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【総　説】

Recent Developments in Meat Science in Europe: Analytical Approaches for Tracking and Tracing

F. Schwägele, W. Jira, S. Münch, K.-H. Schwind

Max Rubner-Institut (MRI), Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Meat, Kulmbach Location, Germany

Abstract

With respect to global food supply consistent standards based on sound science and robust controls are necessary to ensure consumers' health and to maintain consumers' confidence. The *General European Food Law*, Regulation (EC) 178 (2002) with its Article 18 provides procedures to implement reliable traceability systems in the food and feed supply chains in Europe. Food safety and quality require various analytical approaches along the whole food chain, downstream (tracking) from primary production to the consumer and upstream (tracing) from the consumer to primary production. Recent developments in meat science in Europe are reported in this paper.

Keywords：Traceability, Meat, Meat Products, Analytical Approaches

1. Introduction

With respect to the global distribution of feed, food and ingredients, the different countries in our world have never before been more interdependent with respect to their food supply. Consequently, a common approach with consistent standards based on sound science and robust controls is necessary to ensure consumers' health and to maintain consumers' confidence (Wall, 2009).

In this regard, the *General European Food Law*, Regulation (EC) 178 (2002), outlines the general principles and requirements of food law and establishes the *European Food Safety Authority (EFSA)*. Furthermore, it provides procedures in matter of food safety, i.e. among other things the implementation of traceability systems in the food and feed supply chains in Europe.

Article 18 of the Regulation refers to traceability of food, feed, food-producing animals, and any other substance to be incorporated into a food or feed. Traceability shall be established at all stages of production, processing and distribution. Food and feed business operators shall be able to identify any person from whom they have been supplied with these goods. They shall have in place systems and procedures to identify the other companies to which their products have been supplied. Food or feed which is placed on the market in the European Community shall be adequately labelled or identified to facilitate its traceability by relevant documentation or information. For this purpose, all operators shall have in place systems and procedures, making available information to the competent authorities on demand.

Indispensable requirements for every food business are: appropriate process control, biosecurity, adequate traceability as well as good hygiene and manufacturing practices. Food safety and quality require various analytical approaches along the whole food chain (Fig. 1), downstream (tracking) from primary production to the consumer and upstream (tracing) from the consumer to primary production (Schwägele, 2005).

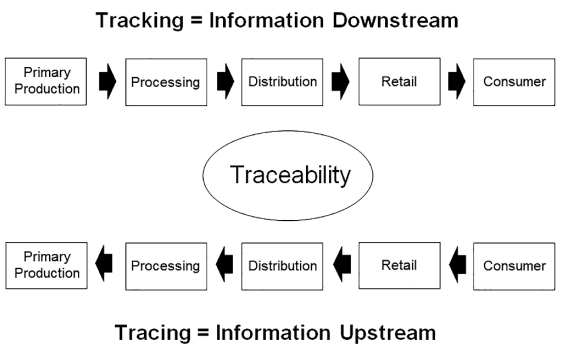

Fig. 1 Tracking and Tracing along the food chain

2. Analytical approaches for tracking and tracing in the meat area

Analytical approaches for tracking and tracing in the meat area are quite diversified. Five different analytical subjects, which were matter of investigation in the laboratories of the Max Rubner-Institut (MRI) in Kulmbach during the last years, are presented below: the mass spectrometric analysis of (2.1) organic residues and contaminants (Andrée *et al.*, 2010), as well as (2.2) heat-induced substances (Pöhlmann *et al.*, 2013), (2.3) the quantitation of animal species by real-time PCR (Binke *et al.*, 2005), the HPLC-MS/MS detection of (2.4) allergens and foreign proteins in meat products (Hoffmann *et al.*, 2017) and of (2.5) microbial transglutaminase in restructured meat (Jira & Schwägele, 2017).

