22%). The lowest RSDs were observed for rye1 (7%) and the highest for barley3 (45%). Whereas no or only slight decreases of the detectability of the grain marker peptides were observed after storage and grilling of emulsion-type sausages, noticeably higher decreases were observed for manufacturing cans for use under tropical conditions in relation to home canned products. Reasons for the decrease of the peak areas of marker peptides in cans for use under tropical conditions might be the higher extent of thermal processing (a temperature of about 120 °C for more than 30 min) compared to home canned products (temperature of at most 100 °C). A temperature of 120 °C may lead to a degradation of amino acids (especially cysteine, lysine and methionine) in proteins (Sielaß, 1996). Furthermore, at higher temperatures (especially in the presence of carbohydrates being abundant in grain flours), target proteins can form advanced glycation end products (AGEs) or insoluble aggregates (Davis, Smales, & James, 2001). But also a suppression by the meat matrix might be a reason for the decreased peak areas of grain marker peptides in samples of cans for use under tropical conditions since the measurement of three peptides (IEDEQALAMQLQK, VQLLH-TQNTSLINTK, and DLEEATLQHEATAAALR) originating from the main muscle protein myosin showed noticeably higher peak areas (in mean about a factor of 3 to 6; $N = 7$, each) for samples of cans for use under tropical conditions compared to home canned products.

4. Conclusions

The HPLC-MS/MS method developed enables the simultaneous mass spectrometric detection of the six most important grain species in meat products. The method is suitable to uncover, for example, economically motivated adulterations by substituting high quality meat

proteins with lower cost grain proteins and to detect gluten and other grain proteins in the low mg/kg range. Three marker peptides for each grain species within the group of cereals containing gluten were detectable in sausages – also fulfilling the quality criteria mentioned in Section 3.3 – at protein concentrations of ≤ 5 mg/kg for oats and rye and ≤ 10 mg/kg for barley and wheat. As a consequence, the determined LOD for wheat (≤ 1 mg/100 g meat product) is at or just below the level of the reference dose of 1 mg wheat protein (Taylor et al., 2014), if the above mentioned portions (100 g) are consumed. Considering the different percentages of gluten to the total content of cereal protein (barley: 55%, oats: < 16%, rye: 43%, wheat: 79%) (Schalk et al., 2017), the following LODs related to gluten resulted: barley: 6 mg/kg, oats: < 1 mg/kg, rye: 2 mg/kg and wheat: 8 mg/kg. The protein extraction was performed using a novel extraction buffer (TRIS-HCl (pH 8.2)/ethanol (1:1) at 90 °C), thus extraction temperatures were used which were noticeably higher than the temperature of 60 °C most commonly used for the mass spectrometric detection of allergens in foods (Nitride et al., 2018). The three to four marker peptides used for each grain species originated from at least two different target proteins ensuring a higher robustness against influences of processing. The effects of processing on the detectability of the marker peptides proven in this study are particularly important regarding a further development to a quantification method. The possibility of quantifying allergens in food by HPLC-MS/MS was reported previously (New, Schreiber, Stahl-Zeng, & Liu, 2017). In this context, more detailed investigations concerning the influence of different grain varieties or grain protein sources (e.g. bran or germ) and different types of processing of the meat products have to be considered. Furthermore, it would also be relevant to check the detectability of grain marker peptides in raw sausages during storage due to the activity of proteolytic enzymes during the maturing process (Petrova, Aasen, Rustad, & Eikevik, 2015) and, consequently, a possible enzymatic degradation of the target proteins. An extension of the method developed to important millet species (*Panicum milliaceum*, *Sorghum bicolor*) showing high annual production rates (28 million tonnes; FAO, 2014) as well as to other relevant non-meat proteins such as soy and milk is also desirable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.09.041>.

