MASS SPECTROMETRIC DETECTION OF WHEAT PROTEINS IN MEAT PRODUCTS

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Abstract – A screening method for the detection of wheat proteins in meat products applying High Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS) was developed. Target proteins were lipid transfer proteins (LTPs) and gamma-gliadins. After tryptic digestion two LTP peptides known from literature and three new gliadin marker peptides were measured by HPLC-MS/MS. For a matrix calibration, emulsion-type sausages with 0, 1, 6, 32, 160, 800, and 4000 ppm gluten were produced in cans. The cans of each batch were heated as home cannings (F value 0.41), full stable cans (F value 4.84), and cans under tropical conditions (F value 14.54). The limit of detection (LOD) of the method was about 6 mg/kg gluten, considering the two most intensive marker peptides for all types of cans, which was considerably better than the LOD when using the LTP marker peptides.

Key Words – gluten, emulsion-type sausages, HPLC-MS/MS.

I. INTRODUCTION

Some kinds of wheat proteins are known to produce an allergic response. Another disease, the lifelong enteropathy induced by the absorption of undigested gluten proteins known as celiac disease is caused by gliadins (1), which represent about 50% of the total protein of wheat (2). Wheat protein can be added to meat products (especially emulsion-type sausages) due to technological (thickener, emulsifier or ingredient of a food additive) and economical (foreign protein) reasons. According to Commission Regulation No 41/2009 foods containing 20 mg/kg gluten or less are defined as "gluten-free". Many approaches were performed to analyze the gluten levels in food (3).

State of the art is the Enzyme-linked Immunosorbent Assay (ELISA) as reference method to determine the gluten content in meat products. The disadvantages of the ELISA methods are the single allergen-detection and the immense influence of matrix and processing conditions such as heating (4,5). Lukaskova et al. (6) analyzed spiked meat products with different ELISA methods and stated that only one special gliadin screening test was able to reliably detect wheat gluten contents below the level of 20 mg/kg. They also found that ELISA kits reproducibly led to false negative results in sausage samples.

Therefore Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) based methods for the detection of allergens in food were developed (7). Sealey-Voyksner et al. (8) managed to detect gliadin peptides with limits of quantification (LOQ) in the range of 0.01 – 0.1 mg peptide/kg food via HPLC-MS/MS. However, a complicated digestion protocol including three different enzymes (pepsin, trypsin, and chymotrypsin) was used, and no data concerning the LOQ of the total amount of gluten were given. Heick et al. (9) detected wheat proteins in different types of food using non-specific lipid transfer proteins (nsLTPs) applying a HPLC-MS/MS method. However, LTPs are not specific for wheat. Furthermore, the protease inhibitor/seed storage/LTP proteins contribute to only about 4% to the total amount of wheat proteins (2) and are thus probably unfavorable target proteins for accomplishing the required detection limits.

The main objective of this study was to develop an analytical method for the mass spectrometric detection of wheat proteins after tryptic digestion in meat products, using new characteristic marker peptides resulting from gliadins. For reasons of comparison, also two LTP peptides known from literature were included (9). For the method development, emulsion-type sausages with different concentrations of wheat flour were produced. Home cannings, full stable cans, and cans under tropical conditions were used to investigate the influence of thermal processing on the detectability of wheat proteins in meat products.

II. MATERIALS AND METHODS

Production of emulsion-type sausages

The basic formulation of a batch applied to a 3L bowl chopper was 44.1-49.1% fresh pork, 26.4% back fat, 22.5% ice, 1.8% salt (containing sodium nitrite ($NaNO_2$); 0.4%), and 0.2% dipotassium hydrogen phosphate (K_2HPO_4). Wheat flour type 405 (10% protein) was added as follows (Tab. 1):

Table 1 Batches of sausages with wheat protein

Batch	Fresh Pork [%]	Wheat flour (gluten) [mg/kg]
0 (control)	49.1	0 (0)
1	49.1	16 (1.28)
2	49.1	80 (6.4)
3	49.1	400 (32)
4	48.9	2000 (160)
5	48.1	10000 (800)
6	44.1	50000 (4000)

The sausage meat was stuffed into 200g cans. The cans of each batch were heated as home cannings (F value 0.41), full stable cans (F value 4.84), and cans under tropical conditions (F value 14.54).

LC-MS/MS-Detection of wheat proteins

Pressurized Liquid Extraction (PLE). For the defatting of the samples 2 g homogenized meat product were filled into 20 mL cells, which were equipped with disposable glass-fiber filters. The PLE extraction was performed with a Speed Extractor E-916 obtained from Büchi (Flawil, Switzerland) and acetone as solvent. Two static cycles were accomplished (operating conditions: 30 °C, 50 bar, static time 15 min and purge time 10 min). After extraction, the defatted and dehydrated meat product was removed from the 20 mL cells and dried at room temperature for at least 2 h.

