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Doctoral Dissertation

Development of mass spectrometric methods for the identification and quantification of allergenic ingredients in food

submitted by

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in partial fulfilment of the requirements for the academic degree

Doktorin der Bodenkultur (Dr.nat.techn.)

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Affidavit

I hereby declare that I have authored this dissertation independently, and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included.

I further declare that this dissertation has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Vienna, 08. November 2021 Kathrin LAUTER

Preface

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Table of content

Abstract

The doctoral thesis deals with the “Development of mass spectrometric methods for the identification and quantification of allergenic ingredients in food”. Due to the different existing food allergen labelling regulations a set of model food allergens was selected, namely milk, egg, peanut, hazelnut and walnut. This set covers food allergens from animal and plant origin. As immunoanalytical tools are at the moment the commercially available state-of-the-art to measure food allergens but with some methodological restrictions, the analytical focus of this work was the development of an in-laboratory confirmatory method for a selection of food allergens. The development included a common extraction protocol for a clinically-validated matrix and further the development of a validated multianalyte mass spectrometric method for in-laboratory confirmation for the selected food allergens. Therefore, the analytical targets, respectively proteins and peptides were identified and collected with focus on further Mass spectrometric multiplex analysis. As clinically relevant matrix chocolate dessert was chosen, produced and spiked with the selected food allergens. Novel allergen extraction protocols were developed to improve a flexible and reliable multiallergen extraction from the selected matrix. Further in-food proteolytic digestion models were developed for the obtained food extracts prior to MS-analysis. The MS-method was developed accordingly including marker identification, marker selection and MS-parameter optimisation. The final targeted LC-MS/MS method was validated for the detection and quantification of the five above mentioned allergenic ingredients.

Kurzfassung

Die Dissertation befasst sich mit der "Entwicklung massenspektrometrischer Methoden zur Identifizierung und Quantifizierung von allergenen Zutaten in Lebensmitteln". Aufgrund der verschiedenen bestehenden Vorschriften zur Kennzeichnung von Lebensmittelallergenen wurde eine Reihe von Modellallergenen ausgewählt, nämlich Milch, Ei, Erdnuss, Haselnuss und Walnuss. Dieses Set an Lebensmittelallergenen deckt den tierischen und pflanzlichen Ursprung ab. Da kommerziell erhältliche immunoanalytische Methoden derzeit den Stand der Technik bei der Messung von Lebensmittelallergenen darstellen, jedoch mit einigen methodischen Einschränkungen verbunden sind, lag der analytische Schwerpunkt dieser Arbeit auf der Entwicklung einer laborinternen Referenzmethode für die ausgewählten Lebensmittelallergene. Die Entwicklung umfasste ein gemeinsames Extraktionsprotokoll für eine klinisch validierte Matrix und die Entwicklung einer validierten massenspektrometrischen Multianalyt-Methode für die laborinterne Bestätigung der ausgewählten Lebensmittelallergene. Daher wurden entsprechende analytische Targets, d. h. Proteine und Peptide, identifiziert und gesammelt, wobei der Schwerpunkt auf der weiteren massenspektrometrischen Multiplexanalyse lag. Als klinisch relevante Matrix wurde Schokoladendessert ausgewählt, hergestellt und mit den ausgewählten Lebensmittelallergenen versetzt. Neuartige Allergenextraktionsprotokolle wurden entwickelt, um eine flexible und zuverlässige Multiallergenextraktion aus der ausgewählten Matrix zu ermöglichen. Für die gewonnenen Lebensmittelextrakte wurden vor der MS-Analyse weitere Modelle für den proteolytischen Verdau in Lebensmitteln entwickelt. Die MS-Methode wurde entsprechend entwickelt, einschließlich Markeridentifizierung, Markerauswahl und MS-Parameteroptimierung. Die endgültige zielgerichtete LC-MS/MS-Methode wurde für den Nachweis und die Quantifizierung der fünf oben genannten allergenen Zutaten validiert.

1 Introduction

1.1 Allergy

Humans are generally exposed to different foreign substances like chemicals, pollen, house dust, and food components, which are normally harmless, but they can elicit in some sensible individuals hypersensitivity known as allergy (1). This hypersensitivity is mediated by the interaction of two components: allergens (antigens, which elicit allergic reactions), and antibodies (immunoglobulin) (2,3); and consists of two stages, sensitization and elicitation. By the initial contact with the protein, internalization of the foreign protein and production of the antibody takes place. Upon re-exposure, a variety of mediators such as histamines are released that lead to inflammatory reactions and tissue damages known as allergic reactions (4). They include a variety of clinical symptoms appeared in skin, respiratory- and gastrointestinal tract for example urticaria (hives), rhinoconjunctivitis, angio-oedema, hypotension, pruritus, atopic dermatitis, colic, vomiting, diarrhoea, asthmatic wheeze (2,5), and in severe cases the anaphylactic shock.

Table 1: Gell and Coombs (6) classification of allergic reaction types

Type	Alternative name	Mediator	Appearance in	Clinical symptoms
I	Immediate hypersensitivity	IgE	< 30 min	• Urticaria (hives) • Allergic rhinitis and asthma • Gastroenteritis • Anaphylactic shock
II	Antibody dependent cytotoxic hypersensitivity	IgG/IgM	Minutes to several hours	• Autoimmune reactions • Hemolytic anemia • Thrombocytopenia • Goodpasture's syndrome
III	Immune complex hypersensitivity	IgG	3-10 hours	• Arthus reaction • Systemic lupus erythematosus • Serum sickness • Vasculitis
IV	Delayed-type hypersensitivity	T-cells	8-48 hours	• Contact dermatitis • Chronic transplant rejection • Multiple sclerosis • Atopic dermatitis • Allergic rhinitis and asthma

Gell et al. (6) classified the allergic reactions in four groups, see Table 1. Characteristic for type I is a rapid or acute response due to the recognition of allergens by immunoglobulin E (IgE), which leads to elevated levels of this antibody (7). Type I is the only IgE mediated allergy; three other types are non-IgE mediated. Type II and III are mediated by IgG or IgM. Type IV is mediated by CD4 helper T-cells; therefore not an antibody mediated hypersensitivity. Since the clinical symptoms of type IV occurs more than 8 hour after exposure to allergen, it is also known as delayed-hypersensitivity (4). Although there is symptomatic treatment for allergy e.g. antihistamine tablets (8), but there is no cure for it, and only the strict avoidance of allergens by sensible individuals can prevent the allergic reactions (9).

1.2 Food Allergy

Food allergy, an adverse food reaction, is an important public health problem that affects children and adults and may be increasing in prevalence. According to several European and American authors (10–13) food allergies affect up to 2% of the adult population and up to 8% of children. A systematic review by Nwaru et al. from 2014 which includes data of 42 studies showed that the prevalence of cow's milk allergy and egg allergy was higher in younger age groups than older age groups, while the prevalence of peanut allergy, tree nut allergy, fish allergy, and shellfish allergy was higher in the older age groups than in the younger age groups (14). It is difficult to estimate the accurate prevalence of food allergy overall in the world because it depends on geographical region and food culture/culinary habits of different folks or nations (3) for example allergy to milk and peanut is the prevalent food allergy in USA and fish allergy is frequently observed in Scandinavian countries, Spain and Japan (1). In the last decade, the prevalence has risen and also the severity of allergy seems to be increasing, especially in industrial countries (15).

The European Academy of Allergology and Clinical Immunology (EAACI) proposed a classification of adverse reactions to food based on mechanisms, which is shown in Figure 1 (15).

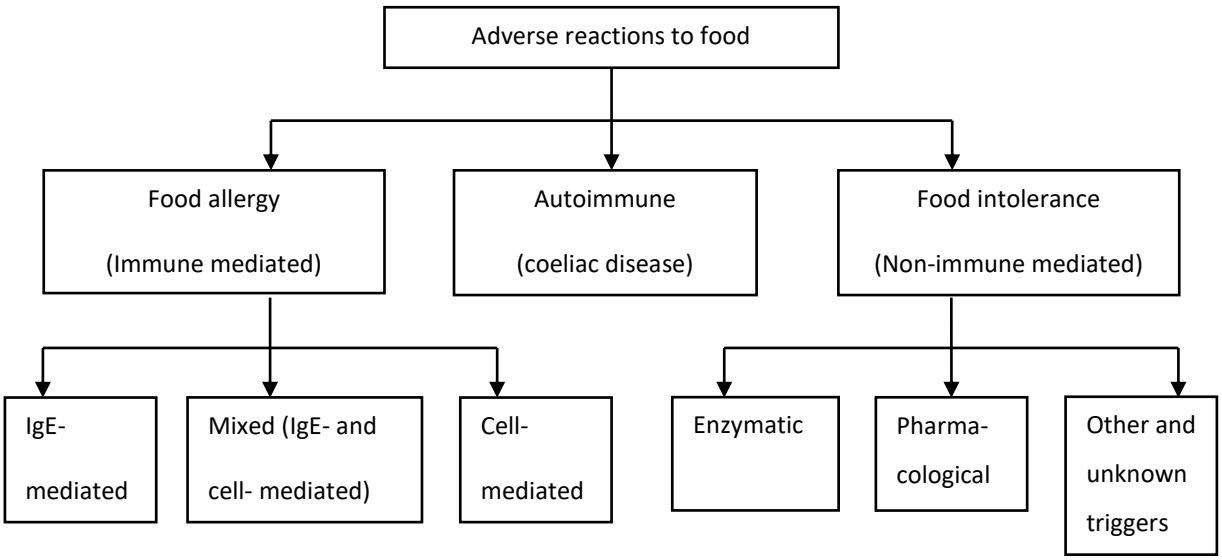


Figure 1:Classification of adverse reactions to food (EFSA NDA Panel, 2014)

Food allergy is defined as an adverse reaction of the immune system that occurs on oral exposure to a given food (16), which can be mediated by food-specific IgE antibodies, by cellular mechanisms or by both (17). The IgE-mediated food allergy may result in immediate reactions (usually within two hours after oral exposure to a given food) and may manifest with a variety of signs and symptoms that can involve one or more target organs, including the skin, the gastrointestinal and respiratory tracts and the cardiovascular system (16). The severity of the reactions can differ from mild (e.g. wheezing, hives or dizziness) to severe (e.g. anaphylaxis). This form includes “typical” food allergies, such as peanut- and cow’s milk allergy. It is not clear why some people develop adverse reaction to certain food or food components. Correlation has been found between the prevalence of food allergy and atopic dermatitis (18) but it does not seem to be clear which promotes the other (“hen-egg-question”). The cell-mediated form of food allergy are typically delayed and occur 2 to 48 hours after ingestion of the offending foods, affecting only the gastrointestinal tract in a mostly chronic way. Most of the resulting disorders (e.g.food protein-induced enterocolitis) resolve before adolescence (19). Coeliac disease is an autoimmune adverse reaction to food triggered by the ingestion of gluten and related to prolamins found in wheat, barley and rye (20). Food intolerances, which are non-immune mediated adverse reactions to food, such as lactose-, fructose- or histamine intolerance, are often caused by enzyme deficiencies or another physical disability to properly metabolize certain food components (18). While food intolerances can be contorolled by limiting the amount of the offending food eaten or administer the missing enzymes prior in form of suplements, with food allergens often strict avoidance is necessary (7,8). The amount of allergen eliciting an allergic reaction can be very small, even trace levels of the offending food can cause reactions, but no sufficient comparable clinical

information is available. Therefore no general threshold value can be given and strict avoidance is the only possibility of risk-free food consumption (21).

For the allergic costumer avoiding offending food means great effort, as labels have to be studied carefully and basic information is necessary to deal with labeling regulations. Some food, respecticely their components, may not be easily identified as such because they may be listed according to their function, e.g. lecithin derived from soy bean as emulsifier of lysozyme as preservative (7).

Currently there are fourteen foodstuffs, see Table 2, listed by the European Union (EU Directive 2007/68/EC Annex IIIa), which had to be labelled only on prepacked food and the way of presenting the labelling was quite open. Since December 2014 the EU Food Information for Consumers Regulation (EU FIC) came into force. Allergen information has to be on the label, if they are used as ingredients in a pre-packaged, sold loose or served food (outside home). As there is no cure for food allergy, allergic individuals must avoid what they are allergic to and be vigilant at all times, especially when purchasing food products or when dining away from home.

Table 2: Food allergens and products thereof listed in the EU Directive 2007/68/EC Annex IIIa

Cereals containing gluten	Soybeans	Sesame seeds
Crustaceans	Milk	Lupin
Eggs	Nuts	Molluscs
Fish	Celery	Sulphur dioxide and Sulphites*
Peanut	Mustard	

***does not belong to the group of actual food allergens, but was added to the list due to its close relation with release of histamine.**

1.3 Food Allergens

Sometimes trace amounts of offending food can cause adverse reactions in susceptible individuals and the threshold levels are different from patient to patient, which makes food allergy a very individual issue. The food allergens are classified into major and minor allergens. If 50% of specific IgE bind the allergen, it defines as major allergen; ≤ 10% refers to minor allergens. The major food allergens are seldom eaten raw. Normally they undergo different kind of processing such as grinding, drying, heating, chilling, fermentation, hydrolysis and purification, before they are bought by consumers (pasteurization or sterilization of milk and roasting of nuts are two special examples), or they are cooked before eating (5). All these processes can alter the structure of allergens and so change the allergenicity. It can result in decreasing of the allergenicity by destruction of proteins and changing of the 3-D structure or interaction of epitopes due to cleavage of the allergens, or

respectively increasing the allergenicity as a result of revealing hidden epitopes and therefore a better accessibility (22). It has to be considered, that certain proteins are more resistant to chemical or thermal treatment and denaturating conditions such as pH changing or high pressure; for example, the prolamin superfamily, whose proteins are capable to build disulphide bonds because of presence of six or eight cysteine residues. Some other proteins have the ability to refold themselves again after these treatments and so retrieve their allergenicity (15).

Generally, any molecule that enables to elicit the production of antibodies and can react with them is an allergen; however these molecules are mainly from biological sources and the majority of them are proteins (3,23), which share some functional and physicochemical properties. It was discussed if these molecular characteristic modifications play a role for their allergenicity. For example, the allergens show often the ability to form disulfide bonds and also different types of ligands, to aggregate or form oligomers, and they are usually glycoproteins. Precisely considered, all of these named properties may lead to stability of allergens; it means they can maintain their natural 3-D structure or they can refold after thermal or proteolytic treatment (24). These normally heat- and digestion resistant allergens can elicit symptoms in the gastrointestinal tract and also sever anaphylactic reactions. However those allergens, which are labile to gastric digestions and heat treatment, trigger symptoms that are often restricted to oral allergy syndrome and mild local symptoms in skin and respiratory tract (25,26). Another common property of allergens is their ability to interact with cell membranes or some lipid structures that result in plant protection against pathogens (24).

Food allergens include proteins or glycoproteins that have a molecular weight of 5-100 kDa (27), but the antibodies can recognise only a specific part of them, which is known as epitope (28). Theoretically, any protein could act as allergen and sensitize the immune system; however, 90% of most severe IgE mediated food allergic reactions are elicited by eight main commodities: cereals containing gluten, crustaceans, hen's egg, fish, peanuts, soybeans, cow's milk, and tree nuts (3,7). In early childhood, egg white and cow's milk are the major allergens (15), but in most of cases the children can outgrow these allergies (23) and can tolerate them at the age of three (29). Many of these major allergens are well investigated (3); the amino acid sequences are identified, the proteins are characterised and even 3D structures are known in some cases. This information is collected in several allergen databases such as listed in Table 3.

Table 3: Actively updated current allergen sequence databases (30)

Name	Maintained by	URL	Update frequency
WHO/IUIS Allergen Nomenclature Database	WHO/IUIS Allergen Nomenclature Sub-Committee	www.allergen.org	Continuous
AllergenOnline (FARRP Allergen Database)	Food Allergy Research and Resource Program, Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE, USA	www.allergenonline.org	Annual
Comprehensive Protein Allergen Resource (COMPARE)	Protein Allergens, Toxins and Bioinformatics Committee, Health and Environmental Sciences Institute	comparedatabase.org	Annual
Allergome	Allergy Data Laboratories, Latina, Italy	www.allergome.org	Continuous
AllerBase	Bioinformatics Centre, Savitribai Phule Pune University, India	bioinfo.net.in/AllerBase/Home.html	Weekly

In other protein databases like UniProt (31) valuable data can be found about allergens and their sequences and functions.

Table 4: Classification of food allergens according to their origin (25)

Origin	Major family	Subfamilies	Function	Some examples
Plant	Prolamin	Prolamin superfamily	Storage protein	Tri a 19, Sec c 20
		2S albumin	Storage protein	Sin a 1, Ber e 1
		nsLTP	Plant defence system	Pru p 3, Mal d 3
		α-amylase/ protease inhibitor	Plant defence system	Hor v 15, Sec c 1
	Cupin superfamily	7S globulin	Storage protein	Ara h 1, Jug r 2
		11S globulin	Storage protein	Ara h 3, Cor a 9
	Bet v 1		Storage protein	Mal d 1, Pru av 1
	Profilin		Regulator protein	Mal d 4, Pru av 4
Animal	Tropomyosin		Regulator protein	Pen i 1, Hom a 1
	Parvalbumin		Ca^{2+} -binding	Gad c 1, Sal s 1
	Caseins		Ca^{2+} -binding	Bos d 8

According to sequence identity (30% and more) or to structure homology and similar functions, the proteins were divided into different families, or into superfamilies if lower sequence similarity but common evolutionary origin appears (8).

Pfam 25.0, a database that collected and classified proteins to different families, showed 12273 protein families in March 2011 (32). However, comparing the data obtained from different allergen databases proved that the allergens are restricted to certain families and not randomly distributed among the various protein families (15). Additionally 29 protein families include more than one food allergen (33). It seems that this restriction is valid also for the other allergens, for example pollen allergens belong only to 29 protein families (15). This structure homology can apparently be a reason or at least play a role for cross-reactivity between different allergens; more than 50% sequence identity can be decisive for cross-reactivity (25).

The allergen terminology was regulated according to the accepted taxonomic name of their source: the genus is represented by its three first letters, followed by a space, and then the species is shown with its first letter, again a space, and finally a sequential number of entered new allergen (34). Writing in italics is used for indication of allergen encoding genes (35). The food allergen can be classified into allergens from plant or animal sources (Table 4).

1.4 Allergens of Plant Origin

Plant proteins, that can cause allergenic reactions are limited to 27 protein families; however, 65% of plant proteins belong to only 4 families: profilin (actin-binding protein) and Bet v 1 families, prolamin and cupin superfamilies (18,25). Common plant food allergens are summarised in Table 5.

The majority of allergens from Bet v 1 family belong to either Rosaceae fruits such as Mal d 1 in apple, Pru av 1 in cherry, Pru ar 1 in apricot, and Pyr c 1 in pear or Apiaceae vegetables for example Api g 1 in celery and Dau c 1 in carrot (8,15). They show often cross-reactivity to pollen (36) and specially to birch pollen. Since they are heat and digestion labile (15), the mild oral allergy syndromes especially itching and swelling of lips are mostly observed reactions (37).

The prolamin superfamily includes heat- and digestion-resistant proteins with low molecular weight (MW). They have a characteristic eight cysteine skeleton and are rich in α-helices stabilized with disulfide bridges (25). Despite this structure similarity, they show few sequence identities. This superfamily divided into three groups of major food allergens (prolamin seed storage proteins, 2S albumins and non-specific lipid transfer proteins (nsLTPs)) and the less important group of cereal α-amylase/protease inhibitors, which included allergens from cereals such as wheat, barely, rice, rye, and corn (15) and is associated with baker asthma (38). The major allergens Tri a 19 from wheat, Sec c 20 from rye, and Hor v 21 from barley belong to prolamin seed storage proteins (8), which include 50% of total protein contents in cereal kernels (25). These sulphur rich proteins (26) are also rich in proline and glutamine, whose combination gave the name prolamin to this family. Many storage proteins of dicotyledon species belong to 2S albumins (8); and a variety of them are characterised as major allergens in seed and tree nuts, such as Ara h 2 and 6 from peanut, Jug r 1 from walnut, Ber e 1 from Brazil nut, Sin 1 from yellow mustard (15,25), Bra j 1 from oriental mustard, and Bra n 1 from rape (8).

Table 5: Common allergenic foods from plant source and their major allergens

Food		Major Allergens	Protein Family	MW
Tree nuts	Hazelnut	Cor a 1.04	Bet v 1 family	17.4
		Cor a 2	Profilins	14.1
		Cor a 8	nsLTPs	9.5
		Cor a 9	Legumins	59.1
		Cor a 11	Vicilins	45.1
	Brazil nut	Ber e 1	2S albumins	12.2
		Ber e	Legumins	52.3
	Walnut	Jug r 1	2S albumins	16.4
		Jug r 2	Vicilins	48.3
		Jug r 3	nsLTPs	10*

	Almond	Amandin	Legumins	63.0
	Cashew	Ana o 1	Vicilins	61.8
		Ana o 2	13S globulin	50.5
		Ana o 3	2S albumins	16.3
Peanuts		Ara h 1	Vicilins	67.7
		Ara h 2	2S albumins	19
		Ara h 3&4	Legumins	58.3
		Ara h 5	Profilins	14.1
		Ara h 6	2S albumins	16.9
		Ara h 7		18.4
		Ara h 8	Bet v 1 family	17.0
Soybeans		β-Conglycinin	Vicilins	63.2
		Gly m 3	Profilins	14.1
		Gly m 4	Bet v 1 family	16.8
		Gly m Bd 28k	Vicilins	50.4
		Gly m 1&Bd 30k	Papain-like cysteine proteases	42.8
		glycinin	Legumins	55.7
Celery		Api g 1	Bet v 1 homologues	16.3
		Api g 4	Profilins	14.3
		Api g 5	FAD binding oxidases	9.4
Cereals	Wheat	Tri a 19	Prolamins seed storage proteins	53.0
		Tri a Bd 36K	PR-9 plant protein	8.4
		CM3	α-amylase/protease inhibitors	18.2
		α - gliadins		36.5
	Rice	RAP		14.5
	Maize	Zea m 14	nsLTPs	9.1
	Rye	Sec c 1	α-amylase/protease inhibitors	2.9
		Sec c 20	Prolamins seed storage proteins	-
	Barley	Hor v 1&15	α-amylase/protease inhibitors	16.5
		Hor v 21	Prolamins seed storage proteins	33.2
Mustard		Sin a 1	2S albumins	14.2
		Bra j 1		14.6
Sesame		Ses i 1		17.5
		Ses i 2		17.5
		Ses i 3	Vicilins	67.1

The MW (kDa) referred to calculated mass, the experimental masses were mentioned with * (26,39,40)

Like Bet v 1 family, the profilins are not stable to thermal treatment and enzymatic digestion and their symptoms are restricted to oral allergy syndromes (26). Although, the individuals, who are allergic to pollen protein from profilins, are also hypersensitive to a wide range of the other dietary profilin proteins (8). For example, allergic persons to grass pollen show cross-reactivity to peanut, tomato, celery, and carrots (due to profilin-specific IgE) (26); and allergic individuals to tree pollen are also sensible to celery, carrots, apple, pear, and potato (8). Allergens of this group were identified in a wide

range of fruits and nuts like Pyr c 3 & 4 (estimated to be the same) from pear, Pru p 4 (Pru p 4.01 & Pru p 4.02) from peach, Gly m 3 (Gly m 3.0101 & Gly m 3.0102) from soy, Ara h 5 from peanut, Ana c 1 from pineapple, Pru av 4 from cherry, Api g 4 from celery, Mus xp 1 from banana, Cap a 2 from bell pepper, Cuc m 2 from melon, Lyc e 1 from tomato (40), Cor a 2 from hazelnut, and Lit c 1 from litchi (26).