2.1. Organic residues and contaminants

Under the general term "dioxins" usually three different chemical substance classes are summarized. Two of them – the polychlorinated dibenzo-p-dioxins (PCDDs) and the polychlorinated dibenzofurans (PCDFs) – are chlorinated tricyclic ethers. The third substance group, the polychlorinated biphenyls (PCBs), are chlorinated aromatic compounds with a biphenyl skeletal structure. All these three substance classes have a high persistence in the environment and a strong lipophilic character. In the substance class of the PCDDs, 75 congeners are possible, the PCDFs have 135 congeners and the PCBs 209 congeners. Whenever in the molecule ground structure of PCDDs and PCDFs the chemical positions 2, 3, 7 and 8 are chlorinated, the resulting congeners have toxic properties. PCBs can be divided into dioxin-like and non-dioxin-like compounds. If in the PCB ground structure the positions 2, 2', 6, 6' are not (non-ortho-PCBs, 4 congeners) or only once chlorinated (mono-ortho-PCBs, 8 congeners), these compounds are called dioxin-like PCBs (Van den Berg *et al.*, 2006). Against this challenge it is the task of the analysts to quantify 29 congeners with toxic relevance (7 PCDD, 10 PCDF and 12 PCB) within a total of 419 congeners. These 29 congeners have very similar toxicological mechanisms of action. However, the quite differently pronounced toxicities of these congeners have to be considered by toxic equivalent factors (TEFs). The concentrations of the 29 congeners are multiplied with their TEFs to obtain the total toxicity (toxic equivalent concentration; TEQ) (Fig. 2).

$$TEQ = \sum (TEF)_i * c_i$$

TEQ = toxic equivalent concentration (e.g. in ng/kg)
TEF = toxic equivalent factor of the congener i
c_i = concentration of the congener i

Fig. 2 Formula for the calculation of the TEQ

In the years from 2006 to 2008, a status survey of dioxins and PCBs in feed and food of animal origin in Germany was carried out (http://edok.ahb.niedersachsen.de/07/630521395.pdf). In this project, feedstuffs, eggs as well as meat and meat products were investigated and analyzed for dioxins and PCBs. Therefore, about 300 samples of meat (pork, poultry, beef and sheep) and meat products (Bologna type sausage, raw ham, cooked liver sausage and raw sausage) were collected, which ensured a preferably high level of representativeness (Andrée *et al.*, 2010).

The median of the WHO-PCDD/F-TEQ in beef was 0.2 ng/kg fat, in pork and poultry it was 0.09 ng/kg fat. These contents ranged clearly below the maximum limits (Commission Regulation (EC) No 199/2006). In the investigated meat products, the median of the WHO-PCDD/F-TEQ was below 0.1 ng/kg fat. The median content of WHO-PCB-TEQ in beef samples was 0.9 ng/kg fat and therefore in the range of the action level of 1.0 ng/kg fat. Poultry had a median content of 0.11 ng WHO-PCB-TEQ/kg fat, which was one order of magnitude below the action level of 1.5 ng/kg fat (Commission Recommendation (EC) No 88/2006). In meat products the WHO-PCB-TEQ varied from 0.06 ng/kg fat for raw ham to 0.13 ng/kg fat for raw sausages. (Fig. 3)

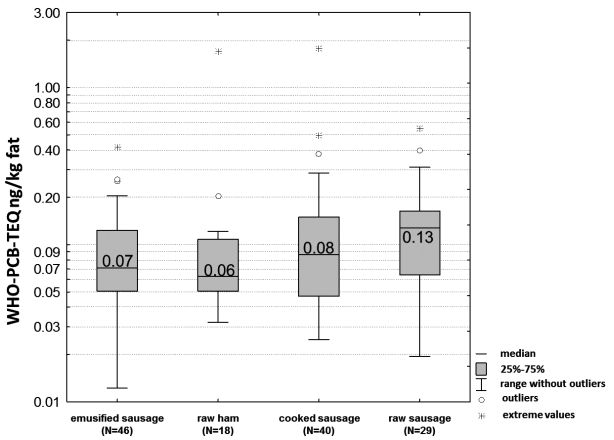

Fig. 3 Box plots of the WHO-PCB-TEQ [ng/kg fat] in different types of meat products (N=133)

2.2. Heat-induced contaminants

In contrast to dioxins and PCBs, polycyclic aromatic hydrocarbons (PAH) are not enriched in meat via a carry-over from feed to the animal. PAH are heat-induced contaminants, which are formed by the incomplete combustion of organic material and consequently also during the smoking of meat and meat products. PAH are a group of about 660 different compounds (Sander & Wise, 1997), some of them showing carcinogenic properties (IARC, 2010). In the European Union, for smoked meat and smoked meat products a maximum level for benzo[a] pyrene of 2 µg/kg and a maximum level of 12 µg/kg for PAH4 (sum content of benzo[a]pyrene, chrysene, benzo[a]anthracene, and benzo[b]fluoranthene; Fig. 4) are established (Commission Regulation (EU) No 835/2011).

