LC-MS/MS-DETECTION OF PEA, LUPINE AND SOY PROTEINS IN MEAT PRODUCTS

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Abstract – A sensitive screening method for the simultaneous detection of pea (*Pisum sativum*), lupine (*Lupinus angustifolius*), and soy (*Glycine maxima*) in meat products applying High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) was developed. After tryptic digestion 3 to 4 marker peptides for each plant protein (3 mass transitions each) were measured by HPLC-MS/MS. For a matrix calibration, emulsion-type sausages containing 0, 1, 6, 32, 160, 800, and 4000 ppm of each raw plant protein were produced. High correlation coefficients (R^2>0.998) between the peak areas of the marker peptides and the contents of plant proteins in the meat products were obtained. The limits of detection (LODs) of the method were about 5 mg/kg for pea protein, 4 mg/kg for soy protein, and 2 mg/kg for lupine protein. No false-positive or false-negative results were obtained. The relative standard deviation (RSD) of repeatability ranged between 1% and 6%.

Key Words –pea, lupine and soy protein, emulsion-type sausages, HPLC-MS/MS.

I. INTRODUCTION

The addition of plant proteins to various types of meat products is a very common practice (1). Vegetable proteins can be added to meat products (especially emulsion-type sausages) for technological reasons like the improvement of the water-binding capacity of meat, the improvement of the textural properties and also for economic reasons like the efficient use of low-quality meats (1). Due to their high protein contents the legumes pea (*Pisum sativum*; 26 % protein in the dry matter [DM]), lupine (*Lupinus spp.*; 36-48% protein DM), and soy (*Glycine maxima*; 41% protein DM) are important cost-effective sources of foreign proteins in meat products.

In addition to the conscious use of pea, lupine, and soy as meat adulterations these plant proteins can also be unintentionally transferred into meat products via contaminations of spice mixtures or other food additives and processing aids. In this context even small amounts of pea, lupine, and soy proteins in the ppm range may be relevant to human health due to the potential allergenicity of these plant proteins.

According to Commission Directive 2007/68/EC (2) lupine and soy belong to the list of food ingredients which must be indicated on the label of meat products as they are likely to cause adverse reactions in susceptible individuals.

Various analytical procedures were developed to detect allergens in food (3). Common used analytical methods are the enzyme linked immunosorbent assays (ELISAs), for example to detect soybean or lupine proteins in meat products (4, 5). Furthermore various analytical methods for the indirect detection of soy (6), lupine (7), and pea (8) in meat products applying real-time polymerase chain reaction (PCR) were developed.

In the last years also analytical methods based on High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) for the determination of food allergens were developed (9, 10), which have the potential of a direct and simultaneous detection of different proteins.

Therefore the main objective of this study was to develop an analytical method for the simultaneous mass spectrometric detection of pea, lupine and soy proteins in meat products using characteristic tryptic marker peptides in order to comply with the EU food-labeling legislation and also to have reliable methods to proof meat adulterations.

For the method development, emulsion-type sausages with six different concentrations of pea protein isolate, lupine flour (*Lupinus angustifolius*), and soy protein isolate (1, 6, 32, 160, 800, and 4000 mg/kg) and also sausages without the addition of the mentioned plant proteins (blank values) were produced.

II. MATERIALS AND METHODS

Production of emulsion-type sausages

The basic formulation of a batch applied to a 3L bowl chopper was 47.9-49.1% fresh pork, 26.4% back fat, 22.5% ice, 1.8% salt (0.4% $NaNO_2$), and 0.2% K_2HPO_4. Pea protein isolate (75.6% protein), Lupine flour (*Lupinus angustifolius*; 33% protein), and soy protein isolate (64.8% protein) were added as follows (Tab. 1):

Table 1 Batches of sausages with pea, lupine and soy

Batch	Fresh Pork [%]	pea, lupine and soy ingredient [mg/kg for each]
0 (control)	49.1	0
1	49.1	1.28
2	49.1	6.4
3	49.1	32
4	49.1	160
5	48.9	800
6	47.9	4000

The sausage meat was stuffed into artificial casings (caliber 60 mm) and heated for 1h at 70°C.

LC-MS/MS-Detection of target proteins

Pressurized Liquid Extraction (PLE). 2-2.5 g homogenized meat product was filled into 20 mL cells. The PLE was performed with a Speed Extractor E-916 from Büchi and acetone as solvent. Two static cycles were accomplished (30 °C, 50 bar). After extraction, the defatted meat product was removed from the 20 mL cells and dried for at least 1 h at room temperature (RT).