Characteristic for this group are four disulfide bridges that could be the reason for their thermal and digestion resistance (25). It is worthy of mention that Pru p 3 is more stable under acidic rather than neutral condition; it cannot refold after heat treatment under neutral conditions, but apparently at pH 3 (41). The cross-reactivity between pollen and food allergens from this family has been seldom observed (25). But Pastorello et al. (42) found a very high cross-reactivity among the nsLTP food allergens from the Prunoideae subfamily (peach, apricot, plum and apple), whose similarity is about 95%, and also between maize and peach (despite their botanically unrelated family). The individuals, who suffer from peach allergy, show also allergic reactions to maize; something that is apparently due to structure homology of nsLTP proteins from maize and fruits from Rosaceae family (15). Pastorello et al. (43) observed high cross reactivity between Cor a 8 and Pru p 3 as well, but it was denied by Gaier et al. (41). They could show that Pru p 3 was recognised by polyclonal anti-Mal d 3 antibody from rabbit serum, however not by anti-Cor a 8.

The cupin superfamily, whose proteins include characteristic β-barrel structural domains (26), is classified into vicilin 7S seed storage globulin family and legumin 11S globulin protein family (15) according to their sediment coefficient factors. The 7S globulins are normally trimeric and 11S are hexameric proteins (4).

Allergens belonging to the legumins are rarely glycosylated. Since cysteines are absent in vicilins, they cannot build disulfide bridges (8,26). These allergens could be characterized in many nuts, seeds and legumes. Some major allergens could be identified as vicilin, e.g. Ara h 1 in peanuts, β-conglycinin in soybean, Ana c 1 in cashew nuts, Jug r 2 in walnut (4), Len c 1 in lentils, Ses i 3 in sesame (8), and Cor a 11 in hazelnut. Some legumins, also characterised as major allergens, are: Cor a 9 in hazelnut, Cocosin in coconut (40), Ara h 3 and 4 in peanut (assumed to be the same allergen (26)), glycinin in soy, Amandin (almond major protein) in almond (4). Although there is homology between 11S globulin proteins from different legumes, this sequence homology is more definitive for 7S globulin proteins, where the variable domains are mainly found within the N- and C- terminal regions and not in the inside parts of the sequences; whereas in 11S proteins only the β-polypeptide is conservative and the α-polypeptide is variable (44). This homologous structure can cause allergic cross reactivity among these nuts; however it is not a guaranty for cross reactivity (45). Goetz et al. (46) could show strong cross-reactivity of hazelnut to walnut and pecan; and moderate cross-reactivity to cashew, Brazil nut,

pistachio, and almond. Also de Leon et al. (47) could evidence the cross-reactivity between peanut and three tree nuts (almond, hazelnut, and Brazil nut). In another study, Koppelman et al. (48) showed the homology between glycinin- and Ara h 3-epitops. This could explain why IgE from peanut-allergic individuals bind to both subunits (26) and often cross-reactivity between soy and peanut was reported (49).

1.4.1 PEANUT

Peanut is a member of the legume family that includes pea, bean, soybean, lupine, lentil and fenugreek. Ara h 1 (vicilin, 63.5 kDa) is a 7S globulin that belongs to the cupin superfamily; Ara h 2 (conglutin, 17 kDa) is a member of the prolamin superfamily; Ara h 3 (glycinin, 57 kDa) is a 11S globulin of the cupin superfamily; they are the major peanut allergens (50). Ara h 4 is an isoallergen form of Ara h 3. Peanut contains around 29% protein; Ara h 1 is the most abundant protein and accounts for approximately 12-16% of the total protein (48). About 10% of the total protein content is Ara h 2, whereas Ara h 3/Ara h 4 are also abundant allergens (51).

Minor allergens are Ara h 5 (profilin,15 kDa) and two conglutinin-homologous proteins Ara h 6 (15 kDa) and Ara h 7 (15 kDa) (50). Ara h 2 has high sequence homology with Ara h 6. As a result of their stability to heat and gastrointestinal digestion, many allergens of the prolamin superfamily may account for severe allergic reactions. Furthermore, Ara h 8 (17 kDa) has shown a cross-reactivity with birch pollen allergens, (i.e. Bet v 1) (52). Ara h 9 (9.8 kDa) is a ns-LTPs. Ara h 10 (16 kDa) and Ara h 11 (14 kDa) have recently been recognised and belong to the oleosin superfamily. Allergenic oleosins are found in legumes, nuts, and seeds.

1.4.2 HAZELNUT

Allergens of hazelnut can be classified as pollen-related and non-pollen-related. The first hazelnut (Corylus avellana) allergen identified was Cor a 1. Cor a 1 (17 kDa) and Cor a 2 (14 kDa), which are similar to other plant profilins, are both homologues with the major pirch pollen allergen Bet v 1 (53). Cor a 8 (9 kDa) and Cor a 14 (15-16 kDa), 2S albumins, represent two members of the prolamin superfamily associated with hazelnut allergy (44, 55). Severe forms of hazelnut allergy are related to two allergens of the cupin superfamily: Cor a 9 (40 k Da), a 11S legumin, and Cor a 11 (48 kDa), a 7S vicilin (20). The role of Cor a 12 (17 kDa) and Cor a 13 (14-16 kDa), two oleosins identified as hazelnut allergens, remains to be established (55).

1.4.3 WALNUT

There are several walnut allergens that can provoke allergic reactions. According to Teuber *et al.* (1998 and 1999) Jug r 1 (14 kDa) is a protein that belongs to the 2S albumin family with its subunits joined by disulfide bridges and Jug r 2 (44 kDa) that belongs to the vicilin-like protein family. Teuber *et al.* (1998) found Jug r 1 to be a major allergen in patients' sera tests. Two other walnut allergens are Jug r 3 (9 kDa), a lipid-transfer protein, and Jug r 4 (58 kDa), an 11S legumin-like globulin. Jug r 1 and Jug r 3 appear to be the most potent allergens of walnut (56). (20)

1.5 Allergens of Animal Origin

The major food allergens from animal origin are milk, egg, and different species of sea animals, whose allergens are restricted to even fewer protein families compared to the plant allergens (8,15). Common animal food allergens are summarized in Table 6. Generally, three protein families: caseins, parvalbumins, and tropomyosins were reported to be predominated in animal food allergens (25).

The major seafood allergens belong either to parvalbumin or tropomyosin families. Both of them are resistant to enzymatic digestion and thermal denaturation (25). Thermostable tropomyosins, with highly conserved domains and identified sequences that lead to cross-reactivity (8), could be found in both important food allergens: mollusc and crustacean. Parvalbumins from calcium-binding EF hand protein family can be found in high concentrations (up to 5 mg/g) in white muscles of fish (15). Although they generally show high resistance to heat treatment, enzymatic and chemical denaturation, the ability of IgE binding is reduced strongly after processing (57). Nonetheless it seems that enough epitopes persist after cooking to trigger allergic reactions (24) and the allergenicity risk is not eliminated necessarily after enzymatic digestion. The cross-reactivity between the parvalbumin from fish and amphibians was reported (25).

Table 6: Common allergenic foods from animal source and their major allergens

Food		**Major Allergens**	**Protein Family**	**MW**
Milk		Bos d 4	Glycoside hydrolase family 22	14.2
		Bos d 5	Lipocalins	19.9
		Bos d 6	Serum albumins	69.3
		Bos d 7	Immunoglobulins	-
		Bos d 8	Caseins	25.1
		Lactoferrin	Transferrins	78.1
Fish	Cod	Gad c 1	Parvalbumins	11.4
		Gad m 1		-

	Carp	Cyp c 1		11.4
	Salmon	Sal s 1		11.9
Crustaceans	Shrimp	Pen i 1	Tropomyosins	34*
		Par f 1		39*
		Pen a 1		32.7
		Met e 1		34*
	Crab	Cha f 1		30.4
	Sessile	Bal r		38*
	Krill	Eup s 1		38*
		Eup p 1		38*
	Lobster	Pan s 1		31.7
		Hom a 1		32.9
Molluscs	Oyster	Cra g 1& 2		26.9
	Snail	Tur c 1		16.8
		Hel as 1		32.6
		Hel a 1		36*
	Squid	Tod p 1		38*
	Mussel	Per v 1		-
	Abalone	Hal m 1		38*
Egg		Gal d 1	Serine protease inhibitors	22.6
		Gal d 2	Serpins	42.8
		Gal d 3	Transferrins	77.8
		Gal d 4	Glycoside hydrolase family 22	14.3
		Gal d 5	Serum albumins	69.9

The MW (kDa) referred to calculated mass, the experimental masses were mentioned with *. In case of caseins, the MW of β-casein was given [21] [25] [26] [27]

1.5.1 BOVINE MILK

Bovine milk contains 3–3.5% of proteins that can be divided into two main classes: caseins (80%) and whey proteins (20%) (58). The major allergens are the following: four casein allergens (Bos d 8, 20–30 kDa) and the two whey proteins beta-lactoglobulin (Bos d 5, 18 kDa) and alpha-lactalbumin (Bos d 4, 14 kDa). Also the other milk proteins with low concentration trigger allergic reactions in susceptible individuals (59), for example Bos d 6 (bovine serum albumin), which consist of 1% of whole milk protein, is the other milk allergen with minor designation.

The casein fraction, which is from the coagulum (curd) fraction, comprises of four proteins coded by different genes carried on the same chromosome: αS1-casein (Bos d 9, 23.6 kDa), αS2-casein (Bos d 10, 25 kDa), β-casein (Bos d 11, 24 kDa) and κ-casein (Bos d 12, 19 kDa). In a majority of cases of milk allergy, caseins, beta-lactoglobulin and alpha-lactalbumin, are the key sensitizing molecules. Cow's milk allergens retain their allergenicity after thermal processing, the reason for still being dangerous

for consumers with milk allergy. Casein is reported to be more thermostable, whereas β-LG demonstrates a thermo-labile behaviour. (7,20,60,61) A significant reduction in allergenicity to milk was observed after boiling at 100°C for at least 10 minutes. Norgaard *et al.* (1996) showed inactivation of beta-lactoglobulin by using this protocol whereas caseins were still capable to induce positive reactions in the skin prick test.

1.5.2 HEN'S EGG

The major allergen identified in egg white is ovomucoid (Gal d 1, 28 kDa) from Kaza-type serine inhibitor family (11% of egg white) (15). Since ovomucoid is highly glycosylated, it is resistant to enzymatic digestion (25). The other major allergens of egg white are ovalbumin (Gal d 2, 44 kDa), ovotransferrin (Gal d 3, 77 kDa) and lysozyme (Gal d 4, 14 kDa)(1,8). The thermo-stabile glycosylated ovalbumin with 54% is the most abundant protein of egg white. Ovotransferrin with 12-13% is a minor allergen, which can bind iron and build disulfide bonds, nevertheless it is not very stable to denaturants, but resistant to heating. Lysozyme is only a minor allergen (3.5% of egg white). It shows the highest thermo-stability at pH range 3.5-5. The hens' egg allergens cross react almost only with the egg allergens from other avian (1).

The major allergenic component of egg yolk is alpha-livetin (identical to chicken serum albumin, 70 kDa) (22). Allergic reactions to egg white occur more often than to egg yolk (62,63), with the predominant egg white allergen being ovomucoid (63,64). Hen's eggs are used in several processes, therefore posing a threat for both food and drink consumption (61). Egg allergens are not only an issue in baking but also in the wine industry, where red wines, rich in tannins, are treated with egg white for fining purposes.

1.6 Legislative background and the lack of reference doses

In order to help patients avoid such offending foods, a list of priority allergenic foods was identified by the Codex Alimentarius Commission [European Regulation (EU) No. 1169/2011], which recommended these allergenic foods to be listed on ingredient labels of pre-packaged foods irrespective of the level at which they might be included in a recipe (65). These recommendations have now been implemented into local food-labelling regulations across Europe, with an amendment in 2014 in the European Union (EU) [amendment No. 78/2014], including a list of ingredients and products made with these ingredients which can cause allergies or intolerances in sensitive individuals upon oral consumption. Worldwide similar regulations were implemented with slightly varying lists of allergenic foods depending on the regional/national differences of the allergic population.

Although mandatory allergen labelling has helped allergic consumers to avoid problem foods, accidental contamination of foods with allergenic ingredients not declared (e.g. happening due to the use of common food-processing lines), still poses uncertainties for the consumers. As a result of such unintended allergen presence, precautionary allergen labels (PALs) are often applied to warn consumers of the potential risks such allergens might pose (66). “May contain x” or “made on shared equipment with x” are examples for common used PALs. The lack of agreed reference doses has resulted in inconsistent application of PAL by the food industry and in levels of contamination that prompt withdrawal action by enforcement officers (66). The excessive use of PAL (67) and the poor relationship between the presence or absence of PAL and actual reaction risks (66) result in the fact that some food-allergic individuals are even ignoring these advisory statements (68). Current regulations do not address the use of precautionary statements, which continues to be an issue for allergenic consumers and the food industry. It is recommended that consumers heed precautionary statements and refrain from purchasing or consuming products that contain or may contain their allergen. However, studies have shown that some consumers are making purchasing decisions based on the type of precautionary statements used on a product, which is becoming a growing concern for health authorities. The food industry also faces challenges in terms of the use of precautionary statements and the need for allergen thresholds, which poses another issue for consumers, as many of them do not understand the concept or use of thresholds. In order to address this issue and move forward, a risk-based approach is needed, which includes the collaboration between all key stakeholders, including food scientists, industry, government, clinicians and researchers, patient groups and consumers.

An expert panel established updated Reference Doses for 11 allergenic food residues as a part of the VITAL (Voluntary Incidental Trace Allergen Labelling) program of The Allergen Bureau of Australia &

New Zealand (ABA) (69). Since 2011 they updated their suggestion of reference doses considering new data from clinical studies. The reference doses of VITAL 3.0, listed in Table 7 as well as those of the previous model VITAL 2.0, orientate on the ED_{01} (eliciting dose in mg protein predicted to provoke a reaction in 1% of the individuals with a specific food allergy) and the 95% lower confidence interval of the ED_{05}.

A year later a European group (70) investigated threshold dose distributions for the 5 major allergenic foods hazelnut, peanut, celeriac, fish, and shrimp in European study populations to contribute to the development of reference doses for allergens.

Table 7:The following table gives an overview of the previous reference doses according to VITAL 2.0 and the next generation reference doses according to VITAL 3.0.

Allergenic commodity	Reference dose in mg of protein	
	VITAL 2.0	VITAL 3.0
Peanut	0.2	0.2
Hazelnut	0.1	0.1
Walnut & Pecan	0.1	0.03
Milk	0.1	0.2
Egg	0.03	0.2
Soy flour	1.0	0.5
Wheat	1.0	0.7
Cashew & Pistachio	2.0	0.05
Mustard	0.05	0.05
Lupin	4.0	2.6
Sesame seed	0.2	0.1
Crustacea	10	25
Fish	0.1	0.3
Celery	none	0.05
Hazelnuts, Almonds, Brazil nuts & Macadamia	0.1	0.1

1.7 Detection methods for allergens in food

Food allergy is more often elicited by eating processed foods containing allergenic material due to cross contamination during ingredients storage or unproper cleaning of the factory than by eating pure allergenic foods. During the manufacturing process allergens undergo various physical and chemical modifications such as unfolding, aggregation, hydrolysis, or covalent modification. (9,71,72) Regarding the above-mentioned reasons, it is of high relevance to detect allergens in food reliably independent from the processing state of a food. The detection methods must not necessarily target

the allergen itself; the detection of each component, which is characteristic for the allergenic food, can deduce the presence of this allergen (15). Detection of major contents of soluble proteins that likely include the allergenic protein leads to increased sensitivity of the assay and makes the determination of trace amounts of the allergen possible. Recently, in the approaches developed for the detection of allergens in food products, the tendency to use a marker rather than the allergenic protein itself rises. Theoretically, these markers can be any component, which is allergen specific (5); but these markers are often peptides (normally a conserved part of protein sequence that preferably does not occur in the other proteins). To guarantee the specificity of the marker, extensive database searching is necessary. Using multi-analyte methods, which target various markers and measure different transitions, is also advisable to increase the sensitivity of the detection method.

1.7.1 ELISA and PCR

The two main used methods for allergen detection along the food chain and food production are Enzyme linked immunosorbent assays (ELISA) (immunobased) and Polymerase chain reaction (PCR) (DNA-based) methods. Since the immunobased methods are rapid, sensitive and selective, they were used in recent years for developing several test kits. Especially fast ELISA test kits and immunochromatographic tests make the food monitoring for allergen ingredients or cross-contamination easy (73). A major characteristic of immunological methods is the fact that the detected epitopes are usually not known and cross-reactivity with matrix components as a main drawback can result in false positive results. The reliability of detection strongly depends on specificity and stability of the employed antibodies and can be affected by the changes induced on protein structure by thermal or other technological treatments. Typical limits of detections (LOD) of the tests based on ELISA kits are in the range of 1–5 ppm (74). The performance of immunological methods (ELISA and lateral-flow devices) can be adversely affected by issues of cross-reactivity (false positives), hook effects (false negatives), and extensive food processing (75,76).

The ELISA detects the protein component of an allergenic food, so the actual threat. In comparison to that, PCR as an DNA based assay, can be seen as indirect measurement of the hazard. Nevertheless, PCR is an alternative for those foods where no ELISA is available or for foods, where the matrices render the allergen undetectable by ELISA.

1.7.2 LC-MS/MS

Seeking a reference method out of the pool of available methods, at the moment mass spectrometry (MS) seems the only possibility for protein measurement. It overcomes the pitfall of the lack of metrologically traceable analytical values regarding the quantification of allergenic foods and therefore it is also benchmarked to serve as reference method in legal cases of dispute.

Two approaches can be used: the analysis of intact proteins (77) or the analysis of prior tryptic digested proteins (51,78–81). Both approaches do have their advantages and limitations. As intact proteins vary in molecular weights within a range from 10 kDa to more than 150 kDa, the measurement of the whole molecules by mass spectrometry is limited as the accuracy of mass detection works more precise for smaller molecules.

Instead of measuring the molecule itself, the ions are measured by MS analysis, because the manipulation of the direction and motion of ions is easily manageable by applying electric and magnetic forces. The basic concept of MS, which involves three main steps, starts with the first step, the ionisation in an ion source, where an electron or proton is removed from the analyte and the ion is produced. The fragmentation of these ions and building of product ions is also possible. In the second step, these ions are separated and measured with a mass analyser according to their mass to charge (m/z) ratio. In the last step, the obtained data is amplified and displayed in form of mass spectra (82).

The publication ratio for the detection of food allergens, especially the quantitative detection, lies more on the side of peptide measurements. Selectivity and specificity, always in discussion for the immunoanalytical approach, is also in mass spectrometry a major requirement. Both can be increased, if multiple target peptides are chosen which leads into the direction of a multianalyte method based on SRM (selected reaction monitoring) measurements for simultaneous determination of different peptides. In the last years, the number of applications of MS techniques in this field has considerably increased for unambiguous identification and accurate quantification of proteins and peptides (77–79,83–85). LC coupled by electrospray ionization (ESI) source to MS/MS detection is a powerful technology for the simultaneous quantification of multiple peptides in complex matrices. It represents a direct detection method for defined allergen sequences providing highly multiplexed allergen detection. (51,83,86–89)

The target quantification of signature peptide(s) as a surrogate marker for the precursor protein using LC–MS technology has several proven advantages, including specificity, sensitivity, and a broad dynamic range that may span four or five orders of magnitude (90). The disadvantage of this method

is the need of expensive special equipment; its advantage is the possibility of simultaneous detection of different allergens in a single run (5).

Targeted approaches in MS relay on the availability of protein sequences from databases like listed in Table 3. Protein sequences often derive from genomes, but in general, there is a lack of such sequences especially for plant derived allergenic foods not included in the “Big Eight”. The number of identifiable species-specific peptide markers is tremendously restricted by required performance characteristics for MS analysis like published by Downs & Johnson 2018. More recent, after the research work of this theses has been performed, the “Allergen Peptide Browser” (92) was published, an interactive database that facilitates peptide selection for targeted selected reaction monitoring (SRM) / multiple reaction monitoring (MRM) experiments using an aggregation of mass spectrometry data published in the field of food allergen detection.

However, it has to be stated that databases are only as good as the already introduced data and they are subjected to permanent development, changes and revisions. Therefore, the databases have to be searched continuously for any appearing new entries, improvements or changes.

The targeted MS/MS approach can be quantitative when gravimetrically added isotope-labelled target analytes are used as an internal standard. This helps to mitigate the effect of instrumental fluctuation, such as interference in SRM ion transition and signal suppression caused by matrix components due to the measurement of relative intensities of the peak areas. Synthetic analogues of the signature peptides used for calibration enable the concentration determination of unknown natural peptides. While mass spectrometers and chromatographic systems from different vendors can provide different measured responses (93), both the use of common calibrants as well as the peptide ratio measurement allow comparing measurements in different laboratories, under different conditions and using different equipment. More importantly, the use of labelled molecular analogues in targeted MS analysis provides traceable results (94).

1.7.2.1 Sample Preparation

There are some factors, which affect the quantitative results. One is the choice of an appropriate extraction buffer that affects the extraction yield: the higher extraction efficiency result in more reliable quantification results. The aim of extraction is the solubilisation of target proteins for further use as immunogens or standards for calibration or for analysis. The proteins can be solubilised in different buffers (albumins in aqueous buffers, globulins in saline buffers, and prolamins in a mixture of water and alcohol); therefore, there is not a universal extraction buffer for all food allergens (95).