In a research project, the contents of PAH4 in smoked Frankfurter-type sausages were investigated, depending on the smoke generation method applied (Pöhlmann *et al.*, 2013). In addition, also desirable phenolic compounds (guaiacol, 4-methylguaiacol, syringol, eugenol and trans-isoeugenol) were analyzed. These compounds are of considerable importance for the organoleptic properties of smoked meat products (Kjallstrand & Petersson, 2001) and show antimicrobial (Davidson & Branden, 1981) and antioxidative (Toth, 1982; Wittkowski, 1985) properties. Within the mentioned study, a total of 63 smoking experiments were performed. The smoke was generated by smouldering with different air supplies (smouldering smoke), by leading overheated steam through wood chips (steam smoke), by friction of a log (friction smoke), and by heating plates (touch smoke). The type of smoke generator had a noticeable influence on the contents of PAH and phenolic compounds. The highest mean content of PAH4 (2.6 mg/kg) was observed for sausages when intensive smouldering smoke was applied, the lowest (0.3 mg/kg) in friction-smoked sausages. The highest mean sum content of the five phenolic compounds was observed for sausages smoked with steam smoke (45 mg/kg), whereas the contents in friction- (15 mg/kg) and touch- (18 mg/kg) smoked products were relatively low.

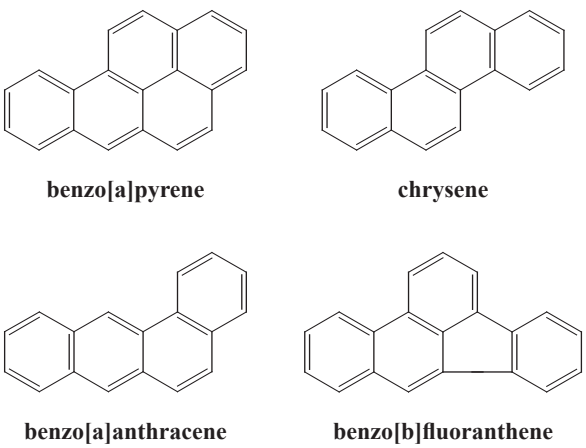

Fig. 4 Structural formulae of benzo[a]pyrene, chrysene, benzo[a]anthracene, and benzo[b]fluoranthene

2.3. Animal species detection and quantitation by real-time PCR

Real-time polymerase chain reaction (PCR) is a widely used molecular biological technique to amplify various target DNA molecules. A specific thermocycler enables to record the increasing fluorescence intensity caused by various dyes in parallel to DNA-amplification. Applying suitable multiplex-PCR systems, it is possible to identify and quantify different species in a single assay simultaneously in dependence on the number of lasers and channels in the thermocycler.

To trace fraud and adulteration in meat products, the animal species labelled in the ingredient list can be in parallel detected and quantified by the use of real-time PCR amplifying target as well as reference DNA fragments. For the relative quantitation (Pfaffl *et al.*, 2001) of goat tissue in a meat product the ratio of the copy number of an amplified single copy target gene fragment (nucleic beta-casein gene) to the copy number of an amplified reference gene fragment (nucleic myostatin gene), which is common for mammals and poultry (Laube *et al.*, 2002), is applied.

For canned meat products, it can clearly be demonstrated that DNA fragmentation increases depending on the applied heat intensity (Fig. 5). Home canned cans (lane 2) with only relatively low energy input show high molecular DNA on top of the gel, whereas extremely heated products (lane 6) reveal DNA fragmentation at a high extent leading to low molecular DNA visualized near the bottom of the gel.