Protein Extraction. 50 mg of the defatted and dehydrated meat product were filled into 1.5 mL microtubes (polypropylene). After addition of 0,5 mL TRIS-HCl (1M, pH 8.2) the samples were shaken for 2 hours at 70 °C and cooled to RT. The extract was centrifuged for 20 min at 8000 rpm.

Tryptic Digestion. 20 µL Trypsin solution (0.1 µg/mL in 50 mM acetic acid) were added to 100 µl protein extract and incubated at 37 °C for 16 h. The digestion was stopped by addition of 2 µl concentrated formic acid and centrifuged for 10 min at 10000 rpm.

Solid-Phase Extraction. The supernatant of the tryptic digestion was loaded on a Strata-X SPE column (30 mg / 1 mL), which was conditioned with 1 mL acetonitrile (ACN) and 1 ml water. After washing with 1 mL water, elution was performed with 500 µl of 80% ACN. The eluate was collected in 1.5 mL microtubes, prefilled with 5 µL of dimethyl sulfoxide and was concentrated to 5 µL by SpeedVac. The concentrated eluate was dissolved in 50 µL of solvent A.

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 10% B for 3 min, followed by a gradient to 30% B in 19 min, another gradient to 100% B in 2 min. An isocratic step at 100% B continued for 7 min. At the end of the run the column was allowed to equilibrate at 10% 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 (Target Protein)	**m/z**	**Product Ions**	**CE [V]**
ELTFPGSVQE INR (pea 1)	745.4 (+2)	999.5 (y9), 500.3 ($y9^{++}$), 573,8 ($y10^{++}$)	35/35/ 35
LSSGDVFVIPAG HPVAVK (pea 2)	598.3 (+3)	875.5 (y9), 513.3 (y5), 988.6 (y10)	27/36/ 26
LTPGDVFVIPAG HPVAVR (pea 3)	615.7 (+3)	903.5 (y9), 541.3 (y5), 1016.6 (y10)	30/36/ 30
QQEQQLEGE LEK (lupine 1)	729.9 (+2)	575.3 (y5), 704.3 (y6), 817.4 (y7)	37/34/ 34
ISSVNSLTLP ILR (lupine 2)	706.9 (+2)	498.3 (y4), 712.5 (y6), 1026.6 (y9)	30/33/ 34
NTLEATFNTR (lupine 3)	583.8 (+2)	838.4 (y7), 709.4 (y6), 951.6 (y8)	29/30/ 24
TLTSLDFPILR (lupine 4)	638.4 (+2)	760.4 (y6), 1061.6 (y9), 960.6 (y8)	28/28/ 29
HFLAQSFNTNE DIAEK (soy 1)	622.0 (+3)	818.4 (y7), 919.4 (y8), 1033.5 (y9)	27/27/ 24
EAFGVNMQIVR (soy 2)	632.3 (+2)	760.4 (y6), 916.5 (y8), 859.9 (y7)	30/34/ 30
FYLAGNQEQEF LK (soy 3)	793.9 (+2)	283.1 (a2), 424.2 (b3), 638.7 ($y11^{++}$)	45/37/ 34

III. RESULTS AND DISCUSSION

A HPLC-MS/MS method for the simultaneous detection of pea, lupine, and soy in meat products was developed. Therefore 3 to 4 characteristic marker peptides for each plant protein (3 mass transitions each) were measured after tryptic digestion. Fig. 1 shows a chromatogram of the selected marker peptides of pea, lupine, and soy in an emulsion-type sausage with afterwards added pea protein isolate, lupine flour, and soy protein isolate (2% each).

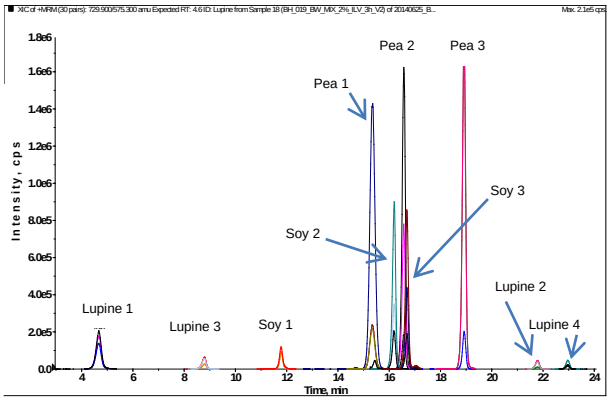

Fig. 1. Chromatogram of the marker peptides for pea, lupine, and soy in a meat product (2% of each type added)

The limits of detection (LODs) of the method were about 5 mg/kg for pea protein, 2 mg/kg for lupine protein, and 4 mg/kg for soy protein, determined by the analysis of the sausages mentioned in Table°1.