Adding of additives such as surfactants or reducing agents to extraction buffers can improve the extraction yield (15). However, it is important that these additives do not manipulate the results, for example the use of fish gelatine is not advisable, if the results deal with fish allergens or another allergen, that can cross react with fish allergens. The other factor is the food matrix that can either influence the detection of analyte or make the extraction difficult (5); an example is chocolate where polyphenols can mask the peanut proteins and reduce the extraction yields down to 50%. To overcome this problem, usage of skim milk powder is often recommended.

1.7.2.2 Liquid Chromatography

Liquid chromatographic separation systems are the common used methods for the analytical and preparative separation of food proteins that are often coupled to a UV or fluorescence detector (96). In high-performance liquid chromatography (HPLC) using smaller particles as stationary phase results in lower plate height values and enhanced chromatographic separation efficiencies. Recently commercialised ≤ 2 µm particles can shorten the run time and improve the separation of analytes and matrices. Although the MS instruments have a high selectivity, without sufficient sample clean-up and chromatographic separation, the matrix components can impair the accuracy of a quantitative method. These co-eluting components can either affect the evaporation of the droplets or compete for electrical charge against the analytes, which leads to ion suppression (97). This can be solved by coupling of a separation technique such as gas chromatography (GC), HPLC, or capillary electrophoresis (CE) to MS (82). Whatever the separation approach is, it adds an additional dimension to the analytical measurement (98). For example, with hyphenation of MS to HPLC, the background signals can be removed and the concentration of analytes increase. This results in higher signal-to-noise ratios. It has to be mentioned that in this case the complete chromatographic resolution is not necessary and time-consuming purification and fractionation steps can be avoided (96).

1.7.2.3 Electrospray Ionisation

Ionisation is the first and most challenging step in the MS analysis. Different types of ion sources have become available over the years (82); nonetheless since the techniques for soft ionisation without excessive fragmentation were lacking, MS analysis was restricted for a long time only to small and thermostable elements (99). The development of soft ionization techniques such as electrospray (ESI) and matrix-assisted laser desorption (MALDI) changed the situation and resulted in the increasing use of MS for the analysis of large, non-volatile, and chargeable molecules such as proteins (98). Because

of the sensitivity of these methods, their high mass range and their capacity to analyse complex mixtures without separation, and also because of their capability to extend by collision-induced fragmentation, these methods were used for characterisation of large biomolecules to get information about the molecular structure and post-translational modifications (96).

ESI is currently the most universal and versatile ionisation technique, because a wide range of analytes can be ionized by ESI. ESI showed also the most successful interface for LC/MS applications. A schematic of a typical ESI is shown in Figure 2. The analytes are injected directly from either an infusion pump or HPLC into the ionisation source through a stainless-steel capillary tube. The tip holds a high voltage comparing to the electrode, which surrounds the area with atmospheric pressure. This potential difference causes the production of an electric field, which converts the injected analytes to small charged droplets (82). The desolvation of these droplets is achieved by either heating, differential pumping (96) or employing an uncharged counter current gas flow such as N_2. The eluents are often aqueous buffers containing organic solvent and low molarities of weak volatile acid or base to promote the ionisation of the samples. The in atmospheric pressure produced ions enter the high vacuum mass analyser through an orifice. With interfacing the electrospray to different mass analysers such as time-of-flight (TOF), quadrupole mass filter, ion traps, and Fourier transform ion cyclotron resonance, accuracies of 0.001% in mass determination can be achieved (96), however the combination of ESI to a quadrupole mass analyser is the most successful one (82).

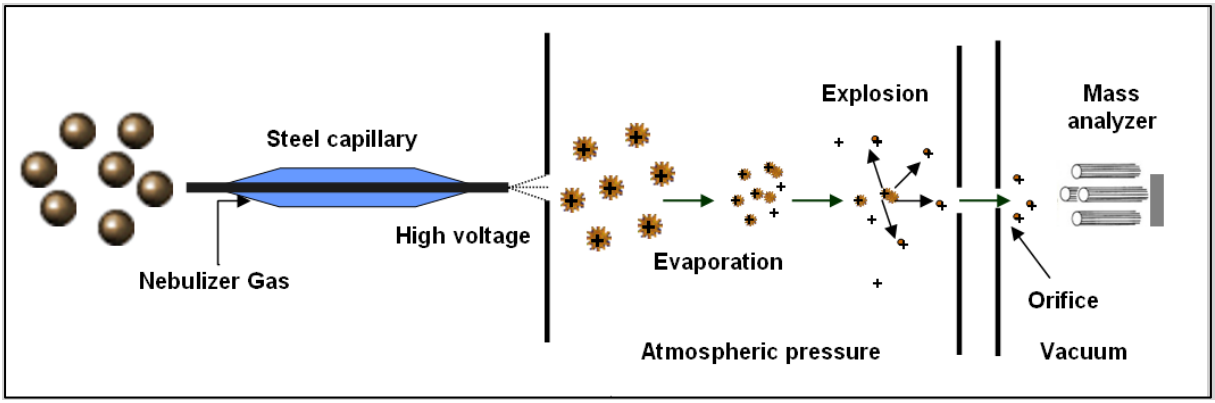


Figure 2: Basic component of electrospray ionization

1.7.2.4 Quadrupole mass Analysers

The mass analyser is the heart of a mass spectrometer. Tandem MS capability, low cost and small size are the desirable characters of a mass analyser. There are different types of mass analyser; however, the quadrupole devices are the common used types. A quadrupole (Q) consists of four metal rods, where every apposite pair is electrically connected to direct current (dc) and radio frequency (rf) power supplies (Figure 3). They produced a high frequency oscillating electric field, along which the ions have to pass with vibratory motions. It can be adjusted by applying certain dc and rf potentials, this field ions with a specific *m/z* can pass. These potentials are changed to obtain the mass spectrums, but their ratio is constant (82). Depending on the polarity-adjustment of the instrument, both positively and negatively charged ions can be detected (96).

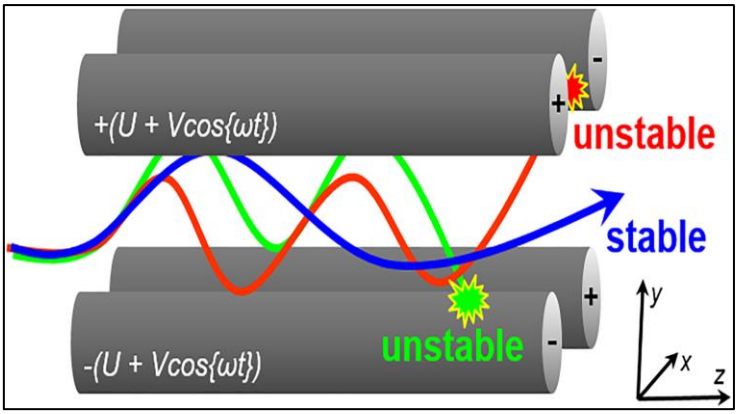


Figure 3: Quadrupole mass analyser (100)

1.7.2.5 Tandem mass spectrometry

In the recent years, tandem mass spectrometry has been used more and more for the identification and quantification of different compounds in complex mixtures. It is related to the coupling of two or more stages of mass analysis (MS_n) (82). Each stage provides an additional dimension of isolation, selectivity and structural information. If quadrupole and sector instruments are combined, the procedure occurs subsequently in the following spaces of the device, which are called tandem-in-space (Figure 4); TOF and quadrupole are two examples of this type tandem MS. In the other technique (tandem in-time), all of the processes are performed sequentially in the same region. Ion traps (IT) belong to this group (98). In MS/MS, the accurate masses of compounds can be determined and a precursor ion at a defined m/z can be selected for fragmentation in the collision cells. The generated product ions are trapped and scanned at high sensitivity in the detector (59). With analysis of these fragments, the detailed structure of peptides can be inferred. The MS/MS in product ion mode can be used to determine the amino acid sequence of the peptides, in SRM mode for quantitative analysis

with very high sensitivity and selectivity (99). In MS/MS, for accurate fragment information from a certain precursor, ion activation and dissociation are necessary. They are used to increase the number of precursor ions with energies over the dissociation threshold. The collision induced dissociation (CID), also known as collisionally activated dissociation (CAD), is the common used technique for ion activation and dissociation [57].

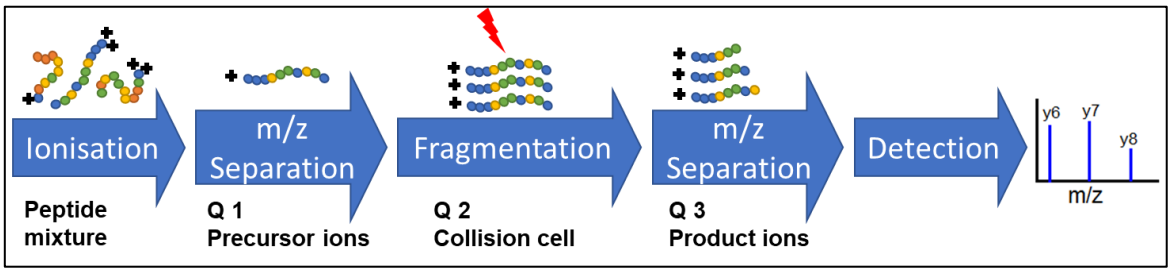


Figure 4: Basic concept of MS/MS

The combination of LC with tandem mass spectrometry is easily possible (96). The commonly used tandem mass spectrometers for the detection of trace amounts of elements are triple quadrupole instruments (QqQ), ion traps (IT), and quadrupole-time-of-flight (Q-TOF); among them, QqQ and QIT have high sensitivity, short dwell time, and wide linear range, if they are operated in selected reaction monitoring (SRM) mode. Therefore, they are the most suitable one for the quantification and screening of target and multi-target analysis (97).

1.7.2.6 Quantification approaches of peptide marker in foods

The quantification at protein level can be performed with external or internal standards (74). The external standardization is made using calibration curves, where the measured intensities are plotted versus the concentrations of the analytes. To enhance the accuracy of these curves, multiple determinations are often performed. The standard curves are normally linear over a wide range of concentrations. By internal standardization, the standard at a known concentration is added to the sample, before any clean-up step to make the same change of the concentration in sample and standard. Since the isotopomers have the identical ionization efficiency but different mass as the target analyte, they can be used as special internal standards (98).

For quantification at peptide level, three methods were described: tagging, isotopically labelled synthetic peptides, and a label free method. All of these methods can be used for relative or absolute quantification. In tagging methods, the protein or peptide is labelled with heavy (^{13}C) or light (^{12}C) stable isotopes and can act as internal standard. They are classified into metabolic, chemical, and

enzymatic labelling; and are appropriate for biomarker identification. The next method uses as reference isotope-labelled synthetic peptides, which include ^{13}C or ^{15}N, and therefore differ to the endogenous peptides with a certain mass. In this method, the choice of peptides is essential; it must be specific to the target protein, to avoid false positive results. The peptides have to be stable in solution. This means that some amino acids, such as methionine and cysteine, which can be oxidised, shall not be included in the peptide. In the recently developed label free method, the sample preparation is simplified and isotopes are not used. The quantification is performed either with the measurement of spectra counting or on the ion signal intensity (101).

1.8 State of the art: Marker Peptides for food allergens with LC-MS/MS

The aim of this section is to summarize available data about peptide collections used for the determination of five food allergens, milk, egg, peanut, hazelnut and walnut, respectively. Within the 7th Framework Programme "Towards evidence-based risk management of food allergies", the funded project iFAAM – "Integrated Approaches to Food Allergen and Allergy Risk Management" – includes to deal with the developments in allergen analysis, that have taken place, with a view to identifying target analytes which are likely to be stable to food processing procedures and would be suitable for multiplexed analysis. The decision was made to collect this list of peptides as base for further method development. Johnson *et al.* (89) listed eight criteria, which are important for protein/peptide target selection. E.g. for the collected peptides the protein sequences are known and available and peptides have to be unique for the regarding species. All the reviewed publications used typical allergenic proteins in different commodities. The allergenic proteins are mainly seed storage proteins or the main protein fractions e.g. caseins for milk. The used peptides are not subjected to post-translational modifications and some seem stable to storage and/or processing hydrolysis. As similar peptides were used in the publications the extractability is given, which is the basis for digestibility. For the peptides itself mostly 2+-charged ions were selected, which show a good fragmentation in MS-analysis. Typical peptide fragmentation generates b or y ions of different mass to charge ratios (see Figure 5).

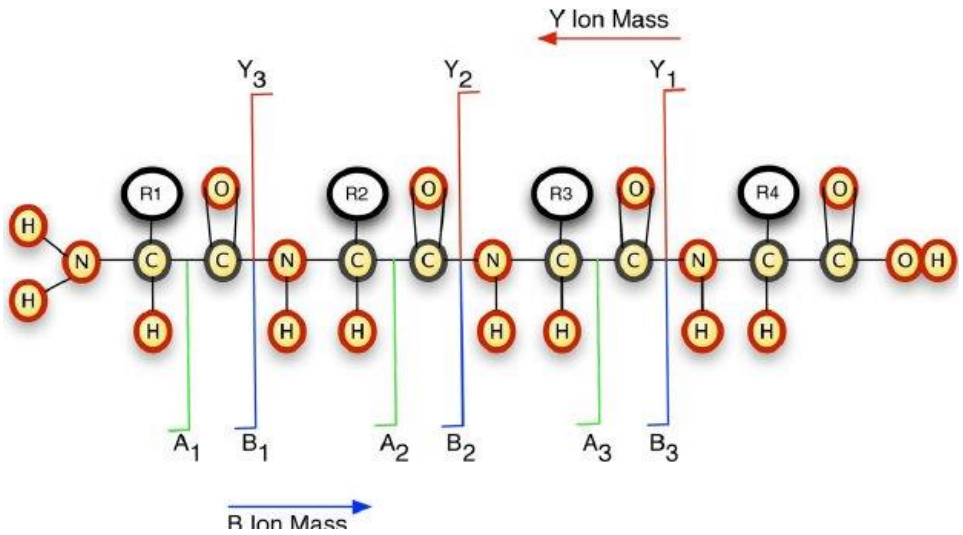


Figure 5: Typical peptide fragmentation generates b or y ions of different mass to charge ratios, and also a ions. Correspondence of the experimentally determined masses to the molecular masses of the amino acid residues can be used to derive the sequence of the parent ion. Figure taken from (102)

The size of the peptides varies from 5 up to 25 amino acids. Reproducibility within the particular published methods was proven for the digests and for the retention times of the chromatographic separation. The uniqueness of the peptides is given for nearly all listed peptides; exceptions will be mentioned in detail in the sections below. The analytical target components are milk and egg as well as peanut, hazelnut and walnut. The following lists contain the collected data and peptide sequences for the five allergenic compounds from the respective publications. Data was collected according to specific mass spectrometric data for the found tryptic peptides like peptide sequence, parent ion [m/z], charge [+], product ions [m/z]. If additional information was available, this is listed. If spiked or incurred food samples or processed food samples were used, the type of samples was added with the respective detection level. The enzyme used for digestions prior to MS analysis was uniquely the protease trypsin. Where available the used mass spectrometers additionally with the used operating conditions were collected from the corresponding papers. In the following, investigated marker peptides available in the literature from 2004-2018 will be described.

1.8.1 Bovine milk

The most promising peptides for quantitative analysis using LC-MS/MS were peptides from the casein fraction. As listed in Table 1 there were 16 peptides available in the literature. Two studies, one by Newsome et Scholl (2013) (110) who analysed incurred cookies and one by *Monaci et al.* (2014) (87) who worked with spiked cookies even show validation data of their final methods. While Newsome *et al.* (2013) achieved a LOQ below 3 mg nonfat drymilk/kg with marker peptides FFVAPFPEVFGK and YLGYLEQLLR from Bos d 9, *Monaci et al.* (2014) determined LOQs even down to 0.5 mg milk powder protein/kg. This comparison shows very well the crucial role of the processing state of a sample regarding the LOQ of a method. Analysing incurred material means the allergen source is added to the matrix before the baking process, which results in a massive loss of extractability of the regarding protein from the matrix for further detection by LC-MS/MS. Parker et al. (2015) (108) confirmed that these two peptides are fit for detection in raw and processed food, and also showed, that protein recovery is strongly influenced by the state of food-processing. Quantifying the peptides YLGYLEQLLR (alpha-s1-casein) and LSFNPTQLEEQCHI (ß-lactoglobulin), they achieved protein recoveries between 70-110% in unprocessed but only 50-65% in processed foods. It has to be mentioned here, that LSFNPTQLEEQCHI poses some risk in being used as marker peptide for quantification, as under-quantification can occur if the cysteines are not fully methylated during sample preparation.

During the last fifteen years the peptides FFVAPFPEVFGK and YLGYLEQLLR deriving from alpha-S1-casein have been used continuously during method development for LC-MS/MS. YLGYLEQLLR is not unique for cow's milk, but is also present in buffalo, yak, sheep and goat milk. As most people would also react to the milk of these other animals, it still remains a promising marker peptide. During this period mass spectrometers used in the different studies changed from better resolving QTOF-instruments at the beginning to the triple-quadrupole instruments, which offer the magnificent advantage in reducing background and increasing signal with multiple reaction monitoring leading to higher sensitivity of the method.

In 2015, Chen *et al.* (112) analysed cookies spiked with β-casein. By detecting their chosen signature peptide VLPVPQK from Bos d 10 they reached an LOQ of 0.5 mg/kg β-casein in cookie.

In a study performed by Tolin *et al*. (2012) (114) milk peptides were also detected in red wine samples where caseins were used as fining agents. Due to missing quantitative data from this study, these peptides were not included into Table 8. (103) validated a method and provided quantitative data for the presence of milk allergens in red wine showing a LOD of 0.5 mg/L of Bos d 9 and even 0.1 mg/L of Bos d 11 in red wine.

De Angelis et al. 2017 (105) even made a step further by investigating an ultrafiltration approach and a size exclusion column prefractionation approach before the sample was passed to tryptic digest and HPLC-MS/MS analysis. For caseinate peptides LODs were found to be approximately 0.05 mg/L.

No peptides were available for serum albumin (Bos d 6). The high molecular weight gives potential for several theoretical peptides, however the relative abundance of serum albumin in milk is too low to be able to generate results in LC-MS/MS. Only 6 peptides deriving from whey proteins Bos d 4 and Bos d 5 were selected by Ansari *et al.* (2011) (60), Lutter *et al.* (2011) (107) and Parker *et al.* (2015) (108). None of these peptides were validated, but can be used as confirmatory milk peptides for milk and dairy products at the level low as 1.1 mg/kg and in baby food, infant cereals and breakfast flakes up to the level of 5 mg/kg.

(105) developed a multiallergen method where they showed clearly, that lowest LODs (0.5 mg milk proteins/kg food) could be achieved even in incurred food by using marker peptides from alpha-caseins, as this is a very heat-stable protein. In comparison for their chosen peptide markers for whey proteins LOD was only at 5 mg protein/kg food.

As summarised in Table 8, six proteins seemed important and clinically relevant for peptide selection for the mass spectrometry analysis: Bos d 4 and Bos d 5 (whey proteins) and Bos d 9, 10, 11 and 12 (caseins). Peptides that belong to these six proteins are found to be part of major and minor epitopes or are characterised as immuno-dominant epitopes. Schulmeister *et al.* (2009) (106) synthesised 11 different peptides from Bos d 9. It was found that FFVAPFPEVFGK was a part of a major epitope as the frequency of IgE recognition was found to be about 70% of the screened milk allergic patients' sera.

Table 8: An overview of published peptide sequences for detection of the bovine milk allergens using LC-MS/MS. Parent ion charge state was +2 for all peptides except for those indicated. If available from the literature, Limit of Detection (LOD) and Limit of Quantification (LOQ) are shown. Electrospray ionization (ESI) was the standard for all instruments except for those indicated.