Nevertheless, if the lengths of indicator and reference gene fragment are similar to those shown for goat (beta-casein gene fragment specific only for goat: 161 bp / myostatin gene fragment, unitary for all animal species:

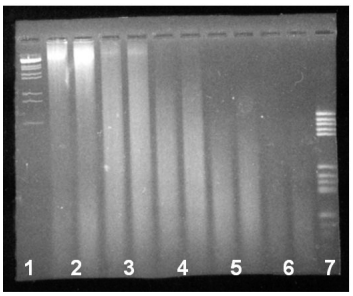

Fig. 5 Increasing DNA fragmentation depending on heat treatment (bp = base pairs; kbp = kilobase pairs); Binke, R. (2005)

	Quota [% Goat]	Actual [%] unheated	Actual [%] HCC	Actual [%] NC	Actual [%] TC
Emulsified Sausage 1	100*	109 ± 9	93 ± 11	114 ± 16	122 ± 22
Emulsified Sausage 2	100	77 ± 23	90 ± 16	82 ± 8	79 ± 18
Emulsified Sausage 3	50	57 ± 4	59 ± 17	56 ± 11	47 ± 8
Emulsified Sausage 4	20	13 ± 1	20 ± 6	19 ± 1	21 ± 7
Emulsified Sausage 5	2	1.9 ± 0.4	2.0 ± 0.7	2.0 ± 0.8	2.7 ± 1.2

* = without pig fat (25 % plant oil)

Table 1 Validated test system beta casein (161 bp) / myostatin (154 bp) for the quantification of goat meat at varying percentages in different heat treated meat products. For emulsion -type sausage 1, plant oil was used instead of pig fat (HCC = home canned can; NC = normal can; TC = can for use under tropical conditions)

Tissue	Animal Species	Samples	DNA [g/kg]
Liver	Pig	5	4.0 - 4.4
Muscle	Pig, Cattle, Goat	148	0.3 - 0.5
Connective tissue	Pig, Goat	20	0.4 - 0.5
Fat	Pig, Goat	58	~ 0.1
Milk	Goat	12	~ 0.01

Table 2 DNA contents of various animal tissues

154 bp), a reliable relative quantitation is possible, regardless of the heat treatment of the meat products (Table 1).

There are only minor differences with respect to the resulting percentages in the unheated products and the cans for use under tropical conditions.

However, the influence of the natural DNA content of different types of tissue on the quantification must always be taken into consideration. In Table 2, the DNA contents of various animal tissues are shown.

2.4. Analysis of allergens and foreign proteins by HPLC-MS/MS

The addition of plant proteins to various types of meat products is a very common practice (Lopez & Alegre, 2009). 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 (Gujral *et al.*, 2002), the improvement of the textural properties (Ulu, 2004) and also for economic reasons like the efficient use of low-quality meats (Lopez & Alegre, 2009). Due to their high protein contents (Belitz *et al.*, 2004) the legumes lupine (Lupinus spp.; 36-48% protein in the dry matter), pea (Pisum sativum; 26% protein in the dry matter), and soy (Glycine maxima; 41% protein in the dry matter) are important cost-effective sources of foreign proteins in meat products.

On the other hand, even small amounts of lupine, pea, and soy proteins in the ppm range may be relevant to human health of persons suffering from an allergy due to the potential allergenicity of these legume proteins. Reference doses for lupine (4 mg protein) and soy (1 mg protein) have been established by the VITAL (Voluntary Incidental Trace Allergen Labeling) Expert Panel (Taylor *et al.*, 2014). Peanut-allergic patients show extensive serological crossreactivity with lupine, pea, and soy (European Food Safety Authority, 2014; Jensen *et al.*, 2008).

Various analytical procedures were used to detect allergens in food (Kirsch *et al.*, 2009). The most common analytical methods are the enzyme linked immunosorbent assays (ELISAs). Furthermore various analytical methods for the indirect detection of the proteins of soy (Köppel *et al.*, 2012), lupine (Ecker *et al.*, 2013), and pea (Brezna *et al.*, 2006) in meat products applying real-time PCR were developed. Due to the fact that PCR is only an indirect method and that ELISA methods have the disadvantage to be, in most cases, only applicable for a single allergen-detection, analytical methods based on High Performance Liquid Chromatography-Tandem Mass Spectrometry (HPLC-MS/MS) have been developed over the last years for the determination of food allergens (Faeste *et al.*, 2011; Monaci & Visconti, 2009).