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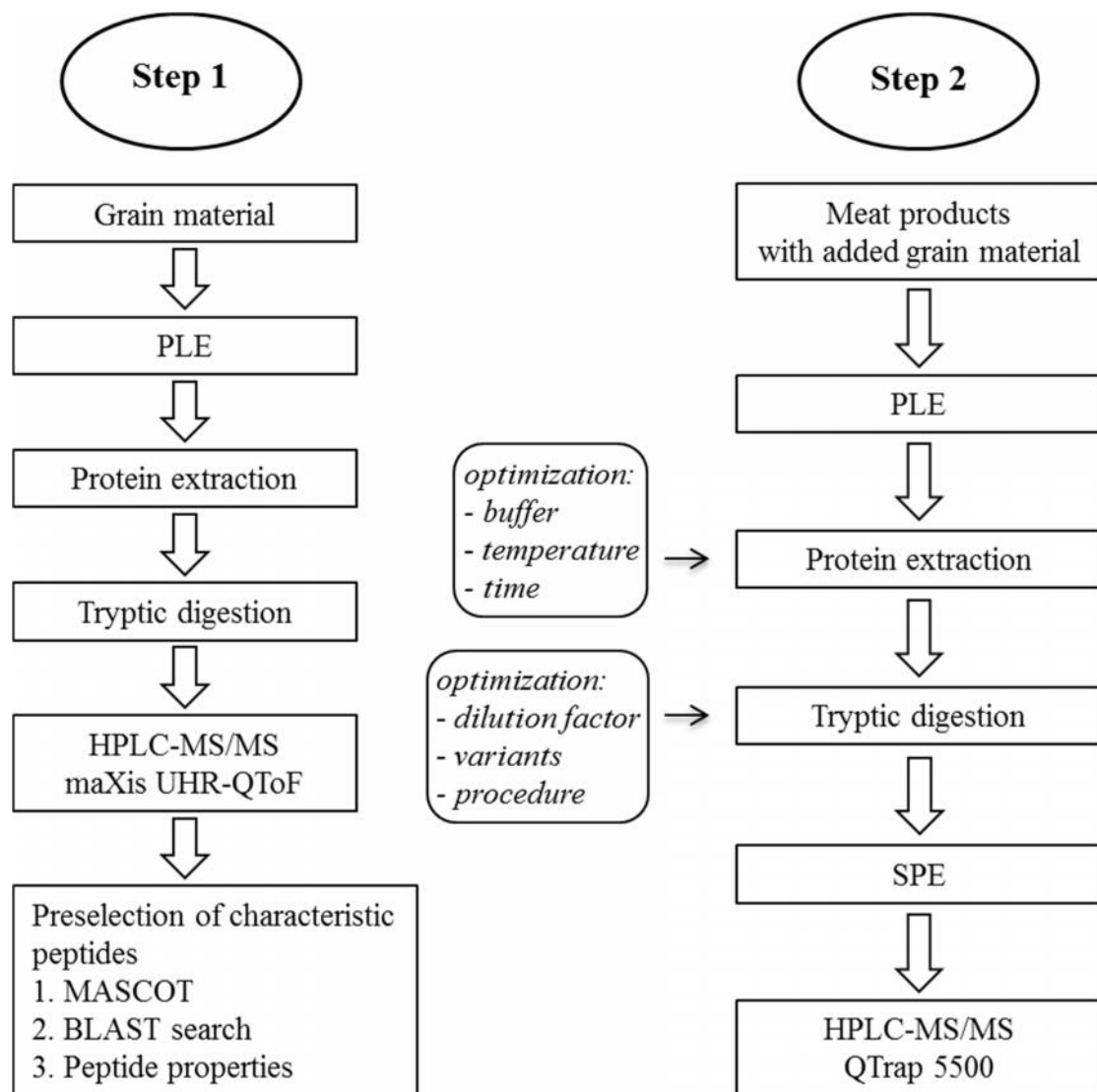


Figure S1: Experimental workflow for the preselection of characteristic peptides for barley, maize, oats, rice, rye and wheat in grain material (maXis UHR-QToF; step 1) and the subsequent selection of suitable marker peptides and optimization of the method in meat products (QTrap 5500; step 2) (according to Hoffmann et al., 2017)

