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LC-MS/MS-DETECTION OF MILK PROTEINS IN MEAT PRODUCTS

Wolfgang Jira* and Fredi Schwägele

Analysis Division, Max Rubner-Institut (MRI), E.-C.-Baumann-Str. 20, 95326 Kulmbach, Germany

*wolfgang.jira@mri.bund.de

Abstract – A screening method for the detection of traces of milk proteins (ppm range) in meat products applying Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) was developed. Target protein of the analytical method was alpha-S1-casein. After tryptic digestion two characteristic marker peptides (YLGYLEQLLR and FFVAPFPEVFGK) were measured by HPLC-MS/MS. For a matrix calibration emulsion-type sausages with 0, 1, 3, 5, 10, 25, and 50 ppm milk protein were produced in cans. The cans of each batch were heated as home cannings (F value 0.41), full stable cans (F value 5.02), and cans under tropical conditions (F value 14.78). The limit of detection (LOD) of the method was significantly below 1 ppm milk protein for all types of cans. The determination coefficients for the correlation between peak area of the marker peptides and concentration of milk protein ranged between R^2=0.9899 and R^2=0.9997. No false positive and false negative results were obtained. The developed method shall be extended to a multiallergen screening method in meat products.

I. INTRODUCTION

Milk allergy is one of the most common food allergies in infants up to one year of age (1). Milk is used in a multitude of foodstuffs like bakery products, cakes, chocolate, ice cream and also in meat products like liver sausages or Frankfurter-type sausages. About 3% of bovine milk is protein, which is subdivided into the two fractions casein (80% of milk protein) and whey (20% of milk protein). The casein fraction (protein content: about 33 g/kg) contains the most allergenic (2,3) and heat stable caseins alpha-S1 (10.0 g/kg), alpha-S2 (2.6 g/kg), beta (9.3 g/kg), and kappa (3.3 g/kg) (Bos d 8). The whey fraction (protein content: about 5 g/l) contains the two major allergens beta-lactoglobulin (Bos d 5; 8-10%) and alpha-lactalbumin (Bos d 4; 4-5%; thermolabile).

A number of Enzyme-linked Immunosorbent Assays (ELISAs) are available for the detection of milk in foodstuffs (4,5). Both sandwich and competitive ELISA formats are available, which can be specific for caseins and whey proteins, or a combination of both (5). ELISA test kits have several advantages like specificity, sensitivity and simplicity, however thermal processing of food can have negative effects on the detection of milk allergens, because a possible alteration of the protein structure can lead to an alteration of antibody binding (6). Furthermore, thermal processing can result in the formation of insoluble protein aggregates, which can not be detected by various ELISA methods (7).

A comparison of two commercially available ELISA Test Kits and a Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) method for the detection of milk in unprocessed and processed bread showed, that one ELISA test kit did not detect the targeted allergens in processed bread, with the other kit in the thermal treated matrix only 17% of the amount detected in the unprocessed matrix were detected. Applying LC-MS/MS processing of the matrix led to a decrease of the signal intensity for milk of 55% (8).

Weber et al. (1) developed an analytical method for the detection of casein in spiked hot dog samples (without thermal processing of milk protein) after tryptic digestion using LC-MS/MS with the help of two marker peptides (YLGYLEQLLR and FFVAPFPEVFGK) from alpha-S1-casein (LOD: 5 ppm).

The main objective of this study was to develop an analytical method for the mass spectrometry detection of milk allergens in commercially available meat product samples. Due to the reported Lowest Observed Adverse Effect Level (LOAEL) for milk of 0.36 ppm (9) a LOD below 1 ppm was aspired. Furthermore the influence of the degree of thermal processing of the meat product on the detectability of milk protein was investigated. Based on the presented method a LC-MS/MS multi allergen screening

method for meat products is intended to be developed.

II. MATERIALS AND METHODS

Production of emulsion-type sausages

The basic formulation of a batch applied to a 3L bowl chopper was 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). Skimmed milk powder (36 % protein) was added as follows (Tab. 1):

Table 1 Batches of sausages with milk protein

Batch	Skimmed milk powder	Milk protein
0 (control)	0 ppm	0 ppm
1	3 ppm	1 ppm
2	8 ppm	3 ppm
3	14 ppm	5 ppm
4	28 ppm	10 ppm
5	69 ppm	25 ppm
6	139 ppm	50 ppm

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 5.02), and cans under tropical conditions (F value 14.78).

LC-MS/MS-Detection of milk proteins

Pressurized Liquid Extraction (PLE). For the defatting of the samples 2 g homogenised meat product were filled into 20 mL cells, which were equipped with disposable glass-fibre 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 for about 3 hours at room temperature.

Protein Extraction. 100 mg of the defatted and dehydrated meat product were filled into 1.5 mL microtubes (polypropylene). After addition of 1 mL TRIS-HCl (100 mM, pH 8.2) the samples were shaked for 1 hour at 60 °C and subsequently cooled to room temperature. The extract was centrifuged for 20 min at 10,000 rpm.

Tryptic Digestion. To a 100 µL sample of the protein extract 20 µL Trypsin solution (0.1 µg/mL in 50 mM acetic acid) were added and incubated at 37 °C for 3 h. The digestion was stopped by addition of 2 µl concentrated formic acid. Subsequently the digest was centrifuged for 1 min at 8000 rpm.

Solid-Phase Extraction. The supernatant of the tryptic digestion (about 120 µL) was loaded on a Strata-X SPE column (30 mg / 1 mL), which was conditioned with 1 mL acetonitrile (ACN) and 1 ml water before. After washing with 1 mL water, elution was performed with 500 µL of 80% acetonitrile in water. The eluate was concentrated to a volume of 50 µL by SpeedVac.