In contrast to bakery products only a very limited number of mass spectrometric methods exist for the de-

tection of plant proteins in meat products. To the best of our knowledge until now just one mass spectrometry-based method for the detection of soy in meat products is existing (Leitner *et al.*, 2006). Methods for the HPLC-MS/MS-detection of lupine and pea in meat products are lacking. In order to comply with the EU foodlabeling legislation and to have reliable methods to proof meat adulterations, there is an urgent need for HPLC-MS/MS methods for the simultaneous detection of plant proteins in meat products.

Therefore, a sensitive screening method for the simultaneous detection of lupine (Lupinus angustifolius), pea (Pisum sativum), and soy (Glycine maxima) in meat products applying HPLC-MS/MS has been developed (Hoffmann *et al.*, 2017). After protein extraction and tryptic digestion, 3 to 4 marker peptides for each plant species were measured by HPLC-MS/MS. The whole analytical procedure was optimized. For example, extraction times of 1, 2, 3, and 4 h were tested (Fig. 6).

The effects of increasing extraction times to the different marker peptides were contrary in parts. Finally, an extraction time of 2 h was chosen as a compromise. For matrix calibration, emulsion-type sausages with 0, 1, 6, 32, 160, 800, and 4000 mg/kg raw legume protein isolates/legume flour were produced. The mentioned legumes were detectable in sausages with concentrations of 6 mg/kg legume protein isolates/legume flour or greater. High correlation coefficients ($R^2 > 0.999$) between the peak areas of the mass transitions of the marker peptides and the contents of legume proteins in the meat products were obtained. The limits of detection (LODs) of the method were about 5 mg/kg meat product for pea protein, 4 mg/kg meat product for soy protein, and 2 mg/kg meat product for lupine protein. No false-positive or false-negative results were recorded. The applicability of the described method was tested by analyzing commercial meat products with and without added legume proteins.

2.5. HPLC-MS/MS-detection of microbial transglutaminase in restructured meat

The protein-based binding system transglutaminase (TG) from *Streptomyces mobaraensis* (Activa, Ajinomoto, Japan) for the restructuring of meat is commercially available in powder form and used as a cold-set binder (Flores, Boyle, & Kastner, 2007). The TG (protein-gluta-