Allergen (Uniprot Accession #)	Peptide sequence	Parent ion m/z	Matrix	State of ingredient	LOD/ LOQ[b]	Instrument	Publication
Bos d 4 (P00711)	VGINYWLAHK	601.2	milk and dairy products		1.1/-	microLC-QQQ-MS^2	(59)
Bos d 5 (P02754)	LIVTQTMK	467.6	milk and dairy products		1.1/-	microLC-QQQ-MS^2	(59)
	IPAVFK	338	milk and dairy products		1.1/-	microLC-QQQ-MS^2	(59)
	VLVLDTDYK	533.3	baby food, infant cereals, breakfast flakes		5-20/-	microLC-QQQ-MS^2	(107)
	TPEVDDEALEK	623.5	baby food, infant cereals, breakfast flakes		5-20/-	microLC-QQQ-MS^2	(107)
	LSFNPTQLEEQCHI	858.407	cereal bar, muffin	incurred with WEP, non-fat dry milk, def. PN	-	nanoLC-QQQ-MS^2	(108)
Bos d 9 (P02662)	FFVAPFPEVFGK	692.8	cookie	spiked with SMP	12.5/-	nanoLC-QTOF-MS^2	(80)
	FFVAPFPEVFGK	692.86	cookie	incurred with SMP	100/-	microLC-QTOF-MS^2	(81)
	FFVAPFPEVFGK	692.6	white wine	fined with caseinate	100/-	microLC-QTOF-MS^2	(81)
	FFVAPFPEVFGK	692.9	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	FFVAPFPEVFGK		milk and dairy products		1.1/-	microLC-QQQ-MS^2	(59)
	FFVAPFPEVFGK		cookie	incurred with NIST NFDM (nonfat dried milk)	< 3/-[a]	microLC-QQQ-MS^2	(110)
	FFVAPFPEVFGK	692.87	cookie	spiked with EP and SMP extract	0.12/ 0.4[a]	microLC-IT-MS^2	(86)
	YLGYLEQLLR	634.3	cookie	spiked with SMP	12.5/-	nanoLC-QTOF-MS^2	(80)

	YLGYLEQLLR	634.34	cookie	incurred with SMP	100/-	microLC-QTOF-MS^2	(81)
	YLGYLEQLLR	634.2	white wine	fined with caseinate	100/-	microLC-QTOF-MS^2	(81)
	YLGYLEQLLR	634.3	bread	incurred with SMP, EWP and def. nuts	5/-[a]	microLC-API-QQQ-MS^2	(87,109)
	YLGYLEQLLR		milk and dairy products		1.1/-	microLC-QQQ-MS^2	(59)
	YLGYLEQLLR		cookie	incurred with NIST NFDM (non-fat dried milk)	< 3/-	microLC-QQQ-MS^2	(110)
	YLGYLEQLLR	634.8	red wine	fortified with the three proteins	0.5/1.0[a]	microLC-IT-MS^2	(103)
	YLGYLEQLLR	634.36	cookie	spiked with EP and SMP	0.14/0.5[a]	microLC-IT-MS^2	(86)
	YLGYLEQLLR	634.36	cereal bar, muffin	incurred with WEP, non-fat dry milk, def. PN	-	nanoLC-QQQ-MS^2	(108)
	HQGLPQEVLNENLLR	587.2[c]	red wine	fortified with the three proteins	-	microLC-IT-MS^2	(103)
Bos d 10 (02663)	NAVPITPTLNR	598.3	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	NAVPITPTLNR	1195.7[d]	cookie	incurred with casein	-	nanoLC-QTOF-MS^2	(111)
	FALPQYLK	490.3	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	FALPQYLK	490.1	baby food, infant cereals, breakfast flakes		5-20/-	microLC-QQQ-MS^2	(107)
	ALNEINQFYQK	684.3	baby food, infant cereals, breakfast flakes		5-20/-	microLC-QQQ-MS^2	(107)
	LNFLK	634.4[d]	cookie	incurred with casein	-	nanoLC-QTOF-MS^2	(111)
Bos d 11 (P02666)	GPFPIIV	742.2[d]	white wine	fined with caseinate	50/-	microLC-QTOF-MS^2	(81)
	GPFPIIV	742.5[d]	milk and dairy products		1.1/-	microLC-QQQ-MS^2	(59)
	GPFPIIV	742.9[d]	cookie	incurred with bovine ß-casein	-	microLC-QQQ-MS^2	(112)
	VLPVPQK	780.6[d]	milk and dairy products		1.1/-	microLC-QQQ-MS^2	(59)
	VLPVPQK	390.9	red wine	fortified with the three proteins	-	microLC-IT-MS^2	(103)
	VLPVPQK	391.0	cookie	incurred with bovine ß-casein	0.5/-[a]	microLC-QQQ-MS^2	(112)

	DMPIQAFLLYQEPVLGPVR	729.2[c]	white wine	fined with caseinate	100/-	microLC-QTOF-MS^2	(81)
	DMPIQAFLLYQEPVLGPVR	730.8[c]	cookie	incurred with bovine ß-casein	-	microLC-QQQ-MS^2	(112)
	AVPYPQR	415.5	baby food, infant cereals, breakfast flakes		5-20/-	microLC-QQQ-MS^2	(107)
	AVPYPQR	415.9	red wine	fortified with the three proteins	0.01/0.03[a]	microLC-IT-MS^2	(103)
	AVPYPQR	416.9	cookie	incurred with bovine ß-casein	-	microLC-QQQ-MS^2	(112)
	EMPFPK	374.9	cookie	incurred with bovine ß-casein	-	microLC-QQQ-MS^2	(112)
	HQGLPQEVLNENLLR	1759.9[d]	cookie	incurred with casein	10/-	nanoLC-QTOF-MS^2	(111)
	ALNEINQFYQK	1367.7 [d]	cookie	incurred with casein	10/-	nanoLC-QTOF-MS^2	(111)
Bos d 12 (P02668)	IPIQYVLSR	626.3	baby food, infant cereals, breakfast flakes		5-20/-	microLC-QQQ-MS^2	(107)
	YIPIQYVLSR	1251.7 [d]	cookie	incurred with casein	10/-	nanoLC-QTOF-MS^2	(111)

[a] method validation was performed

[b] mg of ingredient per kg of matrix

[c] charge state of the parent ion was +3

[d] charge state of the parent ion was +1

1.8.2 Hen's egg

As ovomucoid exhibits trypsin inhibitory activity and is a highly glycosylated protein, many glycosylated peptides remain uncleaved after tryptic digests and its digestion may not be complete.

According to Johnson *et al.* (2011) (89) selected marker peptides should not be subject to any post-translational modifications and the protein must be fully digestible. This might be the reason why Gal d 1 is not used in any assays as the peptides cannot be detected with necessary sensitivity.

In contrast, 10 peptide markers derived from ovalbumin are used in the literature. Additionally, 4 marker peptides derived from Gal d 4 are used in literature. Representing only 3% of egg white protein content and having a molecular mass of 14.3 kDa, Gal d 4 offers good marker peptides for quantitative multiallergen analysis via LC-MS/MS in samples contaminated with whole egg white as most studies have shown (Table 9). The exception is the study by Cryar *et al.* (2013) (116). However, it should be mentioned that the analysed samples were contaminated solely by purified Lysozyme, which makes it the only possible target allergen in this case.

The study of Tolin *et al.* (2012) (114) indicates that LC-MS/MS methods can be superior to immunoanalytical methods. An LC-MS/MS method was developed, which allowed the detection of egg proteins in fined red wines down to 0.5 g/L, the minimum dose commonly adopted for red wine fining of commercial egg white preparation. Immunochemical methods could only detect doses of 5 g/L or higher of fining agent in red wine. Their two chosen peptide markers GGLEPINFQTAADQAR and LTEWTSSNVMEER originating from Gal d 2 are matrix-independent markers, therefore they were also chosen in studies by Azarnia *et al.* (2013) (115), Mattarozzi *et al.* (2014) (104), Monaci *et al.* (2014) (87), Parker *et al.* (2015) (108) and Planque *et al.* (105) with matrices including raw pasta, cookies, muffins and cereal bars. In 2017 a study by Pilolli *et al*. (113) was published where ovalbumin in red wines was analysed with a concentration step via immunoaffinity clean-up of ovalbumin with polyclonal ovalbumin antibodies. With their attempt they proofed that an LOD of at least 0.1 mg/L and an LOQ of at least 0.3 mg/L are possible. They identified the peptide GGLEPINFQTAADQAR to be the most sensitive marker. Additional pre-sample-treatment strategies were investigated in a study by De Angelis *et al*. (104). An ultrafiltration approach was compared to a size exclusion column prefractionation approach before the sample was passed to tryptic digest and HPLC-MS/MS analysis. Results for egg allergens were very promising as very challenging LOD of around 0.05 mg/L could be achieved, see Table 9.

It should also be noted that the two peptide fragments, GGLEPINFQTAADQAR and LTEWTSSNVMEER used by Azarnia *et al.* (2013) (115) for the detection of egg in incurred raw pasta, could not be detected

in cooked pasta, even in the 1000 ppm incurred samples. The results clearly show that egg allergen detection in pasta is heavily affected by matrix and processing. That protein recovery from foods is strongly dependent on the state of processing of the food is also confirmed by the study of Parker *et al*. (76) as already mentioned in the section for milk. About 50% less protein recovery was reached in their study with the most promising marker peptide for egg, NTDGSTDYGILQINSR (lysozyme).

Quantitative results via LC-MS/MS analysis in the literature have been achieved in studies by Mattarozzi *et al.* in 2014 (104) and Monaci *et al.* in 2014 (87) down to an LOD of 0.8 and 0.3 ppm and an LOQ of 2 and 1 ppm allergenic ingredient/matrix. However, the tested samples were only materials spiked after the full processing of the sample, which excludes any assumptions about the influence of the processing state of the sample to the quantitative analysis of the allergens. In a study by Heick *et al.* in 2011, where a bread matrix was incurred with the allergenic ingredient before the baking process, the LOD for their LC-MS/MS analysis was of a factor of 20-40 times higher than of the studies mentioned earlier, supporting the results by Anzarnia *et al.* (2013) (115) that egg allergen detection is affected by processing. Some years later, Planque *et al*. (105) proved that analysing incurred samples influences the LOD of an LC-MS/MS detection method immense and reached LODs for egg white of 3.4 mg egg protein/kg food and for egg yolk 30.8 mg egg protein/kg food.

10 peptides were selected for Gal d 2 and 4 peptides for Gal d 4. If those peptides are compared according to their epitope affiliation, the peptides from Gal d 4 are all parts of allergenic epitopes. Jimenez-Saiz *et al.* (2014) (114) could also show that these fragments remain after simulated gastric and duodenal digests. Out of the 10 peptides used in the literature only 2 could be found to be part of epitopes. By means of sera screening Mine *et al.* (2003) (115) identified 5 immuno-dominant epitopes on ovalbumin, which also included EDTQAMPFRV and DVYSFSLA (DILNQITKPNDVYSFSLASR).

Table 9: An overview of published peptide sequences for detection of the hen's egg allergens using LC-MS/MS. Parent ion charge state was +2 for all peptides except for those indicated. If available from the literature, Limit of Detection (LOD) and Limit of Quantification (LOQ) are shown. Electrospray ionization (ESI) was the standard for all instruments except for those indicated.

Allergen (Uniprot Accession #)	Peptide Sequence	Parent ion m/z	Matrix	State of Ingredient	LOD/ LOQ [b]	Instrumental set-up	Publication
Gal d 2 (P01012)	HIATNAVLFFGR	673.4	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	YPILPEYLQCVK	761.6	bread	incurred with SMP, EWP and def. nuts	42/ -[a]	microLC-API-QQQ-MS^2	(87,109)
	DILNQITKPNDVYSFSLASR	761.6[c]	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	ELINSWVESQTNGIIR	929.5	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	ELINSWVESQTNGIIR	929.99	red wine	fined with EWP	-	nanoLC-QTOF-MS^2	(116)
	GGLEPINFQTAADQAR	844.43	red wine	fined with EWP	-	nanoLC-QTOF-MS^2	(116)
	GGLEPINFQTAADQAR	844.4	raw pasta	incurred with OVA, EWP and whole egg	-	nanoLC-QTOF-MS^2	(117)
	GGLEPINFQTAADQAR	844.9	red wine	fortified with pure protein	0.8/2.0[a]	microLC-IT-MS^2	(103)
	GGLEPINFQTAADQAR	844.42	cookie	spiked with EP and SMP	0.3/1.0[a]	microLC-IT-MS^2	(86)
	GGLEPINFQTAADQAR	844.42	cereal bar, muffin	incurred with WEP, non-fat dry milk, def. PN	-	UPLC-QQQ-MS^2	(108)
	LTEWTSSNVMEER	791.37	red wine	fined with EWP	-	nanoLC-QTOF-MS^2	(116)
	LTEWTSSNVM(ox)EER	799.4	red wine	fined with egg white		nanoLC-QTOF-MS^2	(116)
	LTEWTSSNVMEER	791.4	raw pasta	incurred with OVA, EW and whole egg	-	nanoLC-QTOF-MS^2	(117)
	LTEWTSSNVM(ox)EER	799.36	cookie	spiked with EP and SMP	0.3/1.0[a]	microLC-IT-MS^2	(86)

	VASMASEK	411.9	red wine	fortified with pure protein	-	microLC-IT-MS^2	(103)
Gal d 4 (P00698)	HGLDNYR	437.9	white wine	spiked with peptides of lysozyme	0.95	microLC-QQQ-MS^2	(118)
	FESNFNTQATNR	715.0	white wine	spiked with peptides of lysozyme	0.95	microLC-QQQ-MS^2	(118)
	GTDVQAWIR	523.5	white wine	spiked with peptides of lysozyme	-	microLC-QQQ-MS^2	(118)
	NTDGSTDYGILQINSR	585.28	cereal bar, muffin	incurred with WEP, non-fat dry milk, def. PN	-	nanoLC-QQQ-MS^2	(108)

[a] **method validation was performed**
[b] **mg of ingredient per kg of matrix**
[c] **charge state of the parent ion was +3**

1.8.3 Peanut

There are 4 different peptides that come from Ara h 1, which seem to be promising marker in LC-MS/MS methods. The peptide DLAFPGSGEQVEK was determined in five recently published research papers. Heick *et al*. (2011 a-b) (88,109) presented validation data including a LOD of 11 mg/kg. Using this protocol Shefcheck *et al*. (2006) (79) were able to detect peanut at the level of 2 mg/kg (even in dark chocolate, a very complex matrix containing tannins, that make protein extraction even more difficult). The authors showed that for this level the peptide VLLEENAGGEQEER (Ara h 1) would be especially useful. Shefcheck *et al.* achieved those low LODs by performing the extraction of the proteins and their digestion simultaneously. Most of the studies are completed by two-stage process; extracting the protein first and then digesting it. The peptides GTGNLELVAVR and NNPFYFPSR (no quantitative data) meet only a confirmatory role in the assays where they were implemented.

Due to its allergenic prevalence and thermostability, Ara h 2 is a very interesting allergen to be addressed in LC-MS/MS assays. Although it might be challenging due to the fact that Ara h 2 acts as a trypsin inhibitor, the activity found to increase upon roasting (Burks *et al.* 1998, (51)). Careri *et al.* (2007) (120) reported 2 peptides (Table 10) used in their assay for detection of spiked Ara h 2 in cacao rice crispy. Peptide NLPQQCGLR was shown as more promising for thermally processed foods. Parker *et al*. (76) incurred the samples with defatted roasted peanut flour prior to processing. Chassaigne *et al*. in 2007 (52) identified a peptide which might be a marker independent from the grade of thermal processing, as this peptide was detected in raw, mildly and strongly roasted peanut, but it has not been tested in real food samples. The peptide CCNELNEFENNQR was detected as a marker for thermal modifications and it was recognized in partially and heavily roasted peanut extracts but not in raw peanuts, which disqualifies this peptide as a quantitative representative marker – as it is influenced by the processing state.

For the detection and quantification of Ara h 3/4, ten specific peptides are known from the literature. In the validated assays developed by Careri *et al.* (2007, 2008) (120,122), Pedreschi *et al.* (2012) (123) and Bignardi *et al.* (2013) (124) the following peptides AHVQVVDSNGNR, SPDIYNPQAGSLK and FNLAGNHEQEFLR showed an LOD of 10 mg/kg or even lower. These LODs were obtained only in those samples where the peanut was spiked into the samples after thermal processing. Ara h 3 specific peptide SPDIYNPQAGSLK was demonstrated to be relatively stable under thermal processing and therefore is a promising peptide marker also in incurred foods (Chassaigne *et al.* 2007, (52)). Bignardi *et al.* (2013) (124) showed that LOD and LOQ for the Ara h 3 peptide FNLAGNHEQEFLR were higher for one order of magnitude in chocolate compared to biscuits, mainly blaming the influence of the

complexity of the matrix and the degree of thermal processing. Bignardi *et al.* (2013) enhanced sensitivity for the simultaneous detection of five nut allergens in biscuit and in dark chocolate complex matrices by introducing a rapid size-exclusion solid-phase extraction-based step before liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS2) analysis. Careri *et al.* (2008) (122) proposed an antibody magnetic bead-based method for the selective enrichment of Ara h 3/4 protein in food extracts. With this attempt they reduce background issues in their measurements, but it is even more laborious if several allergens from different allergenic ingredients shall be selected by multi allergen measurement. A study by Planque et al. (2016) (106) looked at the analysis of food samples incurred with peanut, so they implemented the processing of proteins in foods. For the peanut marker peptides FNLAGNHEQEFLR and TANELNLLILR they reached LODs of 2.5 mg peanut protein/kg food.

Finally, the specific peptides for Ara h 6-8 developed in the assay of Latif *et al.* (2013) (119) are not confirmed by any other published LC-MS/MS assay and therefore might be confirmatory peptides to complete an assay, but so far were only tested in a pure peanut sample.

According to Shin *et al.* (1998) (117) the peptides selected (Table 10) for Ara h 1 belong to structurally conserved regions, except the peptide VLLEENAGGEQEER. Peptide VLLEENAGGEQEER overlaps with a known immunologically active epitope, EQEERGQRRW. DLAFPGSGEQVEK overlaps with the immunologically active epitope KDLAFPGSGE (Shin *et al*. (119)). According to the mapped epitopes by Mishra *et al.* (2014) (120) these sequences are referred to IgE-binding. Otsu *et al.* (2014) (121) identified allergenic epitopes on Ara h 2 and Ara h 6. From the 4 listed (Table 10) Ara h 2 peptides only RQQWELQGDR partially overlaps with three different IgE epitopes, HASARQQWEL, QWELQGDRRC, and DRRC-QSQLER (Gaier *et al*. ((41))).

Table 10: An overview of published peptide sequences for detection of the peanut allergens using LC-MS/MS. Parent ion charge state was +2 for all peptides except for those indicated. If available from the literature, Limit of Detection (LOD) and Limit of Quantification (LOQ) are shown. Electrospray ionization (ESI) was the standard for all instruments except for those indicated.

Allergen (Uniprot Accession #)	Peptide Sequence	Parent ion m/z	Matrix	State of Ingredient	LOD/ LOQb	Instrumental set-up	Publication
Ara h 1 (P43237/ P43238)	VLLEENAGGEQEER	786.9	dark chocolate	incurred with Ara h 1	2/-	microLC-QTOF-MS^2	(78)
	VLLEENAGGEQEER	786.88	peanut wo skin	raw, mild, strong roasted	-	nanoLC-QTOF-MS^2	(51)
	DLAFPGSGEQVEK	688.9	dark chocolate	incurred with Ara h 1	2/-	microLC-QTOF-MS^2	(78)
	DLAFPGSGEQVEK	688.85	peanut wo skin	raw, mild, strong roasted	-	nanoLC-QTOF-MS^2	(51)
	DLAFPGSGEQVEK	688.8	bread	incurred with SMP, EWP, defat. nuts	11/-[a]	microLC-API-QQQ-MS^2	(87,109)
	DLAFPGSGEQVEK	688.85	defat. nutmixture	spiked with defat. PNF	-	microLC-Orbitrap-MS^2	(122)
	DLAFPGSGEQVEKL	745.38	peanut with skin	raw	-	nanoLC-Orbitrap-MS^2	(123)
	GTGNLELVAVR	564.4	bread	incurred with SMP, EWP, defat. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	GTGNLELVAVR	564.83	defat. nutmixture	spiked with defat. PNF	-	microLC-Orbitrap-MS^2	(122)
	NNPFYFPSR	571.28	cereal bar, muffin	incurred with WEP, nMP, defat. rPNF	-	nanoLC-QQQ-MS^2	(108)
Ara h 2 (Q6PSU2)	CMCEALQQIMENQSDR	950	cacao rice crispy	spiked with Ara h 2	-	microLC-QQQ-MS^2	(124)
	CCNELNEFENNQR	807	cacao rice crispy	spiked with Ara h 2	5/14[a]	microLC-QQQ-MS^2	(124)
	NLPQQCGLR	543.28	cereal bar, muffin	incurred with WEP, nMP, defat. rPNF	-	nanoLC-QQQ-MS^2	(108)
	RQQWELQGDR	439.23[c]	peanut wo skin	raw, mild, strong roasted	-	nanoLC-QTOF-MS^2	(51)
Ara h 3/4 (Q8LKN1)	YQQQSR	406	breakfast cereals	mixed with nuts	-	microLC-IT-MS^2	(125)
	AHYQVVDSNGDR	649	cacao rice crispy	spiked with Ara h 3/4	-	microLC-QQQ-MS^2	(124)
	AHYQVVDSNGDR	649	breakfast cereals	fortified with roasted PN	3/10[a]	microLC-IT-MS^2	(126)
	AHVQVVDSNGNR	432.5[c]	cookie	incurred with PN	10/-	nanoLC-QQQ-MS^2	(127)
	SPDIYNPQAGSLK	695	cacao rice crispy	spiked with Ara h 3/4	3.7/3.7[a]	microLC-QQQ-MS^2	(124)
	SPDIYNPQAGSLK	695.35	peanut wo skin	raw, mild, strong roasted	-	nanoLC-QTOF-MS^2	(51)

	SPDIYNPQAGSLK	695	breakfast cereals	fortified with roasted PN	3/10[a]	microLC-IT-MS^2	(126)
	SPDIYNPQAGSLK	695	breakfast cereals	mixed with nuts	10/37	microLC-IT-MS^2	(125)
	SPDIYNPQAGSLK	695.4	cookie	incurred with peanut	10/-	nanoLC-QQQ-MS^2	(127)
	SPDIYNPQAGSLK	695	biscuit, dark chocolate	spiked with nuts	-	microLC-IT-MS^2	(128)
	SPDIYNPQAGSLK	695.35	cereal bar, muffin	incurred with WEP, nMP, defat. rPNF	-	nanoLC-QQQ-MS^2	(108)
	AQSENYEYLAFK	732.84	chocolate rice crispy	fortified with raw peanut	-	microLC-QQQ-MS^2	(124)
	FNLAGNHEQEFLR	526	biscuit	spiked with nuts	0.1/0.3[a]	microLC-IT-MS^2	(128)
	FNLAGNHEQEFLR	526	dark chocolate	spiked with nuts	7/25[a]	microLC-IT-MS^2	(128)
	SQSENFEYVAFK	724.84	peanut wo skin	raw, mild, strong roasted	-	nanoLC-QTOF-MS^2	(51)
	RSVNELDLPIL	634.86	peanut with skin	raw	-	nanoLC-Orbitrap-MS^2	(123)
	RPFYSNAPQEIFIQQGR	684.5	bread	incurred with SMP, EWP, defat. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	WLGLSAEYGNLYR	771.4	bread	incurred with SMP, EWP, defat. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	WLGLSAEYGNLYR	771.40	defat. nutmixture	spiked with defat. PNF	27/90[a]	microLC-Orbitrap-MS^2	(122)
	VYDEELQEGHVLVVPQNFAVAGK	848.12	defat. nutmixture	spiked with defat. PNF	26/88[a]	microLC-Orbitrap-MS^2	(122)
Ara h 6 (not mentioned)	KRELMNLPQ	564.81	peanut with skin	raw	-	nanoLC-Orbitrap-MS^2	(123)
Ara h 7 (not mentioned)	ELRNLPQ	435.25	peanut with skin	raw	-	nanoLC-Orbitrap-MS^2	(123)
Ara h 8 (not mentioned)	KPDEEELK	494.25	peanut with skin	raw	-	nanoLC-Orbitrap-MS^2	(123)
	KATVVDGDELTPK	686.87	peanut with skin	raw	-	nanoLC-Orbitrap-MS^2	(123)

[a] method validation was performed
[b] mg of ingredient per kg of matrix
[c] charge state of the parent ion of +3

1.8.4 Hazelnut

Most marker peptides fulfilling these criteria were found for Cor a 9 and Cor a 11, as these peptides have the highest molecular masses and therefore result in a higher number of possible peptides for their quantification in MS-analysis. The studies performed by Ansari *et al*. (2012) (46) and Costa *et al*. (2014) (127) proposed a peptide for Cor a 8 with LOD and LOQ values comparable to those for cupins. The peptides ALPDDVLANAFQISR, QGQVLTIPQNFAVAK and INTVNSNTLPVLR deriving from Cor a 9 seem to be the most promising ones. They were implemented in single-allergen assays by Ansari *et al.* (2012) and Costa *et al.* (2014) and in the multi-allergen assay by Heick *et al.* (2011). They gave LODs and LOQs in a range between 1-5 mg/kg and 3 mg/kg, respectively.

The LOD obtained for complex chocolate matrix in the study performed by Costa *et al.* (2014) was 1 mg/kg. This was comparable to the LOD of 5 and 1.3 mg/kg reported by Heick *et al.* (2011) (88,109) and Bignardi *et al.* (2013) (124), respectively (although the LOQs obtained in these studies were slightly higher as can be seen in Table 11).