Protein Extraction. 100 mg of the defatted and dehydrated meat product were filled into 1.5 mL microtubes (polypropylene). After addition of 1 mL Ethanol/TRIS-HCl (1M, pH 8.2) = 50:50 the samples were shaken for 3 hours at 70 °C and subsequently cooled to room temperature. The extract was centrifuged for 20 min at 10000 rpm, and ethanol was removed by SpeedVac at 100 mbar for 2 h at 60 °C.

Tryptic Digestion. 15 µL DTT (0.2 M) were added to the protein extract and the samples were shaken for 30 min at 60 °C. After cooling to room temperature, 10 µL iodoacetamide (1 M) were added, and the sample was stored for 30 min at room temperature in the dark. Afterwards, 20 µL Trypsin solution (0.1 µg/mL in 50 mM acetic acid) were added and incubated at 37 °C for 18 h. The digestion was stopped by addition of 5 µl concentrated formic acid. Subsequently, the digest was centrifuged for 1 min at 8000 rpm.

Solid-Phase Extraction. The supernatant of the tryptic digestion was loaded on a Strata-X SPE column (30 mg / 1 mL), which was previously conditioned with 1 mL acetonitrile (ACN) and 1 ml water. After washing with 1 mL water, elution was performed with 1.2 mL of 80% acetonitrile in water. The eluate was concentrated to a volume of 120 µL by SpeedVac.

Liquid Chromatography. Separation of peptides was performed with a Dionex UltiMate 3000 RS HPLC. The column temperature was 40 °C, and the injection volume was 10 µL. The analytical column used was a Nucleosil 100-3 C18 HD (125 x 2.0 mm) from Macherey-Nagel. The mobile phase consisted of solvent A: 0.1% formic acid and 3% ACN in water; and solvent B: 0.1% formic acid and 10% water in ACN. The LC run started with 12.5% B for 2 min, followed by a gradient to 18% B in 4 min, to 28% B in 1 min, to 32.5% B in 6 min, and to 100% B in 1 min. An isocratic step at 100% B continued for 7 min. At the end of the run the column was allowed to equilibrate at 12.5% B for 7 min. The flow rate was 250 µL/min.

Mass Spectrometry. Peptide detection was carried out on an AB Sciex QTrap 5500 using the following parameters: Source temperature: 430 °C, ion spray voltage: 5.5 kV, curtain gas flow: 35. Details of the multiple reaction monitoring (MRM) method are shown in Table 2.

Table 2 Parameters of the MRM method

Marker peptide	m/z	Product ions	CE
LTAASITAVC*R (LTP 1)	581.3 (+3)	606.3 (y5)	27
		806.4 (y7)	30
		877.5 (y8)	30
IETPGSPYLAK (LTP 2)	588.3 (+2)	735.4 (y7)	27
		933.5 (y9)	24
APFASIVAGIGGQ (gliadin 1)	594.3 (+2)	502.3 (y6)	19
		686.4 (b7)	17
		927.5 (b10)	17
APFASIVASIGGQ (gliadin 2)	609.3 (+2)	461.2 (y5)	19
		532.3 (y6)	20
		686.4 (b7)	18
APFASIVADIGGQ (gliadin 3)	623.3 (+2)	374.2 (y4)	22
		686.4 (b7)	18
		985.5 (b10)	20

III. RESULTS AND DISCUSSION

In order to check the specificity of the method for wheat, seeds of spelt, einkorn, barley, oat, millet, and rye were analyzed for the presence of the two LTP and the three gliadin marker peptides (Tab. 3).

Table 3 Occurrence of marker peptides in other types of grains

Peptide	Spelt	Einkorn	Barley	Oat	Millet	Rye
Gliadin 1	+	–	–	–	–	–
Gliadin 2	+	–	–	–	–	–
Gliadin 3	+	+	–	–	–	–
LTP 1	+	–	–	–	–	+
LTP 2	+	–	–	–	–	–

A differentiation between wheat and spelt was not possible. In barley, oat, and millet, none of the marker peptides were detected. For einkorn the marker peptide gliadin 3 and for rye the marker peptide LTP 1 were detected.
A chromatogram of the two LTP and the three gliadin marker peptides is shown in Fig. 1.