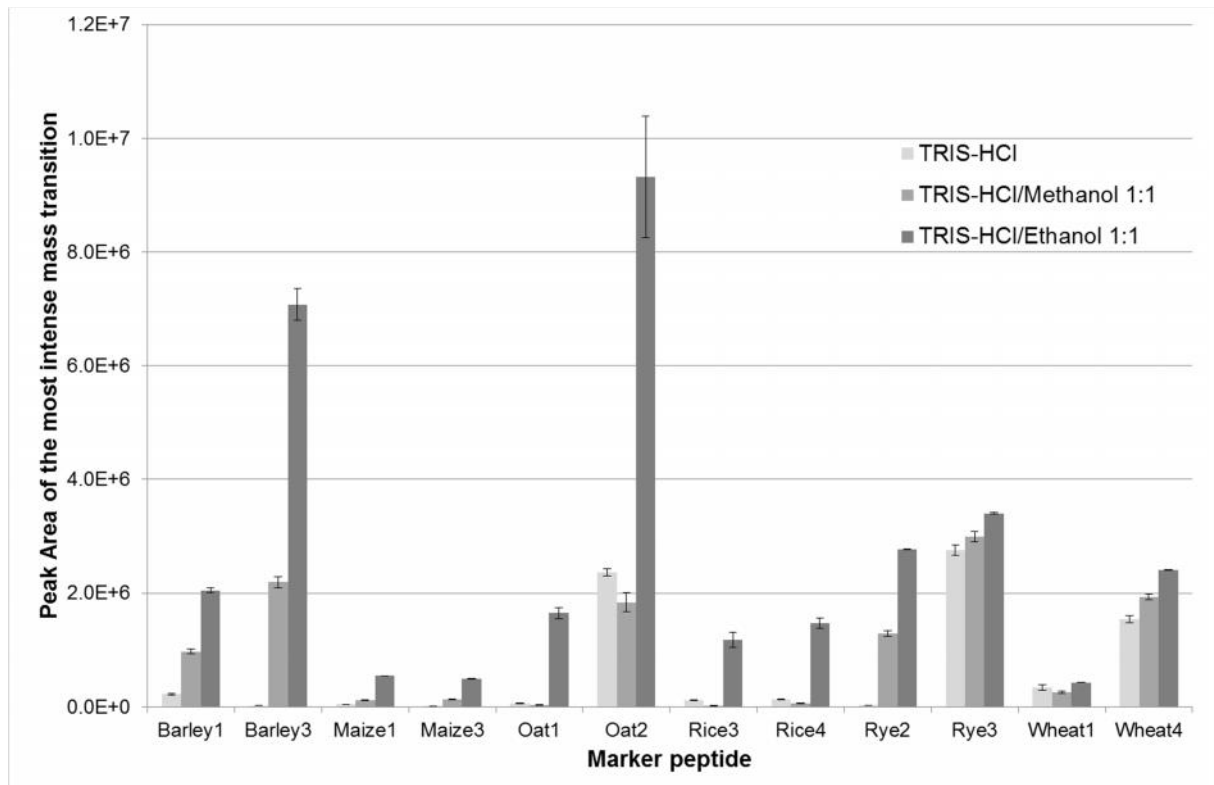


Figure S2. Peak areas with standard deviations ($N = 3$) of the two least sensitive marker peptides for barley, maize, oats, rice, rye and wheat applying different extraction buffers (90 °C) in emulsion-type sausages with 0.1 % grain protein (batch 1-A; QTrap 5500)