If these peptides are assessed as a part of the IgE-recognition site, the list of potentially usable peptides is even smaller. For Cor a 9 there are 6 peptides stated as marker peptides, but only QEWER is part of a strongly reacting epitope whereas WLQLSAER and (ALPDDVLAN)AFQISR are only parts of weakly reacting epitopes (Robotham *et al.* (129)). For the 3 mentioned peptides of Cor a 11 only AFSWEVL(EAALK) can be found as a part of an epitope (Barre *et al*. (130)).

Table 11: An overview of published peptide sequences for detection of the hazelnut allergens using LC-MS/MS. Parent ion charge state was +2 for all peptides except for those indicated. If available from the literature, Limit of Detection (LOD) and Limit of Quantification (LOQ) are shown. Electrospray ionization (ESI) was the standard for all instruments except for those indicated.

Allergen (Uniprot Accession #)	Peptide Sequence	Parent ion m/z	Matrix	State of Ingredient	LOD/LOQ [b]	Instrumental set-up	Publication
Cor a 8 (Q9ATH2)	GIAGLNPNLAAGLPGK	732.2	hazelnut			microLC-QQQ-MS^2	(59)
	GIAGLNPNLAAGLPGK	732.2	chocolate	incurred with HN	1/10	microLC-QQQ-MS^2	(131)
Cor a 9 (Q8GZP6)	ALPDDVLANAFQISR	815.5	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	ALPDDVLANAFQISR	815.6	hazelnut			microLC-QQQ-MS^2	(59)
	ALPDDVLANAFQISR	815.6	chocolate	incurred with HN	1/3	microLC-QQQ-MS^2	(131)
	QGQVLTIPQNFAVAK	807.5	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	QGQVLTIPQNFAVAK	807.8	hazelnut			microLC-QQQ-MS^2	(59)
	QGQVLTIPQNFAVAK	807.8	chocolate	incurred with HN	1/5	microLC-QQQ-MS^2	(131)
	INTVNSNTLPVLR	720.9	bread	incurred with SMP, EWP and def. nuts	5	microLC-API-QQQ-MS^2	(87,109)
	INTVNSNTLPVLR	721.1	hazelnut			microLC-QQQ-MS^2	(59)
	INTVNSNTLPVLR	721.1	chocolate	incurred with HN	1/3	microLC-QQQ-MS^2	(131)
	WLQLSAER	501.9	hazelnut			microLC-QQQ-MS^2	(59)
	WLQLSAER	501.9	chocolate	incurred with HN	-	microLC-QQQ-MS^2	(131)
	ADIYTEQVGR	577	breakfast cereals	mixed with nuts	-	microLC-IT-MS^2	(125)
	ADIYTEQVGR	576.3	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	ADIYTEQVGR	577	dark chocolate	spiked with HN	14/49[a]	microLC-IT-MS^2	(128)

	QEWER	374	breakfast cereals	mixed with HN	30/90	microLC-IT-MS^2	(125)
	QEWER	374	biscuit	spiked with HN	1.3/4.5a	microLC-IT-MS^2	(128)
Cor a 11 (Q8S4P9)	AFSWEVLEAALK	682.7	hazelnut			microLC-QQQ-MS^2	(59)
	AFSWEVLEAALK	682.7	chocolate	incurred with HN	1/10	microLC-QQQ-MS^2	(131)
	LLSGIENFR	524.9	hazelnut			microLC-QQQ-MS^2	(59)
	LLSGIENFR	524.9	chocolate	incurred with HN	1/10	microLC-QQQ-MS^2	(131)
	ELAFNLPSR	524	hazelnut			microLC-QQQ-MS^2	(59)
	ELAFNLPSR	524	chocolate	incurred with HN	1/10	microLC-QQQ-MS^2	(131)

[a] **method validation was performed**
[b] **mg of ingredient per kg of matrix**
[c] **charge state of the parent ion of +3**

1.8.5 Walnut

For Walnut less marker peptides are available in literature. Comparing the two publications of Bignardi *et al.* (2010 and 2013) (121,124) they confirmed, that the introduction of a size exclusion chromatography step (about 10 min) before the enzymatic digestion of the samples can have an improving effect on the final LOD and LOQ of the method. In the case of the peptide LDALEPTNR (Jug r 4) LOD and LOQ was even lower of one order of magnitude. This was not just the case for walnut, but also for hazelnut (Table 11), almonds and cashew which were also analysed in their studies. However, it has to be stated that the peptide LDALEPTNR (Jug r 4), related to walnut, occurs also in Car i 4, a major allergen in pecan nut. This fact degrades it to a marker only fitting for samples where pecan nut can be excluded a priori as contaminant or where a confirmatory marker is included.

A closer look to the peptide markers for Jug r 1 selected by Heick *et al.* (2011a-b) (88,109) shows, that the validated LOD value for DLPNECGISSQR is close to that of Bignardi *et al.* (2013) (124) for Jug r 1 in their first study although they were using an incurred bread matrix, to simulate industrial thermal processing.

Although there are already multiple walnut allergens listed, Table 12 shows that only two allergens were targeted quantitatively with LC-MS/MS, Jug r 1 and Jug r 4. The number of peptides is very scarce compared to milk or peanut. Robotham *et al.* (2009) (125) identified 1 epitope, which was reactive with patient sera, QGLRGEEMEEMV. As listed in Table 12 GEEMEEMVQSAR was one of the targeted peptides for Jug r 1. Also, for Jug r 4 three peptides were targeted. According to Robotham (2009) only EFQQDR is part of a weakly-reacting epitope.

Table 12 An overview of published peptide sequences for detection of the walnut allergens using LC-MS/MS. Parent ion charge state was +2 for all peptides except for those indicated. If available from the literature, Limit of Detection (LOD) and Limit of Quantification (LOQ) are shown. Electrospray ionization (ESI) was the standard for all instruments except for those indicated.

Allergen (Uniprot Accession #)	Peptide Sequence	Parent ion m/z	Matrix	State of Ingredient	LOD/ LOQ b	Instrumental set-up	Publication
Jug r 1 (P93198)	DLPNECGISSQR	688.2	bread	incurred with SMP, EWP and def. nuts	70/ - [a]	microLC-API-QQQ-MS^2	(87,109)
	QCCQQLSQMDEQCQCEGLR	820.2	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
	GEEMEEMVQSAR	698.3	bread	incurred with SMP, EWP and def. nuts	-	microLC-API-QQQ-MS^2	(87,109)
Jug r 4 (Q2TPW5)	LDALEPTNR	516	breakfast cereals	mixed with nuts	55/180[a]	microLC-IT-MS^2	(125)
	LDALEPTNR	515	dark chocolate	spiked with nuts	5/18[a]	microLC-IT-MS^2	(128)
	EFQQDR	412	breakfast cereals	mixed with nuts	-	microLC-IT-MS^2	(125)
	ADIYTEEAGR	563	biscuit	spiked with nuts	0.8/2.6[a]	microLC-IT-MS^2	(128)

[a] **method validation was performed**
[b] **mg of ingredient per kg of matrix**
[c] **charge state of the parent ion of +3**

1.9 MacCoss Lab Software: SKYLINE

Skyline is a freely-available, open-source Windows client application for building Selected Reaction Monitoring (SRM) / Multiple Reaction Monitoring (MRM), Parallel Reaction Monitoring (PRM), DIA/SWATH and targeted DDA quantitative methods and analysing the resulting mass spectrometer data. Its flexible configuration supports All Molecules. It aims to employ cutting-edge technologies for creating and iteratively refining targeted methods for large-scale quantitative mass spectrometry studies in life sciences.(132) The Skyline ecosystem is unique among freely-available, open source mass spectrometry proteomics software in its end-to-end support of the targeted proteomic mass spectrometry workflow. Skyline exports the methods for use in mass spectrometry acquisition on a broad range of instruments from 6 different mass spectrometer vendors. Without need of any file conversion, Skyline then supports importing raw data from most LCMS capable instruments, calculating peak areas in a vendor-neutral manner. Peak area data may be explored within the Skyline document using core analyses, comparing peptide retention times, peak areas, sample groups, underlying chromatograms and even mass spectra when available.(102)

2 Methods

2.1 Bradford Assay (protein determination)

2.1.1 Reagents:

- Coomassie Brilliant Blue G-250 (pure), Sigma- Aldrich
- Ethanol (99.9 %, p.A.), AustrAlco
- Ortho-phosphoric acid (85 %), Fa. Merck
- BSA, Sigma- Aldrich
- NaH_2PO_4, (99.0 %, p.a.), Fa. Merck
- Na_2HPO_4 x 2 H_2O (99.5 %, p.a.), Roth
- NaCl (99.8 %), Roth
- dist. water

2.1.2 Materials:

- ELISA-plates non-binding
- tips, Fa. Eppendorf
- Eppendorf tubes
- magnetic stirrbars
- beakers, erlenmeyer flasks
- measuring cylinders
- pleated filters
- funnel

2.1.3 Equipment:

- pipettes, Fa. Eppendorf
- analytic scale Kern EW 2200-2NM, Fa. Lactan
- magnetic stirrer IKA RCT basic, Fa. Lactan
- photometer for ELISA-plates, Sunrise Basic, Fa. Tecan

2.1.4 Preparation of solutions needed:

PBS-buffer 0.2 mol/L (pH 7.5)

32.22 g $Na_2HPO_4*2H_2O$ (Merck) +2.62 g $NaH_2PO_4*H_2O$ (Merck) +21.18 g NaCl (Merck) → filled up to 1000 mL with ddH_2O; stored at RT

Coomassie-Solution

Dissolve 10 mg Coomassie Brilliant Blue in 5 mL ethanol, mix with 10 ml of phosphoric acid and fill up with distilled water to a volume of 100 mL. Stir the solution overnight at 4 °C. Filter before use through a pleated filter. Store at 4 °C

BSA Stock solution - c = 1000 µg/mL

500 mg BSA are dissolved in 50 mL 0.2 M PBS-buffer

Standard Dilution series

The dilutions of the BSA stock solution for the preparation of the standards for the protein determination with Bradford Assay are prepared according to Table 1 using 0.2 M PBS-buffer for dilution.

Table 13: Dilution series for the standards used for the protein determination with Bradford Assay

	concentration [µg/mL]	solution [µL]	0.2 M PBS-buffer [µL]
1	500	500 stock solution	500
2	250	500 solution 1	500
3	125	500 solution 2	500
4	62.5	500 solution 3	500
5	31.25	500 solution 4	500
6	15.63	500 solution 5	500
7	7.81	500 solution 6	500
8	0	-	500

2.1.5 Procedure

Standards and samples are measured in triplicates and applied onto a 96-well plate (non-binding) like shown in Figure 6. Dilutions of samples are prepared with 0.2 M PBS-buffer. Chocolate dessert extracts were diluted 1:10 before application on the 96-well plate.

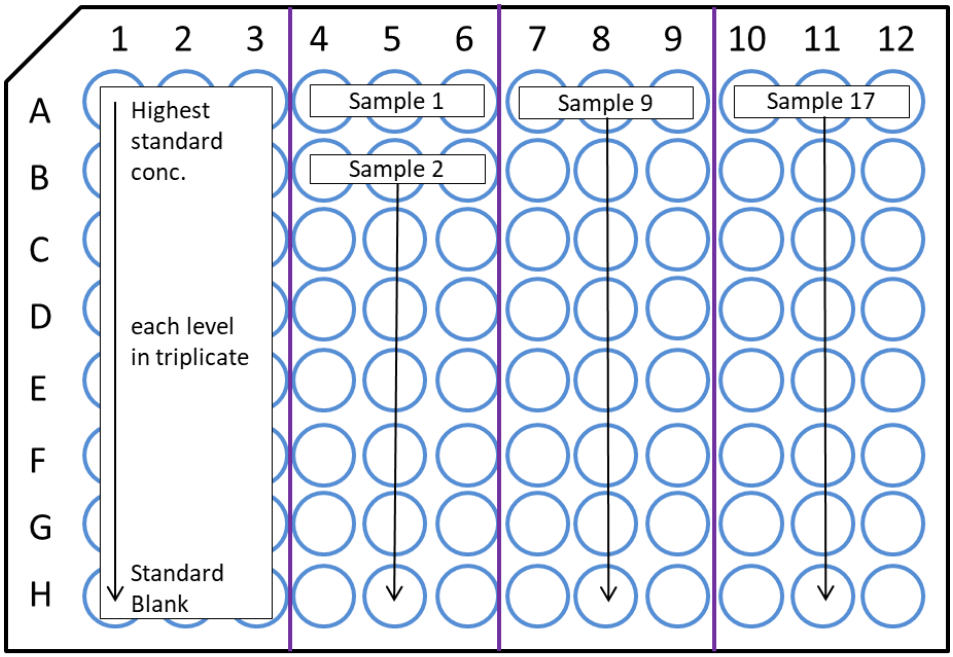


Figure 6: Pipetting Scheme for the protein determination with Bradford Assay

1. Apply 20 µL/well of protein solution or standard (in triplicates)
2. Add 200 µL/well Coomassie-solution
3. Incubate 15 min at room temperature
4. Measure at 595 nm with photometer

For the calculation of the protein concentration within the extracts, a standard curve was prepared by plotting the average absorbance values for each BSA standard versus its concentration (µg/mL) via a cubic spline function. Then the concentrations of the samples were calculated by relating average absorbance of the sample replicates to the standard curve. Dilution factor of 10 for the extracts had to be considered. These calculations were performed with the software Magellan® of the "Sunrise Basic" plate reader from Tecan.

2.2 Chocolate dessert

Chocolate dessert was the model matrix which was used in this research work as a food matrix to develop a multiallergen LC-MS/MS method. This chocolate dessert was spiked with hazelnut, peanut, walnut, milk and egg proteins as allergenic ingredients, details of ingredients can be found in Table 14. The matrix is a starch containing chocolate dessert which is prepared in an un-reconstituted form, meaning no water included. This gives the chocolate dessert base a longer shelf-live and it can be reconstituted before use by addition of water.

2.2.1 Ingredients

Allergenic ingredients were sourced from Austria and from UK and are listed in Table 14. The base materials (see Table 15) for the chocolate dessert were sourced locally at Uni Manchester, as the chocolate dessert was prepared at their production site. The site was equipped with:

- Hobart NCM40 mixer
- Sartorius electronic balances (2 decimal places from gram measurement)
- metal bowls, spoons, spatulas

Table 14: Allergenic ingredients of the chocolate dessert

Material	Source	Protein content [%]
Peanut Flour	Byrd Mill	54.53
Hazelnut flour	Nutranch	31.1
Walnut Flour	Nutranch	68.9
Skimmed Milk Powder	Bioservice	35.1
Egg White Powder	Bioservice	81.5

Protein content of the ingredients was verified via Kjeldahl analysis (n=6.25). Data was provided by Uni Manchester.

Oatmeal was used as substitute for the 0 ppm chocolate dessert base, where no allergenic ingredients were added.

Table 15: Ingredients of the chocolate dessert base. Addition to Table 14.

Ingredient Name	Description	Source
Ultra-TEX-4	Cold swelling starch	National Starch and Chemical, Manchester, UK (sourced direct).
Cadburys Cocoa	Cocoa powder	Birchalls Foodservice, Hapton, UK.
Icing Sugar	Sucrose	Birchalls Foodservice, Hapton, UK.
Maizola	Highly refined maize oil	Birchalls Foodservice, Hapton, UK.
Montanox 60PHA	Emulsifier Polysorbatan 60 (also known as Tween 60)	Seppic AS, Paris, France, obtained from Macphie of Glenbervie Ltd, Stonehaven, UK.
Fine Oatmeal	Oatmeal	Oatmeal of Alford

2.2.2 Recipe

To assure proper homogenization of the different contamination levels, a chocolate dessert blank with 0 ppm allergenic ingredient's protein was prepared (5 kg batch) and a chocolate dessert containing 1000 ppm of each allergenic ingredient's protein (5 kg batch). The blank material was prepared first to minimize the risk of contamination.

According to the weights from the recipe in Table 16 the chocolate dessert was prepared as described in the following.

Table 16: Recipe of the chocolate dessert base containing 0 ppm and 1000 ppm of the active allergenic ingredient's proteins

ppm of allergenic ingredient (reconstituted)	**0 ppm**		**1000 ppm**	
ppm of allergenic ingredient (dessert base)	0 ppm		3333 ppm	
	%	weight (g)	%	weight (g)
Peanut flour	0.00	0.00	0.61	30.56
Hazelnut flour	0.00	0.00	1.08	53.76
Skimmed Milk Powder	0.00	0.00	0.95	47.48
Egg White powder	0.00	0.00	0.41	20.45
Walnut flour	0.00	0.00	0.48	24.19
Starch	16.56	828.00	16.56	828.00
Cocoa	26.49	1324.50	26.49	1324.50
Sugar	22.36	1118.00	22.36	1118.00
Oil	29.89	1494.50	29.89	1494.50
Tween	0.70	35.00	0.70	35.00
Oatmeal	4.00	200.00	0.47	23.55
Total	100.00	5000.00	100.00	5000.00

2.2.3 Preparation of the placebo chocolate dessert base (0 ppm)

Step 1

Preheat the maize oil to 40 °C. (minimum 40 min)

Step 2

Powdered ingredients: Weigh out the powdered ingredients cocoa, starch, icing sugar and toasted oatmeal in quantities specified for placebo dessert into clean metal bowls. Use pre-calibrated scales.

Step 3

Liquid ingredients: Weigh out pre-warmed maize oil and tween-60 in quantities specified for placebo dessert into clean bowls.

Step 4

Powdered ingredient mixing: Add cocoa, starch, icing sugar and toasted oatmeal to clean mixing bowl in the Hobart mixer. Mix for 20 min at low speed (speed 1).

Step 5

Liquid ingredient preparation: Add tween-60 to the warm maize oil and dissolve.

Step 6

Ingredient mixing: Add liquid components from Step 5 to dry ingredient mix from Step 4 in the Hobart mixer. Mix for 1 min at low speed then a further 5 min on medium speed (speed 2).

2.2.4 Preparation of the high dose chocolate dessert base (1000 ppm)

Step 1

Preheat the maize oil to 40 °C. (minimum 40 min)

Step 2

High-dose peanut dessert powdered ingredient weighing: Weigh out cocoa, starch, icing sugar, and toasted oatmeal in quantities specified in Table 16 for high-allergen dose dessert into clean sanitised metal bowls. Seal and put away all non-allergenic ingredients to avoid cross contamination.

Step 3

Allergenic ingredient weighing: Bring the peanut flour out of storage and into production area. Weigh out peanut flour in the quantity specified for high-allergen dose into a clean metal bowl. Seal and remove peanut flour from production area to locked storage. Repeat this step also for hazelnut flour, walnut flour, skimmed milk powder and egg white powder.

Step4

High-dose peanut dessert liquid ingredient weighing: Weigh out pre-warmed maize oil, tween-60 in quantities specified for high-dose allergenic dessert into clean sanitised metal bowls.

Step 5

High-dose peanut dessert powdered ingredient mixing: Add cocoa, icing sugar, peanut flour, walnut flour, hazelnut flour, skimmed milk powder, egg white powder, toasted oatmeal and starch to clean mixing bowl in the Hobart mixer. Mix for 20 min at low speed (speed 1)

Step 6

High-dose peanut dessert liquid ingredient preparation: Add tween-60 to oil and dissolve.

Step 7

Ingredient mixing: Add liquid components from Step 6 to dry ingredient mix from Step 5 in the Hobart mixer. Mix for 1 min at low speed then a further 5 min on medium speed (speed 2).

2.2.5 Preparation of the different ppm-levels of chocolate dessert

According to Table 17 the different contamination levels of the chocolate dessert were mixed stepwise down to lower concentrations of 3, 10, 30 and 100 ppm of allergenic ingredient's protein in chocolate dessert. The 100 ppm material was used during method development and the additional levels were then needed for in-house validation of the method.

Mixing was performed in a Horbat mixer, mixing for 1 min at low speed then a further 5 min on medium speed (speed 2)**.**

Table 17: preparation scheme of the different contamination levels of the chocolate dessert

	weights in g			
resulting level	total mix	0 ppm	active chocolate dessert	
100 ppm	5000	4500	500	(1000 ppm)
30 ppm	4000	2800	1200	(100 ppm)
10 ppm	4500	3000	1500	(30 ppm)
3 ppm	4000	2800	1200	(10 ppm)

Chocolate dessert potting

Weigh out mixed dessert base into supplied pots (see Figure 7). Use a pre-calibrated balance. The target weight for each pot is 15 g dessert with an acceptable tolerance of +/- 0.1 g. Firmly seal the pot lids. Label all high allergen dose pots with appropriate labels.

Figure 7: Potted chocolate dessert

Dessert storage

Store all pots at room temperature if needed immediately. For later use store the pots at 4 °C in a fridge or at -20 °C in the freezer for long term storage. Take care to store placebo, low-dose and high-dose desserts separately.

2.2.6 Reconstitution of the chocolate dessert

Before use for the method development for LC-MS/MS, the chocolate dessert had to be reconstituted to adjust the correct ppm-level of allergenic ingredient's protein. Care was taken **not** to use the same spatula with different pots, to make sure that no cross-contamination between the different levels of spiked food allergen happened. Reconstitution steps 1-4 are visually supported in Figure 8.

Step 1

To reconstitute the chocolate dessert, add 25 mL of deionised water to the 15 g of pre-portioned chocolate dessert base.

Step 2

Mix the content of the pot with a clean metal spatula slowly and gently until the water has been absorbed and incorporated into the dessert matrix to give a smooth (no visible lumps) chocolate matrix. Ensure no splashing or loss of water occurs. Mix thoroughly until the cocoa solids are no longer visible (especially on the sides of the container) and the dessert matrix has thickened. Seal the container.

Step 3

Place desserts in a refrigerator (2-8 °C) for at least 30 min. If there are some small lumps in the dessert these will disappear when chilled.

Step 4

Re-stir with a fresh spatula and use.