Liquid Chromatography. Separation of peptides was performed with a Dionex UltiMate 3000 RS HPLC. The column temperature was 40 °C, the injection volume 2 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 2% B for 3 min, followed by a gradient to 60% B in 18 min, another gradient to 100% B in 3 min. An isocratic step at 100% B continued for 20 min. At the end of the run the column was allowed to equilibrate at 2% B for 7 min. The flow rate was 250 µL/min.

Mass Spectrometry. Peptide detection was carried out on a 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

	YLGYLEQLLR	FFVAPFPEVFGK
RT [min]	16.01	16.65
Precursor [m/z]	634.4 (+2)	692.9 (+2)
Product1 [m/z]	249.2 (a2)	920.5 (y8)
Product2 [m/z]	991.6 (y8)	465.2 (b4)
Prod1/Prod2	1.9	1.3
Prod1 (CE/DP)	28/28	26/28
Prod2 (CE/DP)	27/28	22/24

III. RESULTS AND DISCUSSION

A rapid LC-MS/MS method for the detection of milk proteins in meat products was developed, applying short protein extraction and digestion times (1h and 3h, respectively). Moreover, omission of reducing with dithiothreitol (DTT)

and alkylation using iodoacetamide were time saving.

A chromatogram of the two marker peptides in sausages with 0 ppm and 5 ppm milk protein is shown in Fig. 1.

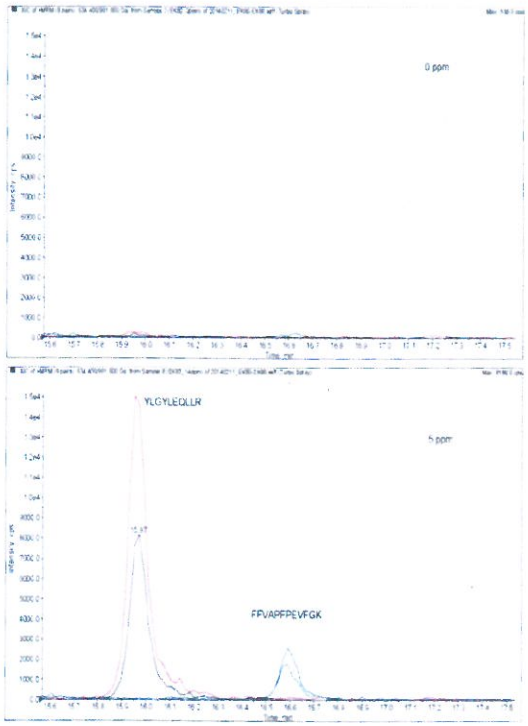

Fig. 1. Chromatograms of the two marker peptides in sausages (full stable cans) with 0 ppm (control) and 5 ppm milk protein

The limit of detection (LOD) of the method was significantly below 1 ppm milk protein for all types of cans. The signal-to-noise (S/N) ratio of marker peptide 1 (YLGYLEQLLR) in the lowest concentration (1 ppm milk protein) was about 50:1 for product 1 (m/z 249.2) and about 80:1 for product 2 (m/z 991.6). Assuming a limit of quantification with a S/N ratio of 10:1 concentrations of about 0.2 ppm milk protein can be detected with the help of this method.

The correlations between peak area and content of milk protein [ppm] for the marker peptides 1 (YLGYLEQLLR) and 2 (FFVAPFPEVFGK) for the different types of cans are shown in Fig. 2. The determination coefficients ranged between R^2=0.9899 (peptide 1 in home cannings) and R^2=0.9997 (peptide 2 in home cannings). No false positive and false negative results were obtained. Between the different thermal treatments of the meat products no relevant differences were observed. Stronger heating did not affect the detection of the two marker peptides negatively. In contrast the detection of marker peptide 2 appeared improved for stronger heated sausages (see Fig. 2).

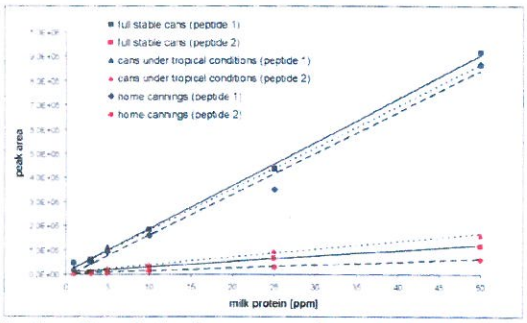

Fig. 2. Correlation between peak area and content of milk protein [ppm] for the marker peptides 1 and 2 in different types of cans

A reliable criterion for the distinct detection of the mentioned marker peptides is the relation of the peak areas of the two selected MRM transitions. The mean relation (N=7) of the peak areas of the two MRM transitions for peptide 1 (634.4->249.2/634.4->991.6) ranged between 1.88 ± 0.06 and 1.96 ± 0.09 and for peptide 2 (692.9->920.5/692.9->465.2) between 1.26 ± 0.03 and 1.32 ± 0.05.

IV. CONCLUSION

The developed analytical method is suitable for the detection of traces of milk protein in meat products below 1 ppm. Thermal processing did not negatively influence the detection of the marker peptides, because the primary structure of proteins is quite stable. Furthermore a limited degree of protein degradation is less critical due to the short peptide sequences of the used marker peptides (10 and 12 amino acids). Based on the presented method a LC-MS/MS multi allergen screening method for meat products should be developed. Therefore the important allergens mustard, celery, egg, soy, and gluten should be taken into consideration. Such a screening method can also be adapted to a detection method for foreign proteins in meat products. The observed good

linearity between peak areas and milk protein concentration provides an excellent basis for implementation of an absolute quantification of milk protein in meat products using stable isotope-labeled peptides.

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