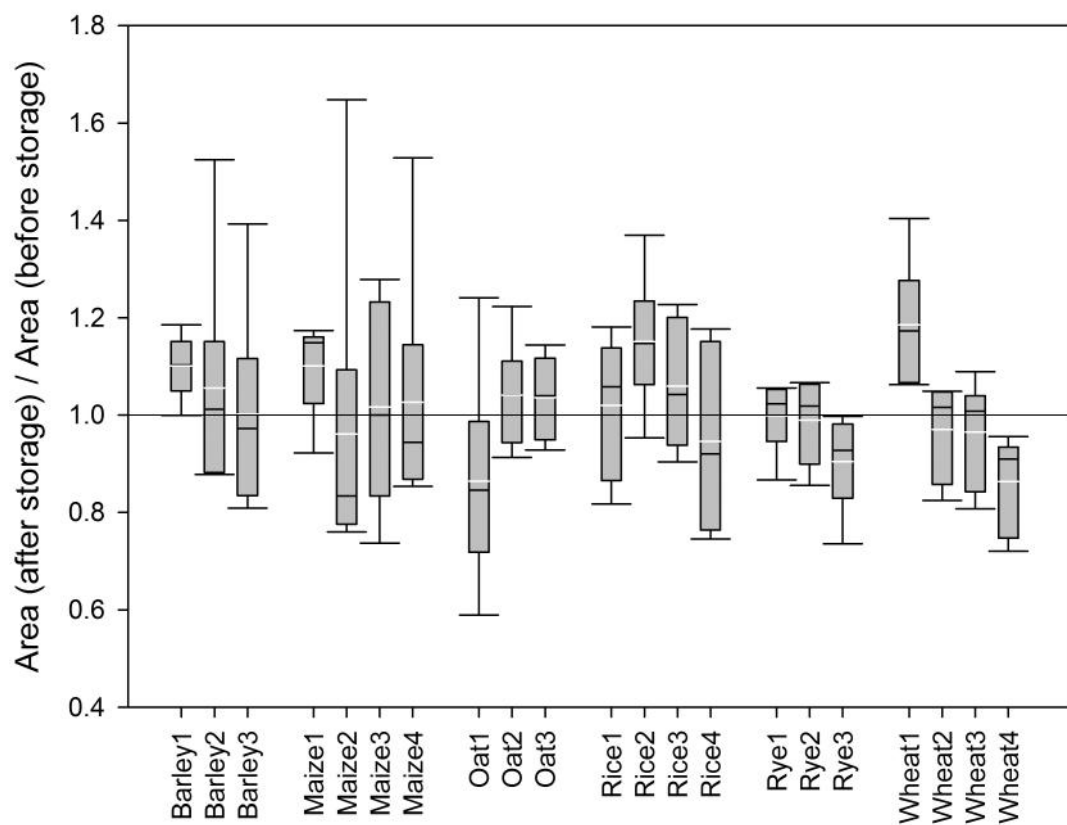


Figure S3: Box Plots of the ratios of the marker peptide peak areas (most intense mass transition, each) of barley, maize, oat, rice, rye and wheat in emulsion-type sausages in casings after storage for six weeks at 2 °C and before storage (N = 6, six concentrations; black line in box: median; white line in box: mean; see Table 1, production series 2)

Table S1

Marker peptides for barley, maize, oats, rice, rye and wheat and their homologies (NCBI)