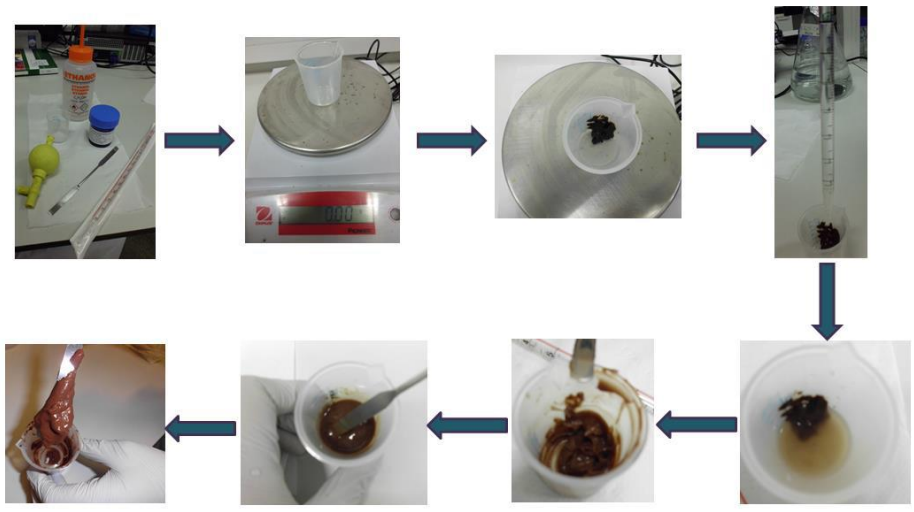


Figure 8: Reconstitution of the chocolate dessert

2.3 In-Silico proteolytic digestion of allergenic ingredients

In an in-silico digestion experiment which was performed with the informatics tool “Skyline”, the set of peanut, hazelnut, walnut, milk and egg allergens (see Table 18) was additionally enzymatically cleaved with 5 different proteases.

- **Trypsin**: cutting K (Lysine) and R (Arginine); c-term
- **Pepsin**: cutting F (Phenylalanine) and L (Leucine); c-term
- **Chymotrypsin**: cutting Y (Tyrosine), F (Phenylalanine), W (Tryptophan) and L (Leucine); c-term
- **GluC**: cutting D (Aspartate) and E (Glutamate); c-term
- **AspN**: cutting D and E; n-term

Table 18: UniProt accession numbers and isoelectric points (pI) of the food allergens included in the in-silico digestion experiment. The pIs were generated by the online tool “expasy.org”.

Allergen	protein	Sequence type	UniProt accession numbers	pI
Jug r 1.0101	2S albumins	Fragment	P93198	5.75
Jug r 2.0101	Vicilins	Fragment	Q9SEW4	6.19
Jug r 3	nsLTPs	Complete	C5H617	9.20
Jug r 4.0101		Complete	Q2TPW5	6.80
Cor a 8.0101	nsLTPs	Complete	Q9ATH2	9.36
Cor a 9.0101	legumins	Complete	Q8W1C2	6.46
Cor a 10.0101		Complete	Q9FSY7	4.97
Cor a 11.0101	vicilins	Complete	Q8S4P9	6.12
Cor a 12.0101		Complete	Q84T21	10.54
Cor a 13.0101		Complete	Q84T91	9.98
Cor a 14.0101		Complete	D0PWG2	6.59
Ara h 1	vicilins	Complete	P43238	6.62
Ara h 2	2S albumins	Complete	Q6PSU2	5.96
Ara h 3/4	legumins	Complete	Q8LKN1	5.52
Ara h 5	profilins	Complete	D3K177	4.69
Ara h 6	2S albumins	Complete	Q647G9	6.13
Bos d 9	αS1 casein	Complete	P02662	4.98
Bos d 10	αS2casein	Complete	P02663	8.55
Bos d 11	β-casein	Complete	P02666	5.26

Bos d 12	κ-casein	Complete	P02668	6.29
Bos d 5	β-Lactoglobulin	Complete	P02754	4.93
Bos d 4	α-Lactalbumin	Complete	P00711	4.92
Gal d 1	Ovomucoid	Complete	P01005	4.75
Gal d 2	Ovalbumin	Complete	P01012	5.19
Gal d 3	Ovotransferrin	Complete	P02789	6.85
Gal d 4	Lysozyme	Complete	P00698	9.36

For the in-silico digest FASTA format files (amino acid sequences) of the allergens, the respective NCBI numbers are listed in Table 18 were imported into Skyline and peptide settings were set the following:

- missed cleavages → 0
- length of peptides → 8-15 AA (according to 1-1.5 kDa)
- potential ragged ends → excluded
- excluded peptides containing Cysteine, Methionine and Glycosylation sites;
- excluded structural modifications → Carbamidomethyl Cysteine

The complete list of enzyme hydrolysis products, containing no "missed cleavages" or sites of enzyme inactivity, was further refined by removing all products containing less than 8 and greater than fifteen amino acids in length. The rational for this was that peptides which contain too few amino acids are not likely to be specific and may be common to many proteins. While peptides containing greater than fifteen amino acids are difficult to synthesise in a pure form, which may hamper the preparation of a calibration standard, and often exhibit unfavourable physical properties for ideal MS detection, such as multiple sites of protonation. Peptides containing the amino acid cysteine and the protein's C and N terminal peptides, as these are often subject to unspecific hydrolysis *in-vivo*, were also removed from the final list. These peptides were de-selected as they may cause issues if used in determining the quantity of protein present as the molar quantity of these peptides may not be representative of the total protein content.

Number of resulting peptides of this in-silico digest for the different proteases were collected and compared to identify the best choice of enzyme for the development of the targeted LC-MS/MS method for the detection of food allergens in chocolate dessert.

2.4 Standard Extraction Protocol

Extraction buffer:

- 50 mM Ammonium bicarbonate buffer, pH 7.8 (ABC_{50}): 0.198 g of NH_4HC_3(ROTH T871) solved in 50 mL of milliQ water → pH adjusted with NH_3OH

Extraction procedure

- Weigh approximately 1.00 g of sample and add 10x volume of the extraction buffer, e.g. 1.02 g sample + 10.2 mL buffer. This was performed in 15 mL Falcons.
- Vortex to obtain a homogeneous slurry
- Incubate and rotate for 30 min at room temperature (RT) (22 °C) using a rotary mixer
- Centrifuge for 15 min at 16,100 × g at 4 °C
- Collect the supernatant in 2 mL Eppis and freeze if not needed immediately
- Before extracts are fortified to the tryptic digestion, protein content was determined with Bradford Assay (see chapter 2.1)

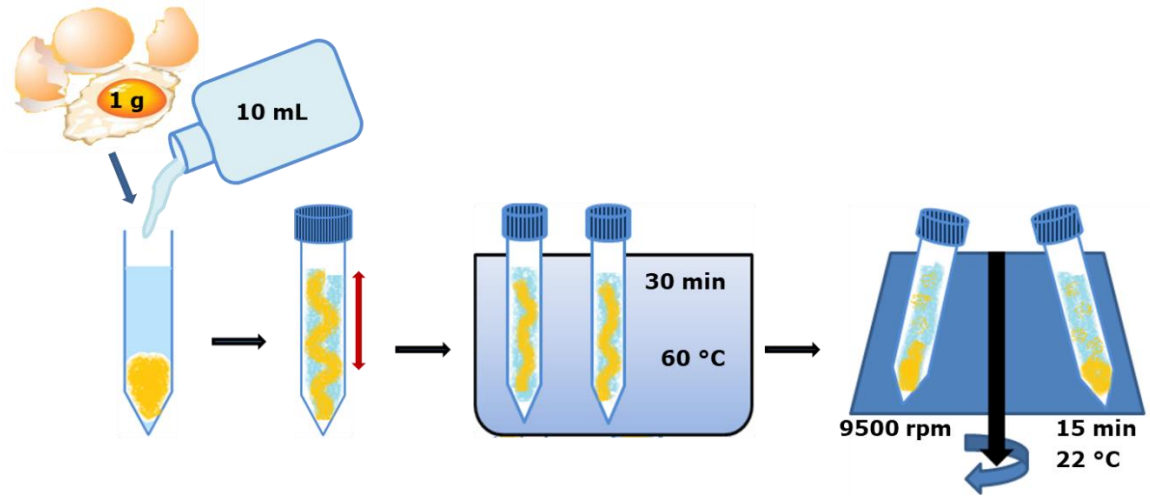


Figure 9: Extraction Scheme of the standard extraction protocol

2.5 Standard Digestion Protocol

Buffers and solutions:

- ABC_{50}: 50 mM Ammonium bicarbonate (ROTH T871) (NH_4HCO_3)
- DTT: 10 mM Dithiothreitol (ROTH 8908.2) in ABC_{50}
- IAA: 55 mM Iodoacetamide (SIGMA I6125) in ABC_{50}
- Trypsin: Trypsin, Sequencing Grade Modified (Promega # V5111)
  0.100 µg/µL in 25 mM Ammonium bicarbonate
- Enolase: Massprep Enolase Digestion Standard (Fa. Waters)
  5 pmol/µL in ABC_{50}
- Fibrinopeptide: [Glu1]-Fibrinopeptide B Standard (Fa. Waters)
  2 µg/mL in milliQ water + 0.1 % formic acid (MS grade)

Digestion Procedure

- Apply 8 µg of protein into a 500 µL Eppi and adjust to a total volume of 60 µL with ABC_{50}
- Add 4 µL of the Enolase of a 5 pmol/µL solution
- Add 20 µL of DTT and incubate at 90 °C, 15 min
- Add 20 µL of IAA and incubate at 25 °C, 15 min in the dark
- Add 8 µL of Trypsin resulting in a protease to protein ratio of 1:10 by weight
- Incubate at 37 °C for 16 h
- Add 1 µL of 50 % Formic acid in water
- Add 4 µL of Fibrinopeptide (6.2 pmol/µL = 10 µg/mL)
- Transfer into a 1.5 mL glass vial equipped with a 100 µL insert for MS measurement
- Analyze samples using a QTrap6500+ from AB Sciex

2.6 Extraction Optimisation Experiments

Extraction optimisation for the targeted LC-MS/MS method to detect food allergens in food with the food model chocolate dessert was performed with reconstituted chocolate dessert containing 100 ppm of allergenic ingredient's protein. The plan consisted of a simple step-by-step experimental layout with 5 stages:

- Buffer
- Detergents
- Temperature
- Time
- Sample-Buffer-Ratio

The standard extraction protocol from chapter 2.4 was modified according to the following parameters.

2.6.1 Extraction buffers

Different relevant extraction buffers fit for LC-MS/MS measurement were tested in the first stage.

a) Tris-HCl → 50 mM Tris-HCl buffer, pH 5.0

Trisma base (sigma) solved in milliQ water; pH adjusted with HCl

b) ABC50 → 50 mM ammonium bicarbonate buffer, pH 7.8

Ammonium bicarbonate - NH_4HCO_3 (ROTH T871) solved in milliQ water; adjust pH with NH_3OH

c) ABC100 → 100 mM ammonium bicarbonate buffer, pH 8.1

Ammonium bicarbonate - NH_4HCO_3 (ROTH T871) solved in milliQ water; adjust pH with NH_3OH

d) TBB → 8 mM Tris, 10 mM Borat buffer pH 8.5

Trisma base (Sigma) and $(NH_4)_2B_{10}O_{16}$ (Sigma-Aldrich) solved in milliQ water

2.6.2 Addition of Detergents

Knowing that the addition of detergents can especially help with the extraction of the seed storage proteins, urea, dithiothreitol (DTT) and sodium deoxycholate (SCD) were chosen as relevant detergents. Chemicals were obtained from Sigma Aldrich.

I. no detergent
II. 0.6 M urea
III. 6 M urea
IV. 0.6 M urea + 50 mM DTT
V. 6 M urea + 50 mM DTT

2.6.3 Extraction time and Extraction temperature

The combinatory effect of extraction time and temperature for protein extraction is well known. Too long extraction with too high temperature might harm proteins and lead to degradation, too low temperature and too low extraction time might lead to insufficient extraction of proteins. Therefore, these two parameters were tested in combination. Extractions were performed in water bath with the regarding temperatures. Buffers were preheated to the respective temperature before application to the samples.

Extraction times:

I. 15 min
II. 30 min
III. 60 min

Extraction temperatures:

a) RT (22 °C)
b) 37 °C
c) 60 °C

2.6.4 Sample-Buffer-Ratio

To determine the best sample-buffer-ratio which gives the highest protein yield in the extracts four different sample-buffer ratios were tested: 1:5, 1:10, 1:20, 1:40.

2.7 Tryptic Digest Optimisation Experiments

Tryptic digest optimization for the targeted LC-MS/MS method to detect food allergens in food with the food model chocolate dessert was performed with reconstituted chocolate dessert containing 100 ppm of allergenic ingredient's protein. The plan consisted of a simple step-by-step experimental layout with 4 stages:

- Trypsin brand
- protease-to-protein ratio
- Temperature
- Addition of detergents

The optimization of trypsin digestion was performed on the basis of the basic protocol, which was also used during extraction optimization. For the tryptic digest optimisation experiments an extract made from the 333 ppm allergenic protein chocolate dessert base was made to have higher concentration of allergenic proteins in the sample and therefore reach higher peptide concentration to see if more peptides can be found and not to be limited due to protein concentration.

2.7.1 Trypsin brand

In the first experiment two different brands of Trypsin were tested. In a recent publication the authors concluded that Sequencing Grade Trypsin from Promega (# V5111) showed the best overall performance out of 6 brands tested (133). Therefore, Sequencing Grade Trypsin from Promega was compared to proteomics grade trypsin from Sigma which was not part of the above-mentioned study.

- Trypsin, Proteomics Grade, BioReagent, Dimethylated (Sigma # T6567)
- Trypsin, Sequencing Grade Modified (Promega # V5111)

2.7.2 Protease-to-protein ratio

The second series of experiments determined the appropriate protein-to-protease ratio of the trypsin chosen at the stage before. Tested protease-to-protein ratios were: 1:5, 1:10, 1:25, 1:50, 1:100.

To achieve the different protease to protein ratios trypsin solutions with different concentrations were used, while volumes were kept constant.

2.7.3 Incubation temperature

Two different temperatures were tested. 37 °C as this is the optimal for most standard applications and is also given as optimum by the provider (PROMEGA) and 55 °C. As the trypsin activity in aqueous buffers declines sharply above 60 °C, we tested temperatures below this value. Additionally, we analysed two protease-protein-ratios (1:5 and 1:10) as results in the previous stage for these two protease-to-protein ratios were quite similar and to test if a higher temperature may allow applying less trypsin.

2.7.4 Addition of Detergents

Described in literature there are some detergents which could have a positive effect on the protein digestion. Therefore, we evaluated the effect of following detergents:

- No detergent
- 0.1 % RapiGest SF (Waters)
- 0.04 % RapiGest SF (Waters)
- 0.5 % Sodium deoxycholate (SDC)
- 10 % Acetonitrile (ACN)

The tryptic digestion was performed as described in the Standard digestion protocol in chapter 2.5 above. Differing from this, ABC_{50} contained the detergents under investigation and was used to adjust the volume to 60 µL.

<u>No detergent</u>

13.3 µg of protein was applied and adjusted to a total volume of 60 µL with ABC_{50}.

<u>0.1 % RapiGest SF (Waters)</u>

1 mg RapiGest SF was reconstituted in 42.3 µL ABC_{50} to gain a concentration of 2.36 % (w/v). 13.3 µg of protein was applied and adjusted to a total volume of 55 µL with ABC_{50} before 5 µL of 2.36 % RapiGest were added to gain a concentration of 0.1 % RapiGest during tryptic digestion.

0.04 % RapiGest SF (Waters)

13.3 µg of protein was applied and adjusted to a total volume of 55 µL with ABC_{50}. 10 µL of 2.36 % RapiGest SF was diluted with 15 µL ABC_{50} to gain a concentration of 0.944 % (w/v) RapiGest SF and 5 µL of this dilution was added to gain a concentration of 0.04 % RapiGest SF during tryptic digestion.

Sodium deoxycholate (SDC)

13.3 µg of protein was applied and adjusted to a total volume of 55 µL with ABC_{50}. 5 mg SDC was dissolved in 42.4 µL ABC_{50} to gain a concentration of 11.8 % SDC (w/v) and 5 µL of 11.8% SDC solution was added to the sample to gain a concentration of 0.5 % (w/v) SDC during tryptic digestion, which is compatible with trypsin activity.

Acetonitrile

13.3 µg of protein was applied and adjusted to a total volume of 48 µL with ABC_{50} and 12 µL Acetonitrile was added. All samples underwent reduction and alkylation as described above before tryptic digestion was carried out for 16 h at 37 °C. Tryptic cleavage was stopped by adding 13 µL 5 % formic acid in water.

The pH of samples with addition of RapiGest SF or SDC was checked to be below 2, to make sure that the surfactants are deactivated. Afterwards the samples were centrifuged and the solid supernatant was removed before MS analysis as it was done in all other stages.

2.8 Evaluation procedure of the optimisation steps

It was planned to perform a 2-step evaluation procedure of the different optimisation stages for the extraction of the protein from chocolate dessert and the tryptic digest of the protein extracts. It should consist of:

I. Determination of the protein yield via 2D-Quant Kit from GE

II. Analyse the digested samples using a QTrap 6500+ from Sciex with a preliminary screening targeted MS/MS method (see chapter 0) with respect to number of proteins and number of peptides per protein detected. Additionally, peak intensities of the total ion chromatogram (TIC) of the three – four strongest y-ions per found peptide were compared.

Finally, the screening method consisted of 142 peptides defined by 604 transitions in total. The transitions were separated into two methods and were measured with a dwell time of 8 msec.

Those peptides represented by at least 2 transitions with a peak height of above 5000 cps giving at least a S/N ratio of 3 were kept for data analysis. As 3 replicates were measured only peptides found in minimum 2 replicates were taken as found. Peak area of the total ion chromatogram (TIC) of the three strongest transitions are displayed in the result figures (Figure 10). Preliminary targeted LC-MS/MS method for optimisation evaluation:

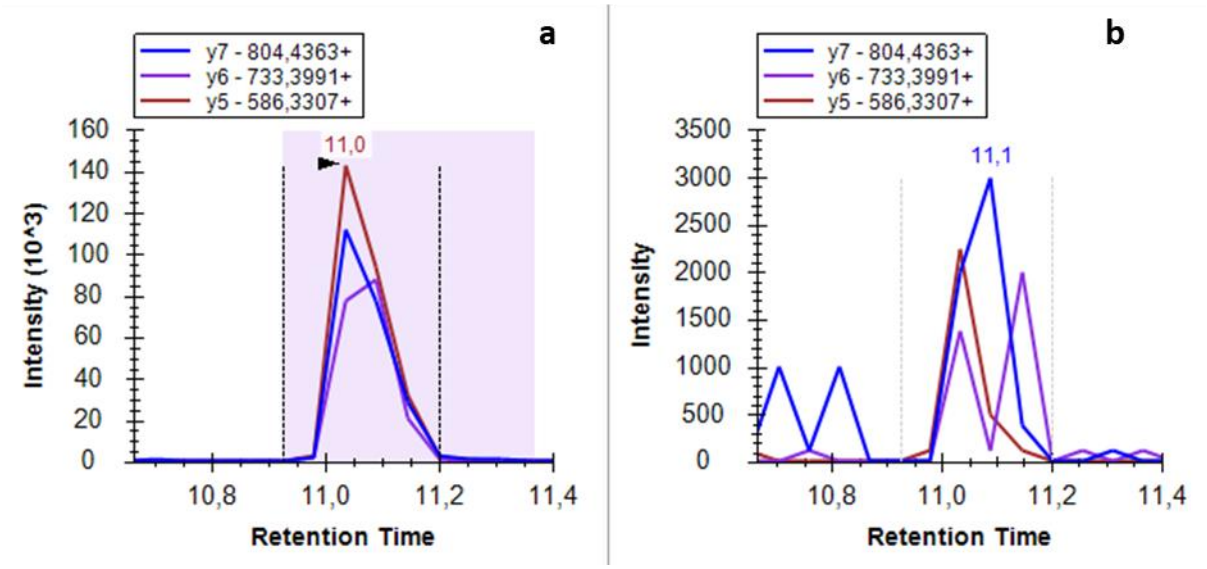


Figure 10: Peak areas of the three strongest y-ions from peptide ELAFNLPSR (Cor a 11) measured in (a) TBB buffer where it was taken as "found" during data evaluation and in (b) TRIS buffer where it was not taken as "found".

Reagents

- MilliQ water
- Formic acid (LC-MS grade), Fa. Merck
- Methanol (hypergrade for LC-MS LiChrosolv®), Fa. Merck

Materials

- Glass vials for LC-MS/MS
- Caps for glass vails incl. Septum

Equipment

- **LC system:** Agilent 1290 Infinity Binary LC System (Agilent); column oven (Agilent 1290 G1316C), binary pump (Agilent 1290 G4220A), autosampler (Agilent 1290 G4226A)
- **LC guard column:** SecurityGuard ULTRA Cartridge UHPLC C18-Peptide for 2.1 mm ID Columns (Phenomenex)
- **LC column:** Aeris PEPTIDE 3.6u XB – C18 column 150*2.1 mm (Phenomenex)
- **MS:** QTRAP® 6500+ System (AB Sciex) equipped with an IonDrive™ Turbo V source
- **Software:** Skyline (MacCoss Lab Software)

2.8.1.1 Generation of LC-MS/MS method

The software used to generate the method-file for the screening method was Skyline. Protein-sequences listed in Table 18, which were the protein sequences that have also been incorporated in the in-silico digest experiment, were transferred into Skyline.

Further the settings for the peptides generation was set to lent between 6-25 amino acids (AAs), last 3 N-terminal AAs were excluded, potential ragged ends were excluded, peptides containing Methionine and Histidine were excluded. As trypsin is not able to cleave a protein if proline is following the lysine or the arginine also those peptides were excluded for the method.

In the transition settings precursor charge of +2 was chosen and only y-ions were taken into count. Product ion selection was chosen from "(m/z > precursor)-1" to 4 ions. That means only those y-ions, starting with the first y-ion which is smaller than the m/z of the precursor and then 4 further (bigger) y-ions were chosen. This resulted in maximum 4 y-ions per peptide. Ion match tolerance was set to 0.5 m/z.

Prediction of collision energy (CE) and declustering potential (DP) for these experiments were done by the software Skyline, as it already has implemented some predefined parameters for proteomics for the most common MS-instruments.

Two Export-files were generated (see Table 19-Table 20) and imported into Analyst 1.6.3, which is the measurement software of the QTRAP 6500+ (AB Sciex Instruments). As the number of concurrent transitions, namely 604 transitions, was too high for putting all in one single run, 2 methods (302 transitions each) were generated with dwell time per transition of 8 msec. This was done to ensure a cycle time of 2.4 seconds, which resulted in 7-8 data points per peak as overall peak width was 0.3 minutes.

Table 19: Transition list of method 1 used for the Optimisation experiments including information about transition, Q1 Mass (precursor), Q3 Mass (transitions), DP (declustering potential) and CE (collision energy). Dwell time was set for each transition to 8 msec.