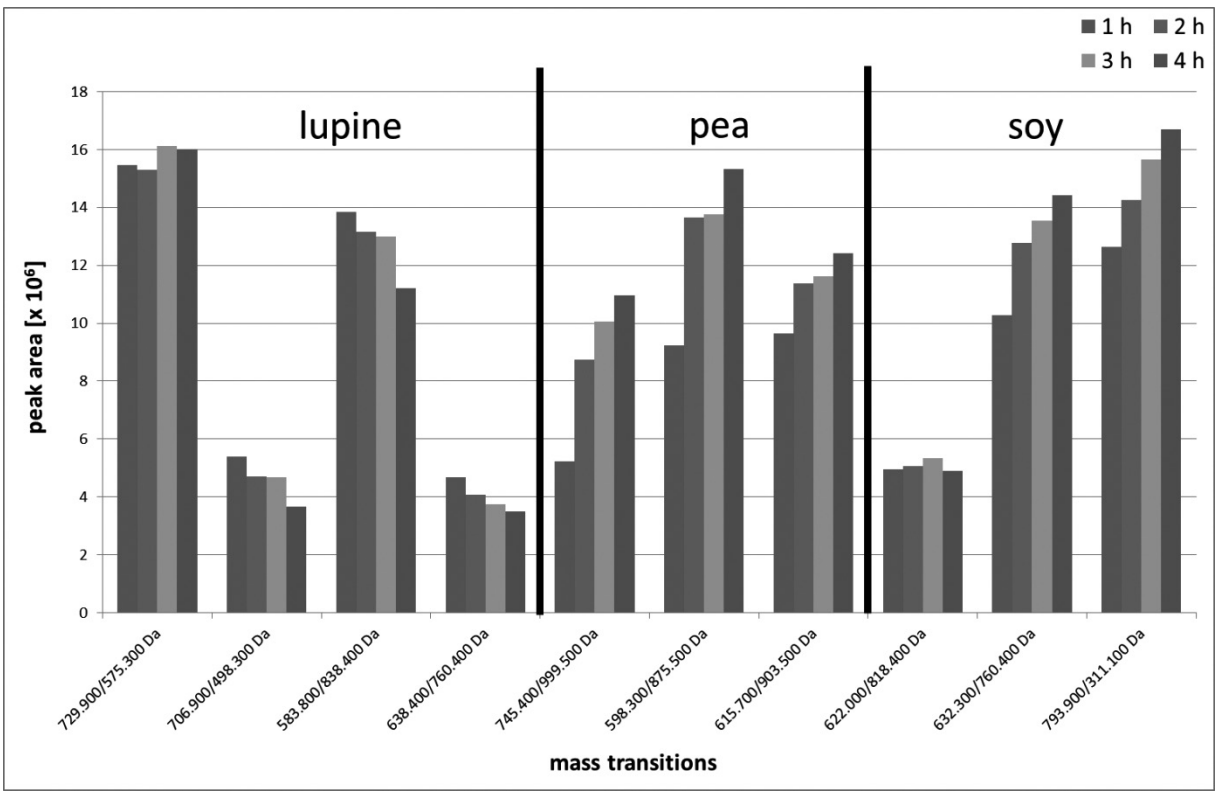

Fig. 6 Peak areas of the marker peptides for lupine, pea, and soy applying different extraction times in emulsion type sausages

mine γ-glutamyltransferase; EC 2.3.2.13) catalyzes the formation of isopeptide bonds between an ε-amino group of a lysine residue and a γ-carboxamide group of a glutamine residue (Yokoyama, Nio, & Kikuchi, 2004), leading to a cross-linking of proteins. The enzyme TG is a monomeric 38 kDa protein containing 331 amino acids and no saccharide or lipid moieties (Kanaji *et al.*, 1993). For the detection of TG in different types of restructured meat (pork, beef, chicken, and turkey), a sensitive HPLC-MS/MS-method was developed using the six unique tryptic marker peptides AETVVNNYIR, EVASVMNR, GAYVITFIPK, LAFASFDEDR, SPFYSALR, and VTPPAEPLDR (Jira & Schwägele, 2017). In comparison to an HPLC-MS/MS-method for the detection of TG using very short (4-6 amino acids) and unspecific TG digestion products of Achromopeptidase lyticus protease (Kaufmann *et al.*, 2012), all tryptic marker peptides used in the developed method were unique and fulfilled the commonly accepted criterion of marker peptides of a size of 6-12 residues (Johnson *et al.*, 2011).

Meat binding experiments were performed with two technical TG mixtures with (Activa EB) and without caseinate (Activa PB). After defatting of the samples of restructured meat with acetone, the protein extraction was performed in TRIS-HCl (1 M, pH 8.2) for 1 h at 100 °C. The tryptic digestion was performed with trypsin for 3 h. Separation of peptides was performed on the HPLC-column Nucleosil 100-3 C18 HD from Macherey-Nagel using a gradient elution with acetonitrile/water. Peptide detection by tandem mass spectrometry was carried out on an AB Sciex QTrap 5500 in the ESI positive mode using the MRM-mode. For each TG marker peptide three mass transitions were used. Extracted ion chromatograms of two TG marker peptides are shown in Fig. 7.

The restructured meat and corresponding blank values (total samples: 62) were analyzed in a raw and heat state. When investigating samples pre-treated with oil marinade, emulsion marinade, seasoning salt as well as breadcrumbs, only very little effects regarding the type of pre-treatment on the detectability of TG were found. Using the four TG marker peptides EVASVMNR, GAYVITFIPK, LAFASFDEDR, and VTPPAEPLDR, no false-positive or –negative results were obtained. The LOD of the developed method was about a factor of 10 below the recommended amount of TG for raw as well as heated restructured meat.