Marker	Peptide sequence	Protein (NCBI)	Homologies (NCBI)
Barley1	IETPGPPYLAK	Alpha-amylase/trypsin inhibitor (P32936.2)	<i>Secale cereale</i>
Barley2	IVPLAIDTR	Hordein (AAA32967.1)	---
Barley3	TLPTMCSVNVPLYR	Hordein (AAA32967.1)	<i>Thinopyrum intermedium</i> , <i>Triticum aestivum</i> , <i>Aegilops tauschii</i> x <i>Triticum turgidum</i>
Barley4	ILPFGIDTR	Hordein (AAZ76368.1)	---
Maize1	LEDLPGCPR	Hageman Factor Inhibitor (ABA34136.1)	<i>Eleusine coracana</i> , <i>Oryza sativa</i> , <i>Sorghum bicolor</i>
Maize2	TNPCGVSAAIIPPYY	Zein (AAA33543.1)	---
Maize3	FSLMNPVLSR	Zein (AAA33533.1)	---
Maize4	QQPIVGGAIF	Zein (CAA39257.1)	<i>Sorghum bicolor</i> , <i>Saccharum officinarum</i>
Oat1	CTGVSVIR	11S Globulin (CAA52763.1)	<i>Brachypodium distachyon</i>
Oat2	ALPVDVLANAYR	12S Globulin (CAA54153.1)	---
Oat3	DFPITWPWK	Avenin (P27919.1)	---
Rice1	LQAFEPPIR	Glutelin (AAA33906.1)	<i>Oryza brachyantha</i> , <i>Zizania latifolia</i>
Rice2	ELGAPDVGHMSEVFR	Seed allergenic protein RA5 (Q01881.2)	---
Rice3	VIQPQGLLVPR	Glutelin (CAA38212.1)	<i>Oryza brachyantha</i>
Rice4	VIEPQGLLVPR	Glutelin (BAC77349.1)	<i>Oryza brachyantha</i>
Rye1	TPFASTVAGIGGQ	Gamma-Prolamin (AEW46799.1)	<i>Triticum aestivum</i> , <i>Aegilops uniaristata</i>
Rye2	SLVLQNLPTMCNVYVPR	75k Gamma-Secalin (ADP95479.1)	<i>Triticum aestivum</i>
Rye3	ILWGIPTLLK	HMW Glutenin (ADC79689.1)	<i>Triticum aestivum</i>
Wheat1	SVAVSQVAR	HMW Glutenin (AKP95633.1)	<i>Aegilops</i> spp., <i>Thinopyrum</i> spp., <i>Triticum</i> spp.
Wheat2	VFLQQQCSPVAMPQSLAR	LMW Glutenin (AFU48612.1)	<i>Aegilops</i> spp., <i>Thinopyrum</i> spp., <i>Triticum</i> spp.
Wheat3	APFASIVAGIGGQ	Gamma-Gliadin (AGZ20273.1)	<i>Aegilops</i> spp., <i>Thinopyrum</i> spp., <i>Triticum</i> spp., <i>Taeniatherum caput-medusae</i> , <i>Australopyrum retrofractum</i> , <i>Psathyrostachys juncea</i>
Wheat4	IFWGIPALLK	HMW Glutenin (BAG12019.2)	<i>Aegilops</i> spp., <i>Thinopyrum</i> spp., <i>Triticum</i> spp., <i>Secale cereale</i> x <i>Triticum aestivum</i>

HMW = high molecular weight; LMW = low molecular weight

Table S2

Occurrence of the marker peptides rye1, rye2, rye3 and barley3 in different commercial wheat cultivars (N = 18)

Origin of samples: University of Hohenheim, State Plant Breeding Institute, Stuttgart, Germany (a)

Bavarian State Research Center for Agriculture (LfL), Freising, Germany (b)

Julius Kühn-Institut, Groß Lüsewitz, Germany (c)

Cultivar	Rye1	Rye2	Rye3	Barley3
Akteur ^a	---	---	---	---
Altigo ^a	---	---	---	---
Axioma ^b	---	---	---	---
Exquisit ^c	---	---	---	---
Faustus ^b	---	---	---	---
Glaucus ^a	---	---	---	---
Julius ^a	---	---	---	---
Kamerad ^b	+	---	---	---
Kerubino ^b	---	---	---	---
Lear ^a	---	---	---	---
Patras ^b	---	---	---	---
Pionier ^a	---	---	---	---
Ponticus ^b	---	---	---	---
Spontan ^b	---	---	---	---
Tengri ^a	---	---	---	---
Thasos ^c	---	---	---	---
Tobak ^a	---	---	---	---
Xerxes ^a	---	---	---	---

Table S3

A) Mean peak areas (N = 2, each) of barley marker peptides (most intense mass transitions; expressed as a percentage of the average of all barley cultivars) in different commercial varieties of barley (winter) and relative standard deviation (RSD, %) (N = 6)

Origin of samples: Bavarian State Research Center for Agriculture (LfL), Freising, Germany

Cultivar	Barley1	Barley2	Barley3	Barley4
California	94.2	117.2	105.4	110.3
Hedwig	93.9	63.9	68.0	71.1
KWS Meridian	100.8	110.6	100.4	97.7
Sandra	84.1	100.1	116.1	121.3
Su Ellen	90.0	85.9	83.5	79.2
SY Tepee	136.9	122.2	126.6	120.3
Mean	100.0	100.0	100.0	100.0
RSD	18.9	21.9	21.4	21.2