ID	Q1 Mass	Q3 Mass	DP	CE
Ara h 1.LEYDPR.+2y5.light	396.70	679,30	60	23.1
Ara h 1.LEYDPR.+2y4.light	396.70	550.26	60	23.1
Ara h 1.LEYDPR.+2y3.light	396.70	387.20	60	23.1
Ara h 1.WGPAGPR.+2y6.light	370.70	554.30	58.1	22.2
Ara h 1.WGPAGPR.+2y5.light	370.70	497.28	58.1	22.2
Ara h 1.WGPAGPR.+2y4.light	370.70	400.23	58.1	22.2
Ara h 1.YGNQNGR.+2y6.light	404.69	645.31	60.6	23.4
Ara h 1.YGNQNGR.+2y5.light	404.69	588.28	60.6	23.4
Ara h 1.YGNQNGR.+2y4.light	404.69	474.24	60.6	23.4
Ara h 1.IVQIEAK.+2y6.light	400.75	687.40	60.3	23.3
Ara h 1.IVQIEAK.+2y5.light	400.75	588.34	60.3	23.3
Ara h 1.IVQIEAK.+2y4.light	400.75	460.28	60.3	23.3
Ara h 1.PNTLVLPK.+2y6.light	441.28	670.45	63.3	24.7
Ara h 1.PNTLVLPK.+2y5.light	441.28	569.40	63.3	24.7
Ara h 1.PNTLVLPK.+2y4.light	441.28	456.32	63.3	24.7
Ara h 1.PNTLVLPK.+2y3.light	441.28	357.25	63.3	24.7
Ara h 1.IPSGFISYILNR.+2y8.light	690.39	1025.58	81.4	33.7
Ara h 1.IPSGFISYILNR.+2y7.light	690.39	878.51	81.4	33.7
Ara h 1.IPSGFISYILNR.+2y6.light	690.39	765.43	81.4	33.7
Ara h 1.IPSGFISYILNR.+2y5.light	690.39	678.39	81.4	33.7
Ara h 1.DQSSYLQGFSR.+2y8.light	644.30	957.48	78.1	32.1
Ara h 1.DQSSYLQGFSR.+2y7.light	644.30	870.45	78.1	32.1
Ara h 1.DQSSYLQGFSR.+2y6.light	644.30	707.38	78.1	32.1
Ara h 1.DQSSYLQGFSR.+2y5.light	644.30	594.30	78.1	32.1
Ara h 1.SSENNEGVIVK.+2y8.light	588.30	872.48	74	30
Ara h 1.SSENNEGVIVK.+2y6.light	588.30	644.40	74	30
Ara h 1.SSENNEGVIVK.+2y5.light	588.30	515.36	74	30
Ara h 1.EGEPDLSNNFGK.+2y8.light	653.80	894.43	78.8	32.4
Ara h 1.EGEPDLSNNFGK.+2y7.light	653.80	779.40	78.8	32.4
Ara h 1.EGEPDLSNNFGK.+2y6.light	653.80	666.32	78.8	32.4
Ara h 1.EGEPDLSNNFGK.+2y5.light	653.80	579.29	78.8	32.4

ID	Q1 Mass	Q3 Mass	DP	CE
Ara h 1.IFLAGDK.+2y6.light	382.22	650.35	59	22.6
Ara h 1.IFLAGDK.+2y5.light	382.22	503.28	59	22.6
Ara h 1.IFLAGDK.+2y4.light	382.22	390.20	59	22.6
Ara h 1.DNVIDQIEK.+2y7.light	537.28	844.48	70.3	28.2
Ara h 1.DNVIDQIEK.+2y6.light	537.28	745.41	70.3	28.2
Ara h 1.DNVIDQIEK.+2y5.light	537.28	632.32	70.3	28.2
Ara h 1.DNVIDQIEK.+2y4.light	537.28	517.30	70.3	28.2
Ara h 1.DLAFPGSGEQVEK.+2y9.light	688.84	930.45	81.3	30.7
Ara h 1.DLAFPGSGEQVEK.+2y8.light	688.84	833.40	81.3	33.7
Ara h 1.DLAFPGSGEQVEK.+2y7.light	688.84	776.38	81.3	33.7
Ara h 1.DLAFPGSGEQVEK.+2y5.light	688.84	632.32	81.3	33.7
Ara h 1.PQSQSQSPSSPEK.+2y9.light	693.83	946.45	81.7	33.8
Ara h 1.PQSQSQSPSSPEK.+2y8.light	693.83	859.42	81.7	33.8
Ara h 1.PQSQSQSPSSPEK.+2y7.light	693.83	731.36	81.7	33.8
Ara h 1.PQSQSQSPSSPEK.+2y6.light	693.83	644.32	81.7	33.8
Ara h 1.EDQEEENQGGK.+2y9.light	631.76	1018.44	77.2	31.6
Ara h 1.EDQEEENQGGK.+2y8.light	631.76	890.39	77.2	31.6
Ara h 1.EDQEEENQGGK.+2y7.light	631.76	761.34	77.2	31.6
Ara h 1.EDQEEENQGGK.+2y5.light	631.76	503.26	77.2	31.6
Ara h 1.GPLLSILK.+2y6.light	420.78	686.48	61.8	24
Ara h 1.GPLLSILK.+2y5.light	420.78	573.40	61.8	24
Ara h 1.GPLLSILK.+2y4.light	420.78	460.31	61.8	24
Ara h 2.DEDSYGR.+2y6.light	421.17	726.31	61.8	24
Ara h 2.DEDSYGR.+2y5.light	421.17	597.26	61.8	24
Ara h 2.DEDSYGR.+2y4.light	421.17	482.24	61.8	24
Ara h 3_4.QILQNLR.+2y6.light	442.77	756.47	63.4	24.8
Ara h 3_4.QILQNLR.+2y5.light	442.77	643.39	63.4	24.8
Ara h 3_4.QILQNLR.+2y4.light	442.77	530.30	63.4	24.8
Ara h 3_4.SPDIYNPQAGSLK.+2y9.light	695.35	977.51	81.8	33.9
Ara h 3_4.SPDIYNPQAGSLK.+2y8.light	695.35	814.44	81.8	33.9
Ara h 3_4.SPDIYNPQAGSLK.+2y7.light	695.35	700.40	81.8	33.9
Ara h 3_4.SPDIYNPQAGSLK.+2y6.light	695.35	603.35	81.8	33.9
Ara h 3_4.TANELQLNLLILR.+2y8.light	755.95	982.64	86.2	36.1
Ara h 3_4.TANELQLNLLILR.+2y7.light	755.95	854.58	86.2	36.1
Ara h 3_4.TANELQLNLLILR.+2y6.light	755.95	741.50	86.2	36.1
Ara h 3_4.TANELQLNLLILR.+2y5.light	755.95	627.46	86.2	36.1
Ara h 3_4.WLGLSAEYGNLYR.+2y8.light	771.39	985.47	87.4	36.6
Ara h 3_4.WLGLSAEYGNLYR.+2y7.light	771.39	914.44	87.4	36.6
Ara h 3_4.WLGLSAEYGNLYR.+2y6.light	771.39	785.39	87.4	36.6
Ara h 3_4.WLGLSAEYGNLYR.+2y5.light	771.39	622.33	87.4	36.6
Ara h 3_4.SQSENFEYVAFK.+2y8.light	724.84	1017.50	84	35
Ara h 3_4.SQSENFEYVAFK.+2y7.light	724.84	903.46	84	35
Ara h 3_4.SQSENFEYVAFK.+2y6.light	724.84	756.39	84	35
Ara h 3_4.SQSENFEYVAFK.+2y5.light	724.84	627.35	84	35

ID	Q1 Mass	Q3 Mass	DP	CE
Ara h 3_4.NNNPFK.+2y5.light	367.19	619.32	57.9	22.1
Ara h 3_4.NNNPFK.+2y4.light	367.19	505.28	57.9	22.1
Ara h 3_4.NNNPFK.+2y3.light	367.19	391.23	57.9	22.1
Ara h 3_4.FFVPPSEQSLR.+2y8.light	653.84	913.47	78.8	32.4
Ara h 3_4.FFVPPSEQSLR.+2y7.light	653.84	816.42	78.8	41.4
Ara h 3_4.FFVPPSEQSLR.+2y6.light	653.84	719.37	78.8	32.4
Ara h 3_4.FFVPPSEQSLR.+2y5.light	653.84	632.34	78.8	32.4
Ara h 5.LGDYLIDTGL.+2y7.light	540.28	794.43	70.5	28.3
Ara h 5.LGDYLIDTGL.+2y6.light	540.28	631.37	70.5	28.3
Ara h 5.LGDYLIDTGL.+2y5.light	540.28	518.28	70.5	28.3
Ara h 5.LGDYLIDTGL.+2y4.light	540.28	405.20	70.5	28.3
Ara h 6.SSDQQQR.+2y5.light	424.70	674.32	62.1	24.1
Ara h 6.SSDQQQR.+2y4.light	424.70	559.29	62.1	24.1
Ara h 6.SSDQQQR.+2y3.light	424.70	431.24	62.1	24.1
Jug r 2.DDDDEENPR.+2y7.light	552.71	874.35	71.4	28.8
Jug r 2.DDDDEENPR.+2y6.light	552.71	759.33	71.4	28.8
Jug r 2.DDDDEENPR.+2y5.light	552.71	644.30	71.4	28.8
Jug r 2.DDDDEENPR.+2y4.light	552.71	515.26	71.4	28.8
Jug r 2.DVDDQNPR.+2y6.light	479.71	744.33	66.1	26.1
Jug r 2.DVDDQNPR.+2y5.light	479.71	629.30	66.1	26.1
Jug r 2.DVDDQNPR.+2y4.light	479.71	514.27	66.1	26.1
Jug r 2.DVDDQNPR.+2y3.light	479.71	386.21	66.1	26.1
Jug r 2.DAESVAVVTR.+2y7.light	523.78	731.44	69.3	27.7
Jug r 2.DAESVAVVTR.+2y6.light	523.78	644.41	69.3	27.7
Jug r 2.DAESVAVVTR.+2y5.light	523.78	545.34	69.3	27.7
Jug r 2.DAESVAVVTR.+2y4.light	523.78	474.30	69.3	27.7
Jug r 2.ATLTLVSQETR.+2y8.light	609.84	933.50	75.6	30.8
Jug r 2.ATLTLVSQETR.+2y7.light	609.84	832.45	75.6	30.8
Jug r 2.ATLTLVSQETR.+2y6.light	609.84	719.37	75.6	30.8
Jug r 2.ATLTLVSQETR.+2y5.light	609.84	620.30	75.6	30.8
Jug r 2.VPAGATVYVINQDSNER.+2y7.light	916.96	862.36	98	41.9
Jug r 2.VPAGATVYVINQDSNER.+2y6.light	916.96	748.32	98	41.9
Jug r 2.VPAGATVYVINQDSNER.+2y5.light	916.96	620.26	98	41.9
Jug r 2.VPAGATVYVINQDSNER.+2y4.light	916.96	505.24	98	41.9
Jug r 2.LLQPVNNPGQFR.+2y8.light	691.88	931.47	81.6	33.8
Jug r 2.LLQPVNNPGQFR.+2y7.light	691.88	832.41	81.6	33.8
Jug r 2.LLQPVNNPGQFR.+2y6.light	691.88	718.36	81.6	33.8
Jug r 2.LLQPVNNPGQFR.+2y5.light	691.88	604.32	81.6	33.8
Jug r 2.EYYAAGAK.+2y7.light	436.71	743.37	63	24.6
Jug r 2.EYYAAGAK.+2y6.light	436.71	580.31	63	24.6
Jug r 2.EYYAAGAK.+2y5.light	436.71	417.25	63	24.6
Jug r 2.SPDQSYLR.+2y6.light	483.24	781.38	66.3	26.3
Jug r 2.SPDQSYLR.+2y5.light	483.24	666.36	66.3	26.3
Jug r 2.SPDQSYLR.+2y4.light	483.24	538.30	66.3	26.3

ID	Q1 Mass	Q3 Mass	DP	CE
Jug r 2.SPDQSYLR.+2y3.light	483.24	451.27	66.3	26.3
Jug r 2.VFSNDILVAALNTPR.+2y9.light	815.45	954.57	90.6	38.2
Jug r 2.VFSNDILVAALNTPR.+2y8.light	815.45	841.49	90.6	38.2
Jug r 2.VFSNDILVAALNTPR.+2y7.light	815.45	742.42	90.6	38.2
Jug r 2.VFSNDILVAALNTPR.+2y6.light	815.45	671.38	90.6	38.2
Jug r 2.FFDQQEQR.+2y6.light	549.25	803.36	71.2	28.6
Jug r 2.FFDQQEQR.+2y5.light	549.25	688.34	71.2	28.6
Jug r 2.FFDQQEQR.+2y4.light	549.25	560.28	71.2	28.6
Jug r 2.ATVVVYVVEGTGR.+2y9.light	675.37	979.52	80.4	33.2
Jug r 2.ATVVVYVVEGTGR.+2y8.light	675.37	880.45	80.4	33.2
Jug r 2.ATVVVYVVEGTGR.+2y7.light	675.37	717.39	80.4	33.2
Jug r 2.ATVVVYVVEGTGR.+2y6.light	675.37	618.32	80.4	33.2
Jug r 2.LLGFDINGENNQR.+2y7.light	745.37	831.37	85.5	35.7
Jug r 2.LLGFDINGENNQR.+2y6.light	745.37	717.33	85.5	35.7
Jug r 2.LLGFDINGENNQR.+2y5.light	745.37	660.31	85.5	35.7
Jug r 2.LLGFDINGENNQR.+2y4.light	745.37	531.26	85.5	35.7
Jug r 2.DFLAGQNNIINQLER.+2y7.light	872.95	885.52	94.8	40.3
Jug r 2.DFLAGQNNIINQLER.+2y6.light	872.95	772.43	94.8	40.3
Jug r 2.DFLAGQNNIINQLER.+2y5.light	872.95	659.35	94.8	40.3
Jug r 2.DFLAGQNNIINQLER.+2y4.light	872.95	545.30	94.8	40.3
Jug r 3.AAATTADR.+2y6.light	388.70	634.32	59.5	22.9
Jug r 3.AAATTADR.+2y5.light	388.70	563.28	59.5	22.9
Jug r 3.AAATTADR.+2y4.light	388.70	462.23	59.5	22.9
Jug r 3.AAATTADR.+2y3.light	388.70	361.18	59.5	22.9
Jug r 4.LDALEPTNR.+2y6.light	514.77	729.39	68.6	27.4
Jug r 4.LDALEPTNR.+2y5.light	514.77	616.30	68.6	27.4
Jug r 4.LDALEPTNR.+2y4.light	514.77	487.26	68.6	27.4
Jug r 4.QSQQGQSR.+2y7.light	459.72	790.38	64.6	25.4
Jug r 4.QSQQGQSR.+2y6.light	459.72	703.35	64.6	25.4
Jug r 4.QSQQGQSR.+2y5.light	459.72	575.29	64.6	25.4
Jug r 4.QSQQGQSR.+2y4.light	459.72	447.23	64.6	25.4
Jug r 4.EFQQDR.+2y5.light	411.69	693.33	61.1	23.7
Jug r 4.EFQQDR.+2y4.light	411.69	546.26	61.1	23.7
Jug r 4.EFQQDR.+2y3.light	411.69	418.20	61.1	23.7
Jug r 4.NFYLAGNPDDEFR.+2y8.light	779.35	949.40	87.9	36.9
Jug r 4.NFYLAGNPDDEFR.+2y7.light	779.35	892.38	87.9	36.9
Jug r 4.NFYLAGNPDDEFR.+2y5.light	779.35	681.28	87.9	36.9
Jug r 4.ENIGDPSR.+2y6.light	444.21	644.34	63.5	24.8
Jug r 4.ENIGDPSR.+2y5.light	444.21	531.25	63.5	24.8
Jug r 4.ENIGDPSR.+2y4.light	444.21	474.23	63.5	24.8
Jug r 4.ENIGDPSR.+2y3.light	444.21	359.20	63.5	24.8
Jug r 4.ADIYTEEAGR.+2y8.light	562.76	938.46	72.1	29.1
Jug r 4.ADIYTEEAGR.+2y7.light	562.76	825.37	72.1	29.1
Jug r 4.ADIYTEEAGR.+2y6.light	562.76	662.31	72.1	29.1

ID	Q1 Mass	Q3 Mass	DP	CE
Jug r 4.ADIYTEEAGR.+2y5.light	562.76	561.26	72.1	29.1
Jug r 4.NEGFEWVSFK.+2y7.light	621.79	942.47	76.4	31.2
Jug r 4.NEGFEWVSFK.+2y6.light	621.79	795.40	76.4	31.2
Jug r 4.NEGFEWVSFK.+2y5.light	621.79	666.36	76.4	31.2
Jug r 4.NEGFEWVSFK.+2y4.light	621.79	480.28	76.4	31.2
Jug r 4.ALPEEVLATAFQIPR.+2y9.light	827.96	1016.59	91.5	44.7
Jug r 4.ALPEEVLATAFQIPR.+2y8.light	827.96	903.50	91.5	44.7
Jug r 4.ALPEEVLATAFQIPR.+2y6.light	827.96	731.42	91.5	38.7
Jug r 4.ALPEEVLATAFQIPR.+2y5.light	827.96	660.38	91.5	38.7
Jug r 4.QESTLVR.+2y6.light	416.73	704.39	61.5	23.9
Jug r 4.QESTLVR.+2y5.light	416.73	575.35	61.5	23.9
Jug r 4.QESTLVR.+2y4.light	416.73	488.32	61.5	23.9
Jug r 4.QESTLVR.+2y3.light	416.73	387.27	61.5	23.9
Gal d 1.DVLVC[CAM]NK.+2y6.light	424.22	732.41	62	24.1
Gal d 1.DVLVC[CAM]NK.+2y5.light	424.22	633.34	62	24.1
Gal d 1.DVLVC[CAM]NK.+2y4.light	424.22	520.25	62	24.1
Gal d 1.DVLVC[CAM]NK.+2y3.light	424.22	421.19	62	24.1
Gal d 2.DILNQITK.+2y6.light	472.77	716.43	65.6	25.9
Gal d 2.DILNQITK.+2y5.light	472.77	603.35	65.6	25.9
Gal d 2.DILNQITK.+2y4.light	472.77	489.30	65.6	25.9
Gal d 2.DILNQITK.+2y3.light	472.77	361.24	65.6	25.9
Gal d 2.PNDVYSFSLASR.+2y7.light	678.33	767.40	80.6	33.3
Gal d 2.PNDVYSFSLASR.+2y6.light	678.33	680.37	80.6	33.3
Gal d 2.PNDVYSFSLASR.+2y5.light	678.33	533.30	80.6	33.3
Gal d 2.PNDVYSFSLASR.+2y4.light	678.33	446.27	80.6	33.3
Gal d 2.LYAEER.+2y5.light	390.70	667.30	59.6	22.9
Gal d 2.LYAEER.+2y4.light	390.70	504.24	59.6	16.9
Gal d 2.LYAEER.+2y3.light	390.70	433.20	59.6	19.9
Gal d 2.VTEQESK.+2y6.light	410.71	721.34	61.1	23.6
Gal d 2.VTEQESK.+2y5.light	410.71	620.29	61.1	23.6
Gal d 2.VTEQESK.+2y4.light	410.71	491.25	61.1	23.6
Gal d 2.VTEQESK.+2y3.light	410.71	363.19	61.1	23.6
Gal d 3.DLTQQER.+2y6.light	445.22	774.41	63.6	24.9
Gal d 3.DLTQQER.+2y5.light	445.22	661.33	63.6	24.9
Gal d 3.DLTQQER.+2y4.light	445.22	560.28	63.6	24.9
Gal d 3.DLTQQER.+2y3.light	445.22	432.22	63.6	24.9
Gal d 3.ISLTC[CAM]VQK.+2y6.light	474.76	748.40	65.7	25.9
Gal d 3.ISLTC[CAM]VQK.+2y5.light	474.76	635.32	65.7	25.9
Gal d 3.ISLTC[CAM]VQK.+2y4.light	474.76	534.27	65.7	25.9
Gal d 3.ISLTC[CAM]VQK.+2y3.light	474.76	374.24	65.7	25.9
Gal d 3.ATYLDC[CAM]IK.+2y6.light	492.25	811.40	67	26.6
Gal d 3.ATYLDC[CAM]IK.+2y5.light	492.25	648.34	67	26.6
Gal d 3.ATYLDC[CAM]IK.+2y4.light	492.25	535.25	67	26.6
Gal d 3.ATYLDC[CAM]IK.+2y3.light	492.25	420.23	67	26.6

ID	Q1 Mass	Q3 Mass	DP	CE
Gal d 3.GAIEWEGIESGSVEQAVAK.+2y10.light	980.49	975.51	102.6	44.2
Gal d 3.GAIEWEGIESGSVEQAVAK.+2y9.light	980.49	888.48	102.6	44.2
Gal d 3.GAIEWEGIESGSVEQAVAK.+2y8.light	980.49	831.46	102.6	44.2
Gal d 3.FFSASC[CAM]VPGATIEQK.+2y9.light	821.40	942.53	91	38.4
Gal d 3.FFSASC[CAM]VPGATIEQK.+2y8.light	821.40	843.46	91	38.4
Gal d 3.FFSASC[CAM]VPGATIEQK.+2y7.light	821.40	746.40	91	38.4
Gal d 3.GDVAFVK.+2y5.light	368.21	563.36	58	22.1
Gal d 3.GDVAFVK.+2y4.light	368.21	464.29	58	22.1
Gal d 3.GDVAFVK.+2y3.light	368.21	393.25	58	22.1
Gal d 3.DEYELLC[CAM]LDGSR.+2y9.light	735.33	1062.52	84.7	35.3
Gal d 3.DEYELLC[CAM]LDGSR.+2y8.light	735.33	933.48	84.7	35.3
Gal d 3.DEYELLC[CAM]LDGSR.+2y7.light	735.33	820.40	84.7	35.3
Gal d 3.DEYELLC[CAM]LDGSR.+2y6.light	735.33	707.31	84.7	35.3
Gal d 3.QPVDNYK.+2y6.light	432.22	735.37	62.6	24.4
Gal d 3.QPVDNYK.+2y5.light	432.22	638.31	62.6	24.4
Gal d 3.QPVDNYK.+2y4.light	432.22	539.25	62.6	24.4
Gal d 3.QPVDNYK.+2y3.light	432.22	424.22	62.6	24.4
Gal d 3.TC[CAM]NWAR.+2y5.light	404.18	706.31	60.6	23.4
Gal d 3.TC[CAM]NWAR.+2y4.light	404.18	546.28	60.6	23.4
Gal d 3.TC[CAM]NWAR.+2y3.light	404.18	432.24	60.6	23.4
Gal d 3.VEDIWSFLSK.+2y8.light	612.32	995.52	75.8	30.9
Gal d 3.VEDIWSFLSK.+2y7.light	612.32	880.49	75.8	30.9
Gal d 3.VEDIWSFLSK.+2y6.light	612.32	767.41	75.8	30.9
Gal d 3.VEDIWSFLSK.+2y5.light	612.32	581.33	75.8	30.9
Gal d 3.AQSDFGVDTK.+2y8.light	534.25	868.40	70.1	22.1
Gal d 3.AQSDFGVDTK.+2y7.light	534.25	781.37	70.1	22.1
Gal d 3.AQSDFGVDTK.+2y6.light	534.25	666.35	70.1	28.1
Gal d 3.AQSDFGVDTK.+2y5.light	534.25	519.28	70.1	28.1
Gal d 3.IQWC[CAM]AVGK.+2y7.light	481.25	848.41	66.2	26.2
Gal d 3.IQWC[CAM]AVGK.+2y6.light	481.25	720.35	66.2	26.2
Gal d 3.IQWC[CAM]AVGK.+2y5.light	481.25	534.27	66.2	26.2
Gal d 3.IQWC[CAM]AVGK.+2y4.light	481.25	374.24	66.2	26.2
Gal d 3.YDDESQC[CAM]SK.+2y7.light	566.22	853.34	72.4	29.2
Gal d 3.YDDESQC[CAM]SK.+2y6.light	566.22	738.31	72.4	29.2
Gal d 3.YDDESQC[CAM]SK.+2y5.light	566.22	609.27	72.4	29.2
Gal d 3.YDDESQC[CAM]SK.+2y4.light	566.22	522.23	72.4	29.2
Gal d 3.LC[CAM]QLC[CAM]QGSGGIPPEK.+2y10.light	822.40	969.50	91.1	38.5
Gal d 3.LC[CAM]QLC[CAM]QGSGGIPPEK.+2y9.light	822.40	841.44	91.1	38.5
Gal d 3.LC[CAM]QLC[CAM]QGSGGIPPEK.+2y8.light	822.40	784.42	91.1	38.5
Gal d 3.YFGYTGALR.+2y8.light	524.27	884.46	69.3	27.7
Gal d 3.YFGYTGALR.+2y7.light	524.27	737.39	69.3	24.7
Gal d 3.YFGYTGALR.+2y6.light	524.27	680.37	69.3	27.7
Gal d 3.YFGYTGALR.+2y5.light	524.27	517.31	69.3	24.7
Gal d 3.EFLGDK.+2y5.light	354.68	579.31	57	21.6