3. Conclusion

Recent analytical developments in meat science referring especially to traceability along the whole food chain are reported in this paper. A status survey on the intake of dioxins and PCBs from meat and meat products, analyzing highly representative samples by GC-MS, shows that the median daily intake of the German consumer (70 kg body weight) concerning these compounds is 4 pg WHO-PCDD/F-PCB-TEQ. This corresponds to only about 3 % of the established tolerable weekly intake (TWI) according to the Scientific Committee on Food (SCF). In Germany about 60 % of meat products are smoked. The use of modern smoke generators leads to low PAH contents far below the maximum levels. In the case of friction smoke the lowest PAH contents are ob-

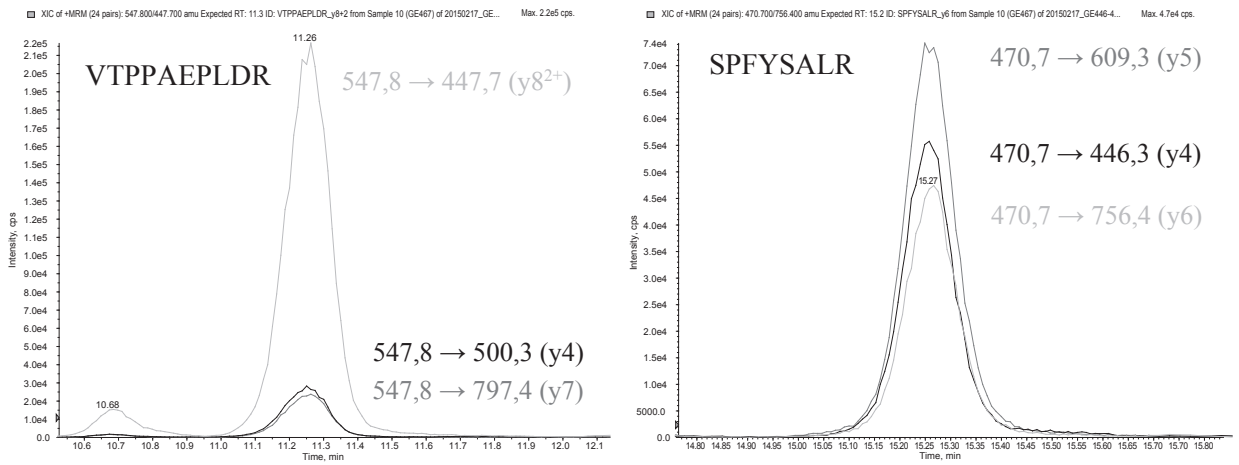

Fig. 7 Extracted ion chromatograms of the TG marker peptides VTPPAEPLDR and SPFYSALR (three mass transitions each)

served. The reduction of the contents of PAH applying modern smoking technologies does not necessarily lead to a simultaneous decrease in the amounts of positive smoke ingredients like phenolic substances. Real-time PCR is a molecular biological technique used to amplify and in parallel to detect or quantify target DNA-molecules. A relative quantitation of the percentage of an animal species in meat products based on a combination of the ratio of a specific system of target gene fragment and reference gene fragment is possible. However, for a quantitation increasing DNA fragmentation by heat treatment as well as the influence of the natural DNA content of different animal tissues has to be considered. Adulterations of meat products can be detected and quantified by means of suitable PCR systems. Even very small amounts of lupine and soy protein in the low ppm range can be dangerous to allergic reacting persons. Using suitable and characteristic marker peptides, the simultaneous detection of lupine, pea, and soy is possible applying HPLC-MS/MS methodology with LODs referring to the reference doses of the VITAL Expert Panel. The development of a multi-method for the simultaneous analysis of various foreign proteins in meat products is intended. Restructuring of meat by microbial transglutaminase is applied in meat industry. On the basis of four characteristic marker peptides, a reliable detection of the use of microbial transglutaminase in meat by means of HPLC-MS/MS appears possible. Heating and other pre-treatment procedures of meat show no negative effect on the detectability of transglutaminase. The developed HPLC-MS/MS method allows a simultaneous detection of transglutaminase and casein, which is present in the commercially available Activa EB formulation.

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