B) Mean peak areas (N = 2, each) of maize marker peptides (most intense mass transitions; expressed as a percentage of the average of all maize cultivars) in different commercial varieties of maize and relative standard deviation (RSD, %) (N = 7)

Origin of samples: Deutsche Saatveredelung AG, Lippstadt, Germany

Cultivar	Maize1	Maize2	Maize3	Maize4
Cathy	95.6	101.0	102.3	87.5
Danubo	88.1	72.8	51.8	129.8
Davas	122.4	115.7	91.4	53.8
Joy öko	67.8	98.5	32.8	62.5
Korynt	101.9	90.2	154.8	158.8
Messago	87.8	77.6	148.1	112.1
Ridley	136.4	144.1	118.7	95.5
Mean	100.0	100.0	100.0	100.0
RSD	23.0	24.3	45.8	37.0

C) Mean peak areas (N = 2, each) of oat marker peptides (most intense mass transitions; expressed as a percentage of the average of all oat cultivars) in different commercial varieties of oats and relative standard deviation (RSD, %) (N = 9)

Origin of samples: Nordsaat Saatzzucht GmbH, Langenstein, Germany

Cultivar	Oat1	Oat2	Oat3
Apollon	92.3	86.9	95.5
Bison	113.0	97.7	95.0
Canyon	96.5	68.9	104.9
Delfin	102.3	81.4	98.8
Harmony	87.2	75.0	93.3
Ivory	97.2	88.4	112.6
Poseidon	81.4	87.5	116.7
Scorpion	85.7	84.1	104.7
Yukon	144.3	230.0	78.5
Mean	100.0	100.0	100.0
RSD	19.1	49.4	11.4

D) Mean peak areas (N = 2; for black rice: N = 1) of rice marker peptides (most intense mass transitions; expressed as a percentage of the average of all rice cultivars) in different commercial varieties of rice and relative standard deviation (RSD, %) (N = 9)

Origin of samples: Reishunger GmbH, Bremen, Germany

Cultivar	Rice1	Rice2	Rice3	Rice4
Basmati	127.1	115.6	129.4	67.5
Black	47.5	53.5	76.5	67.5
Jasmin	113.0	96.3	92.1	82.0
Milk	118.4	110.9	97.6	102.5
Paella	113.5	159.7	99.5	162.0
Red	39.3	42.0	70.4	76.3
Risotto	83.8	137.4	117.7	133.9
Sticky	183.6	87.1	124.1	112.2
Sushi	73.7	97.7	92.7	96.2
Mean	100.0	100.0	100.0	100.0
RSD	44.5	37.1	20.3	31.9

E) Mean peak areas (N = 2, each) of rye marker peptides (most intense mass transitions; expressed as a percentage of the average of all rye cultivars) in different commercial varieties of rye (winter) and relative standard deviation (RSD, %) (N = 7)

Origin of samples: KWS Lochow GmbH, Bergen, Germany

Cultivar	Rye1	Rye2	Rye3
Brasetto	88.2	104.2	120.5
KWS Daniello	118.8	80.0	95.6
KWS Eterno	83.0	81.7	99.1
KWS H51760	107.2	113.9	98.3
KWS H51810	100.8	92.4	80.7
KWS Serafino	115.3	112.1	98.7
KWS Trebiano	86.6	115.7	107.2
Mean	100.0	100.0	100.0
RSD	14.4	15.2	12.0

F) Mean peak areas (N = 2, each) of wheat marker peptides (most intense mass transitions; expressed as a percentage of the average of all wheat cultivars) in different commercial varieties of wheat (winter) and relative standard deviation (RSD, %) (N = 16)

Origin of samples: University of Hohenheim, State Plant Breeding Institute, Stuttgart, Germany (a)
Bavarian State Research Center for Agriculture (LfL), Freising, Germany (b)