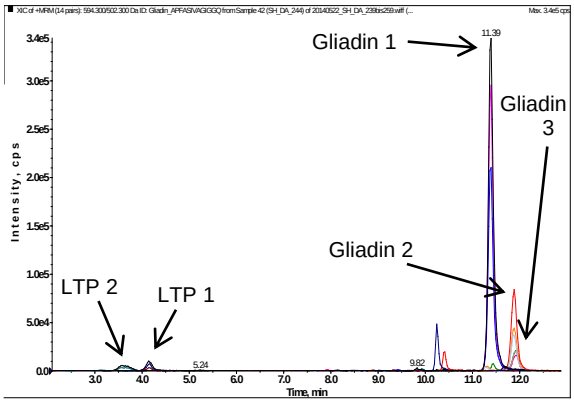

Fig. 1. Chromatograms of the wheat marker peptides in sausages (full stable cans) with 4 g gluten/kg

Wheat protein was detectable in batches 2 (6.4 mg gluten/kg) to 6 (4000 mg gluten/kg). In batch 1 and in the control batch the wheat marker peptides were not detectable. The limit of detection (LOD) of the method was about 6 mg/kg gluten for all types of cans, considering the marker peptides gliadin 1 and 2. The signal-to-noise (S/N) ratio of the most intensive marker peptide APFASIVAGIGGQ (gliadin 1) in batch 2 (6.4 mg gluten/kg) was about 12:1 for product ion 1 (m/z 927.5).

The correlations between peak area and content of gluten [mg/kg] for the most intensive marker peptide APFASIVAGIGGQ (gliadin 1) of the different types of cans are shown in Fig. 2. The determination coefficients ranged between R^2=0.9610 (cans under tropical conditions) and R^2=0.9939 (home cannings). For the most heated samples the lowest peak areas were observed for the marker peptide gliadin 1. Consequently, stronger heating influenced the detectability; however, the detection of gluten in the concentration of 6 mg gluten/kg was possible.

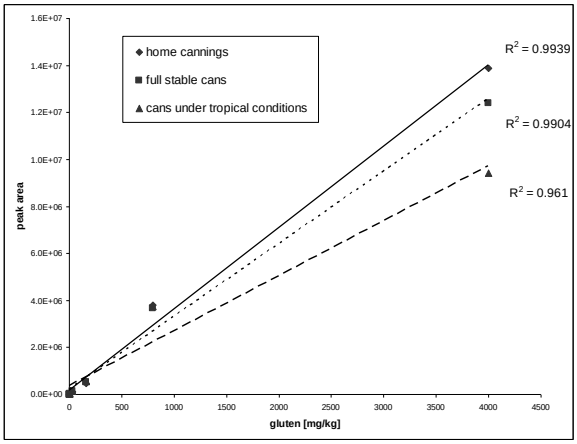

Fig. 2. Correlation between peak area and content of gluten [mg/kg] for the marker peptide gliadin 1 in different types of cans

In order to check the repeatability of the method, seven samples of batches 2 (6.4 mg gluten/kg), 4 (160 mg gluten/kg), and 6 (4000 mg gluten/kg) were analyzed under identical conditions. The standard deviations of the three mass transitions of gliadin marker peptide 1 for the samples of batches 2 and 6 ranged between 7% and 8%. For batch 4, the standard deviations were even lower in the range of 3%.

Table 4 Repeatabilities of the gliadin marker peptide 1 in different batches (N=7)

Gluten [mg/kg]	Product ion (m/z)	Standard deviation [%]
6.4	502.3	8.2
	686.4	7.6
	927.5	7.8
160	502.3	3.4
	686.4	3.3
	927.5	2.9
4000	502.3	7.3
	686.4	7.2
	927.5	7.5

IV. CONCLUSION

After a targeted optimization of the extraction conditions, with the use of Ethanol/TRIS-HCl (1M, pH 8.2) = 50:50 an LOD of about 6 mg gluten /kg considering the marker peptides gliadin 1 and 2 was achieved. When using the new gliadin marker peptides, significantly lower LODs (well below 20 mg gluten/kg) were achieved compared to those obtained by using the analyzed marker peptides LTP 1 (LOD 160 mg gluten/kg) and LTP 2 (LOD 800 mg/kg).

By the investigation of commercially available meat products with and without wheat protein it could be shown that the analytical method is also suitable for a more complex matrix. No false positive and false negative results were obtained.

Based on the presented method, an LC-MS/MS screening method for the simultaneous detection of other types of grain containing gluten (barley, oat, and rye) in meat products should be developed. The advancement of the method ought to be performed with regard to the intolerance to gluten (low ppm range) as well as the problem of foreign proteins (percentage range).

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