The simultaneous determination of different proteins was an analytical challenge and required a series of important compromises of fundamental and interacting parameters. One example was the definition of the extraction temperature (see Fig. 2). Varying extraction temperatures led to opposing effects with respect to the different marker peptides. Increasing temperatures resulted in higher peptide areas regarding to all pea peptides and two of the three soy peptides. The third soy peptide and one of the four lupine peptides were little influenced by the extraction temperature. However, regarding the other three lupine marker peptides a strong decrease of their yield was noticeable with increasing temperatures. Considering all results an extraction temperature of 70 °C was an appropriate compromise due to the suitable results for the critical lupine peptides and higher peak areas for the pea and soy peptides compared to the use of an extraction temperature of 60 °C.

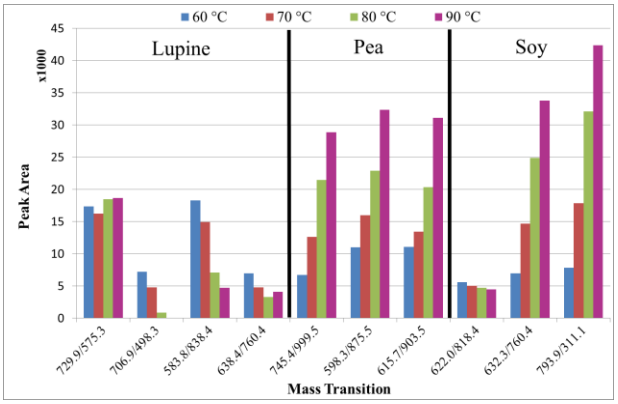

Fig. 2. Peak areas of the marker peptides for pea, lupine, and soy applying different extraction temperatures (60 °C, 70 °C, 80 °C, and 90 °C); mean of repeat determinations

The correlation between the ratio of concentrations of the analytes (marker peptides) to their internal standards and the ratio of peak areas of the analytes to their internal standards is shown in Fig. 3.

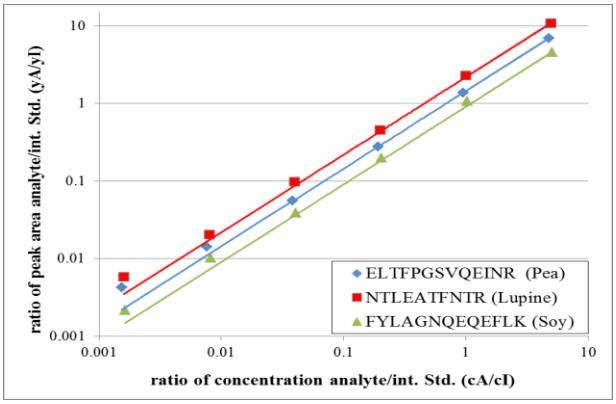

Fig. 3. Correlations between ratio of concentrations of analytes to internal standards and ratio of peak areas of analytes to internal standards in matrix solution (N=3)

The used internal peptide standards were nearly identical to the corresponding analytes, however the amino acid sequence differed in one or two amino acids in terms of one CH_2-group (like isoleucine-valine, leucine-valine or glutamine-asparagine). The internal standards were ELTFPGSVQEVNR for pea, NTLEATFQTR for

lupine, and FYVAGNQEQEFVK for soy. Via a database query (BLAST) it was proven that these compounds do not occur naturally and consequently were appropriate as internal standards. Furthermore they were not detected in the analysed sausages.
The calibration was determined in matrix solution to correct the shift of retention times between pure standards and samples. The determination coefficients of the main marker peptides ranged between R^2=0.9987 (soy), R^2=0.9998 (lupine) and R^2=1.0 (pea) (Fig. 3).

IV. CONCLUSION

All selected marker peptides for pea, lupine, and soy originated from plant storage proteins, which are suitable as analytical target proteins due to their occurrence in high amounts and their high specificity. With the help of the four lupine marker peptides only a reliable determination of the blue lupine (*Lupinus angustifolius*) was possible. Only one lupine marker peptide allowed the detection of white lupine (*Lupinus albus*).
In order to prove the use of not labeled foreign proteins in meat products it was very important to check the effects of the kind of processing of these proteins because this could strongly influence their detectability. So the recovery values of the marker peptides depended on the conditions of the sausage production. This kind of work should be completed as well as investigations of different origins of pea, lupine, and soy proteins respectively their production methods.

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