Cultivar	Wheat1	Wheat2	Wheat3	Wheat4
Akteur ^a	98.0	101.6	118.2	84.8
Altigo ^a	97.0	119.1	110.7	112.5
Axioma ^b	140.3	147.6	135.9	154.1
Faustus ^b	85.0	102.1	90.9	98.1
Glaucus ^a	68.7	94.4	97.3	68.6
Julius ^a	98.2	103.7	95.9	108.2
Kamerad ^b	85.8	23.5	52.4	84.8
Kerubino ^b	114.7	129.8	120.5	130.1
Lear ^a	82.5	99.2	78.1	86.0
Patras ^b	91.4	85.1	82.2	73.4
Pionier ^a	102.3	102.0	98.0	105.1
Ponticus ^b	88.3	87.3	90.5	84.8
Spontan ^b	83.7	81.5	87.7	75.0
Tengri ^a	171.3	133.5	148.2	152.5
Tobak ^a	95.2	106.2	97.6	103.3
Xerxes ^a	97.6	83.5	95.8	78.7
Mean	100.0	100.0	100.0	100.0
RSD	24.7	27.8	23.0	26.5

Table S4: Groups of possible ingredients for the production of sausages and commercial spice mixtures, which were tested regarding cross-reactivity with the grain marker peptides analyzed

Possible ingredients

Group 1

cauliflower (*Brassica oleracea* var. *botrytis*)
 brussel sprouts (*Brassica oleracea* var. *gemmifera*)
 kohlrabi (*Brassica oleracea* var. *gongylodes*)
 broccoli (*Brassica oleracea* var. *italica* Plenck)
 savoy cabbage (*Brassica oleracea* convar. *capitata* var. *sabauda*)
 red cabbage (*Brassica oleracea* convar. *capitata* var. *rubra*)

Group 2

swede (*Brassica napus* subsp. *rapifera*)
 rape (*Brassica napus*)
 chinese cabbage (*Brassica rapa* subsp. *pekinensis*)
 onion (*Allium cepa*)
 spring onion (*Allium fistulosum*)
 wild garlic (*Allium ursinum*)
 leek (*Allium ampeloprasum*)

Group 3

cashew (*Anacardium occidentale*)
 macadamia (*Macadamia spp.*)
 brazil nut (*Bertholletia excelsa*)
 pistachio (*Pistacia vera*)
 peanut (*Arachis hypogaea*)
 hazelnut (*Corylus avellana*)
 almond (*Prunus dulcis*)

Group 4

horseradish (*Armoracia rusticana*)
 radish (*Raphanus spp.*)
 celery (*Apium graveolens*)
 carrot (*Daucus carota*)
 beetroot (*Beta vulgaris*)
 small radish (*Raphanus sativus* var. *sativus*)

Group 5

bean (*Phaseolus vulgaris*)
 soy (*Glycine maxima*)
 pea (*Pisum sativum*)
 lupin (*Lupinus spp.*)
 guar gum (*Cyamopsis tetragonoloba*)

Group 6

cucumber (*Cucumis sativus*)
 pumpkin (*Curcubita spp.*)
 tomato (*Lycopersicon esculentum*)
 zucchini (*Curcubita pepo*)
 paprika (*Capsicum annuum*)
 champignon (*Agaricus spp.*)
 chili (*Capsicum spp.*)

Group 7

parsley (*Petroselinum crispum*)
cress
fennel (*Foeniculum vulgare*)
ginger (*Zingiber officinale*)
asparagus (*Asparagus officinalis*)
marjoram (*Origanum majorana*)
mace (*Myristica fragrans*)

Group 8

white mustard (*Sinapis alba*)
brown mustard (*Brassica juncea*)
black mustard (*Brassica nigra*)

Group 9

yeast (*Saccharomyces cerevisiae*)

Commercial spice mixtures for the use in...

Emulsion-type sausages (Lyoner)
Liver sausage
Frankfurter-type sausages
Bratwurst
Bierschinken
Curry (for various sausages)
Brat/Weißwurst
Gelbwurst
Pressack weiß
Liver sausage
Krakauer/Bierwurst
Rotwurst/Blutwurst/Pressack