ID	Q1 Mass	Q3 Mass	DP	CE
Gal d 3.EFLGDK.+2y4.light	354.68	432.25	57	21.6
Gal d 3.FYTVISSLK.+2y7.light	529.30	747.46	69.7	27.9
Gal d 3.FYTVISSLK.+2y6.light	529.30	646.41	69.7	27.9
Gal d 3.FYTVISSLK.+2y5.light	529.30	547.34	69.7	27.9
Gal d 3.FYTVISSLK.+2y4.light	529.30	434.26	69.7	27.9
Gal d 4.FESNFNTQATNR.+2y8.light	714.83	951.46	83.2	34.6
Gal d 4.FESNFNTQATNR.+2y7.light	714.83	804.40	83.2	34.6
Gal d 4.FESNFNTQATNR.+2y6.light	714.83	690.35	83.2	34.6
Gal d 4.FESNFNTQATNR.+2y4.light	714.83	461.25	83.2	34.6
Gal d 4.NTDGSTDYGILQINSR.+2y8.light	877.42	900.53	95.1	40.4
Gal d 4.NTDGSTDYGILQINSR.+2y7.light	877.42	843.50	95.1	40.4
Gal d 4.NTDGSTDYGILQINSR.+2y6.light	877.42	730.42	95.1	40.4
Gal d 4.NTDGSTDYGILQINSR.+2y5.light	877.42	617.34	95.1	40.4
Gal d 4.GTDVQAWIR.+2y7.light	523.27	887.47	69.3	27.7
Gal d 4.GTDVQAWIR.+2y6.light	523.27	772.45	69.3	27.7
Gal d 4.GTDVQAWIR.+2y5.light	523.27	673.38	69.3	27.7
Gal d 4.GTDVQAWIR.+2y4.light	523.27	545.32	69.3	27.7
Cor a 8.AVNDASR.+2y6.light	366.69	661.33	57.8	22.1
Cor a 8.AVNDASR.+2y5.light	366.69	562.26	57.8	22.1
Cor a 8.AVNDASR.+2y4.light	366.69	448.22	57.8	22.1
Cor a 9.LNALEPTNR.+2y7.light	514.28	800.43	68.6	27.4
Cor a 9.LNALEPTNR.+2y6.light	514.28	729.39	68.6	27.4
Cor a 9.LNALEPTNR.+2y5.light	514.28	616.30	68.6	27.4
Cor a 9.LNALEPTNR.+2y4.light	514.28	487.26	68.6	27.4
Cor a 9.QGQGQSQR.+2y6.light	444.72	703.35	63.5	24.9
Cor a 9.QGQGQSQR.+2y5.light	444.72	575.29	63.5	24.9
Cor a 9.QGQGQSQR.+2y4.light	444.72	518.27	63.5	24.9
Cor a 9.QGQGQSQR.+2y3.light	444.72	390.21	63.5	24.9
Cor a 9.ESEQER.+2y5.light	389.17	648.29	59.5	22.9
Cor a 9.ESEQER.+2y4.light	389.17	561.26	59.5	22.9
Cor a 9.ESEQER.+2y3.light	389.17	432.22	59.5	22.9
Cor a 9.ADIYTEQVGR.+2y6.light	576.29	689.36	73.1	29.6
Cor a 9.ADIYTEQVGR.+2y5.light	576.29	588.31	73.1	29.6
Cor a 9.ADIYTEQVGR.+2y4.light	576.29	459.27	73.1	29.6
Cor a 9.INTVNSNTLPVLR.+2y9.light	720.91	1013.57	83.7	37.8
Cor a 9.INTVNSNTLPVLR.+2y8.light	720.91	899.53	83.7	40.8
Cor a 9.INTVNSNTLPVLR.+2y4.light	720.91	484.32	83.7	37.8
Cor a 9.VQVVDDNGNTVFDDELR.+2y7.light	967.96	893.44	101.7	43.7
Cor a 9.VQVVDDNGNTVFDDELR.+2y6.light	967.96	794.37	101.7	43.7
Cor a 9.VQVVDDNGNTVFDDELR.+2y5.light	967.96	647.30	101.7	43.7
Cor a 9.VQVVDDNGNTVFDDELR.+2y4.light	967.96	532.27	101.7	43.7
Cor a 9.TNDNAQISPLAGR.+2y7.light	678.85	713.43	80.6	33.3
Cor a 9.TNDNAQISPLAGR.+2y6.light	678.85	600.35	80.6	33.3
Cor a 9.TNDNAQISPLAGR.+2y5.light	678.85	513.31	80.6	33.3

ID	Q1 Mass	Q3 Mass	DP	CE
Cor a 9.ALPDDVLANAFQISR.+2y9.light	815.43	1019.56	90.6	44.2
Cor a 9.ALPDDVLANAFQISR.+2y8.light	815.43	906.48	90.6	47.2
Cor a 9.ALPDDVLANAFQISR.+2y7.light	815.43	835.44	90.6	44.2
Cor a 9.QETTLVR.+2y6.light	423.74	718.41	62	24.1
Cor a 9.QETTLVR.+2y5.light	423.74	589.37	62	24.1
Cor a 9.QETTLVR.+2y4.light	423.74	488.32	62	24.1
Cor a 9.QETTLVR.+2y3.light	423.74	387.27	62	24.1

Table 20: Transition list of method 2 used for the Optimisation experiments including information about transition, Q1 Mass (precursor), Q3 Mass (transitions), DP (declustering potential) and CE (collision energy). Dwell time was set for each transition to 8 msec.

ID	Q1 Mass	Q3 Mass	DP	CE
Cor a 10.ITPSWVGFTDGER.+2y8.light	732.86	880.42	84.5	35.2
Cor a 10.ITPSWVGFTDGER.+2y7.light	732.86	781.35	84.5	35.2
Cor a 10.ITPSWVGFTDGER.+2y6.light	732.86	724.33	84.5	35.2
Cor a 10.ITPSWVGFTDGER.+2y5.light	732.86	577.26	84.5	35.2
Cor a 10.LIGEAAK.+2y6.light	351.21	588.34	56.7	21.5
Cor a 10.LIGEAAK.+2y5.light	351.21	475.25	56.7	21.5
Cor a 10.LIGEAAK.+2y4.light	351.21	418.23	56.7	21.5
Cor a 10.NQAAVNPER.+2y6.light	499.75	685.36	67.5	26.8
Cor a 10.NQAAVNPER.+2y5.light	499.75	614.33	67.5	26.8
Cor a 10.NQAAVNPER.+2y4.light	499.75	515.26	67.5	26.8
Cor a 10.NQAAVNPER.+2y3.light	499.75	401.21	67.5	26.8
Cor a 10.PYIQVK.+2y5.light	374.22	650.39	58.4	22.3
Cor a 10.PYIQVK.+2y4.light	374.22	487.32	58.4	22.3
Cor a 10.PYIQVK.+2y3.light	374.22	374.24	58.4	22.3
Cor a 10.DAVVTVPAYFNDAQR.+2y7.light	833.42	913.42	91.9	38.9
Cor a 10.DAVVTVPAYFNDAQR.+2y6.light	833.42	750.35	91.9	38.9
Cor a 10.DAVVTVPAYFNDAQR.+2y5.light	833.42	603.28	91.9	38.9
Cor a 10.DAVVTVPAYFNDAQR.+2y4.light	833.42	489.24	91.9	38.9
Cor a 10.DAGIIAGLNVAR.+2y8.light	585.34	813.49	73.8	29.9
Cor a 10.DAGIIAGLNVAR.+2y7.light	585.34	700.41	73.8	29.9
Cor a 10.DAGIIAGLNVAR.+2y6.light	585.34	629.37	73.8	29.9
Cor a 10.DAGIIAGLNVAR.+2y5.light	585.34	572.35	73.8	29.9
Cor a 10.FEELNNDFVQK.+2y8.light	691.83	977.51	81.6	33.8
Cor a 10.FEELNNDFVQK.+2y7.light	691.83	864.42	81.6	33.8
Cor a 10.FEELNNDFVQK.+2y6.light	691.83	750.38	81.6	33.8
Cor a 10.FEELNNDFVQK.+2y5.light	691.83	636.34	81.6	33.8
Cor a 10.NQIDEIVLVGGSTR.+2y9.light	750.90	901.55	85.9	35.9
Cor a 10.NQIDEIVLVGGSTR.+2y8.light	750.90	788.46	85.9	35.9
Cor a 10.NQIDEIVLVGGSTR.+2y7.light	750.90	689.39	85.9	35.9
Cor a 10.NQIDEIVLVGGSTR.+2y6.light	750.90	576.31	85.9	35.9
Cor a 10.VQQLLK.+2y5.light	364.74	629.40	57.7	22
Cor a 10.VQQLLK.+2y4.light	364.74	501.34	57.7	22
Cor a 10.VQQLLK.+2y3.light	364.74	373.28	57.7	22
Cor a 10.DYFDGK.+2y5.light	372.66	629.29	58.3	22.3
Cor a 10.DYFDGK.+2y4.light	372.66	466.23	58.3	22.3
Cor a 10.FDLTGVPPAPR.+2y8.light	585.32	794.45	73.8	29.9
Cor a 10.FDLTGVPPAPR.+2y7.light	585.32	693.40	73.8	29.9
Cor a 10.FDLTGVPPAPR.+2y6.light	585.32	636.38	73.8	29.9
Cor a 10.FDLTGVPPAPR.+2y5.light	585.32	537.31	73.8	29.9
Cor a 10.ITITNDK.+2y6.light	402.73	691.36	60.5	23.4

ID	Q1 Mass	Q3 Mass	DP	CE
Cor a 10.ITITNDK.+2y5.light	402.73	590.31	60.5	23.4
Cor a 10.ITITNDK.+2y4.light	402.73	477.23	60.5	23.4
Cor a 10.ITITNDK.+2y3.light	402.73	376.18	60.5	23.4
Cor a 10.LSQEEIDR.+2y6.light	495.25	789.37	67.2	26.7
Cor a 10.LSQEEIDR.+2y5.light	495.25	661.32	67.2	26.7
Cor a 10.LSQEEIDR.+2y4.light	495.25	532.27	67.2	26.7
Cor a 10.LSQEEIDR.+2y3.light	495.25	403.23	67.2	26.7
Cor a 10.NQVNDK.+2y5.light	359.18	603.31	57.3	21.8
Cor a 10.NQVNDK.+2y4.light	359.18	475.25	57.3	21.8
Cor a 10.NQVNDK.+2y3.light	359.18	376.18	57.3	21.8
Cor a 10.LESDEK.+2y5.light	360.67	607.26	57.4	21.8
Cor a 10.LESDEK.+2y4.light	360.67	478.21	57.4	21.8
Cor a 10.LESDEK.+2y3.light	360.67	391.18	57.4	21.8
Cor a 10.EDYDEK.+2y5.light	399.66	669.27	60.3	23.2
Cor a 10.EDYDEK.+2y4.light	399.66	554.25	60.3	23.2
Cor a 10.EDYDEK.+2y3.light	399.66	391.18	60.3	23.2
Cor a 11.LLSGIENFR.+2y7.light	524.79	822.41	69.4	24.7
Cor a 11.LLSGIENFR.+2y6.light	524.79	735.38	69.4	27.7
Cor a 11.LLSGIENFR.+2y5.light	524.79	678.36	69.4	27.7
Cor a 11.LLSGIENFR.+2y4.light	524.79	565.27	69.4	24.7
Cor a 11.AFSWEVLEAALK.+2y8.light	682.37	872.51	80.9	33.4
Cor a 11.AFSWEVLEAALK.+2y7.light	682.37	743.47	80.9	33.4
Cor a 11.AFSWEVLEAALK.+2y6.light	682.37	644.40	80.9	33.4
Cor a 11.VFGEQSK.+2y6.light	397.71	695.34	60.1	23.2
Cor a 11.VFGEQSK.+2y5.light	397.71	548.27	60.1	23.2
Cor a 11.VFGEQSK.+2y4.light	397.71	491.25	60.1	23.2
Cor a 11.VFGEQSK.+2y3.light	397.71	362.20	60.1	23.2
Cor a 11.GNIVNEFER.+2y6.light	539.27	793.38	70.4	28.3
Cor a 11.GNIVNEFER.+2y5.light	539.27	694.32	70.4	28.3
Cor a 11.GNIVNEFER.+2y4.light	539.27	580.27	70.4	28.3
Cor a 11.GNIVNEFER.+2y3.light	539.27	451.23	70.4	28.3
Cor a 11.ELAFNLPSR.+2y7.light	523.79	804.44	69.3	27.7
Cor a 11.ELAFNLPSR.+2y6.light	523.79	733.40	69.3	27.7
Cor a 11.ELAFNLPSR.+2y5.light	523.79	586.33	69.3	27.7
Cor a 11.ELAFNLPSR.+2y4.light	523.79	472.29	69.3	27.7
Cor a 11.NQDQAFFFPGPNK.+2y8.light	755.36	953.49	86.2	36
Cor a 11.NQDQAFFFPGPNK.+2y7.light	755.36	806.42	86.2	36
Cor a 11.NQDQAFFFPGPNK.+2y6.light	755.36	659.35	86.2	36
Cor a 11.NQDQAFFFPGPNK.+2y5.light	755.36	512.28	86.2	36
Cor a 11.QQEEGGR.+2y6.light	402.19	675.31	60.4	23.3
Cor a 11.QQEEGGR.+2y5.light	402.19	547.25	60.4	23.3
Cor a 11.QQEEGGR.+2y4.light	402.19	418.20	60.4	23.3
Cor a 12.EVGQEIQSR.+2y7.light	523.27	817.42	69.3	27.7
Cor a 12.EVGQEIQSR.+2y6.light	523.27	760.39	69.3	27.7

ID	Q1 Mass	Q3 Mass	DP	CE
Cor a 12.EVGQEIQSR.+2y5.light	523.27	632.34	69.3	27.7
Cor a 12.EVGQEIQSR.+2y4.light	523.27	503.29	69.3	27.7
Cor a 14.QQNLNQC[CAM]QR.+2y7.light	594.78	932.44	74.5	30.3
Cor a 14.QQNLNQC[CAM]QR.+2y6.light	594.78	818.39	74.5	30.3
Cor a 14.QQNLNQC[CAM]QR.+2y5.light	594.78	705.31	74.5	30.3
Cor a 14.QQNLNQC[CAM]QR.+2y4.light	594.78	591.27	74.5	30.3
Cor a 14.DLPNQC[CAM]R.+2y5.light	451.71	674.30	64	25.1
Cor a 14.DLPNQC[CAM]R.+2y4.light	451.71	577.25	64	25.1
Cor a 14.DLPNQC[CAM]R.+2y3.light	451.71	463.21	64	25.1
Bod d 4.ALC[CAM]SEK.+2y5.light	354.17	636.30	56.9	21.6
Bod d 4.ALC[CAM]SEK.+2y4.light	354.17	523.22	56.9	21.6
Bod d 4.ALC[CAM]SEK.+2y3.light	354.17	363.19	56.9	21.6
Bod d 4.LDQWLC[CAM]EK.+2y6.light	546.26	863.41	70.9	28.5
Bod d 4.LDQWLC[CAM]EK.+2y5.light	546.26	735.35	70.9	28.5
Bod d 4.LDQWLC[CAM]EK.+2y4.light	546.26	549.27	70.9	28.5
Bod d 4.LDQWLC[CAM]EK.+2y3.light	546.26	436.19	70.9	28.5
Bos d 5.VYVEELK.+2y6.light	440.24	780.41	63.2	24.7
Bos d 5.VYVEELK.+2y5.light	440.24	617.35	63.2	24.7
Bos d 5.VYVEELK.+2y4.light	440.24	518.28	63.2	24.7
Bos d 5.VYVEELK.+2y3.light	440.24	389.24	63.2	24.7
Bos d 5.PTPEGDLEILLQK.+2y8.light	726.90	971.58	84.1	35
Bos d 5.PTPEGDLEILLQK.+2y7.light	726.90	856.55	84.1	35
Bos d 5.PTPEGDLEILLQK.+2y6.light	726.90	743.47	84.1	35
Bos d 5.PTPEGDLEILLQK.+2y5.light	726.90	614.42	84.1	35
Bos d 5.IDALNENK.+2y6.light	458.74	688.36	64.6	22.4
Bos d 5.IDALNENK.+2y5.light	458.74	617.33	64.6	25.4
Bos d 5.IDALNENK.+2y4.light	458.74	504.24	64.6	22.4
Bos d 5.TPEVDDEALEK.+2y8.light	623.30	918.44	76.6	31.3
Bos d 5.TPEVDDEALEK.+2y7.light	623.30	819.37	76.6	31.3
Bos d 5.TPEVDDEALEK.+2y6.light	623.30	704.35	76.6	31.3
Bos d 5.TPEVDDEALEK.+2y5.light	623.30	589.32	76.6	31.3
Bos d 6.LVNELTEFAK.+2y7.light	582.32	837.44	73.6	29.8
Bos d 6.LVNELTEFAK.+2y6.light	582.32	708.39	73.6	29.8
Bos d 6.LVNELTEFAK.+2y5.light	582.32	595.31	73.6	29.8
Bos d 6.LVNELTEFAK.+2y4.light	582.32	494.26	73.6	29.8
Bos d 6.DDSPDLPK.+2y6.light	443.71	656.36	63.5	24.8
Bos d 6.DDSPDLPK.+2y5.light	443.71	569.33	63.5	24.8
Bos d 6.DDSPDLPK.+2y4.light	443.71	472.28	63.5	24.8
Bos d 6.DDSPDLPK.+2y3.light	443.71	357.25	63.5	24.8
Bos d 6.PDPNTLC[CAM]DEFK.+2y7.light	668.30	912.41	79.8	32.9
Bos d 6.PDPNTLC[CAM]DEFK.+2y6.light	668.30	811.37	79.8	32.9
Bos d 6.PDPNTLC[CAM]DEFK.+2y5.light	668.30	698.28	79.8	32.9
Bos d 6.PDPNTLC[CAM]DEFK.+2y4.light	668.30	538.25	79.8	32.9
Bos d 6.GAC[CAM]LLPK.+2y6.light	379.72	701.40	58.8	22.5

ID	Q1 Mass	Q3 Mass	DP	CE
Bos d 6.GAC[CAM]LLPK.+2y5.light	379.72	630.36	58.8	22.5
Bos d 6.GAC[CAM]LLPK.+2y4.light	379.72	470.33	58.8	22.5
Bos d 6.GAC[CAM]LLPK.+2y3.light	379.72	357.25	58.8	22.5
Bos d 6.VLASSAR.+2y6.light	352.21	604.34	56.8	21.5
Bos d 6.VLASSAR.+2y5.light	352.21	491.26	56.8	21.5
Bos d 6.VLASSAR.+2y4.light	352.21	420.22	56.8	21.5
Bos d 6.AEFVEVTK.+2y6.light	461.75	722.41	64.8	25.5
Bos d 6.AEFVEVTK.+2y5.light	461.75	575.34	64.8	25.5
Bos d 6.AEFVEVTK.+2y4.light	461.75	476.27	64.8	25.5
Bos d 6.LVTDLTK.+2y6.light	395.24	676.39	59.9	23.1
Bos d 6.LVTDLTK.+2y5.light	395.24	577.32	59.9	23.1
Bos d 6.LVTDLTK.+2y4.light	395.24	476.27	59.9	23.1
Bos d 6.LVTDLTK.+2y3.light	395.24	361.24	59.9	23.1
Bos d 6.YIC[CAM]DNQDTISSK.+2y9.light	722.32	1007.46	83.8	34.9
Bos d 6.YIC[CAM]DNQDTISSK.+2y8.light	722.32	892.44	83.8	34.9
Bos d 6.YIC[CAM]DNQDTISSK.+2y7.light	722.32	778.39	83.8	34.9
Bos d 6.YIC[CAM]DNQDTISSK.+2y6.light	722.32	650.34	83.8	34.9
Bos d 6.QNC[CAM]DQFEK.+2y6.light	534.72	826.34	70.1	28.1
Bos d 6.QNC[CAM]DQFEK.+2y5.light	534.72	666.31	70.1	28.1
Bos d 6.QNC[CAM]DQFEK.+2y4.light	534.72	551.28	70.1	28.1
Bos d 6.QNC[CAM]DQFEK.+2y3.light	534.72	423.22	70.1	28.1
Bos d 6.LGEYGFQNALIVR.+2y9.light	740.40	1017.58	85.1	35.5
Bos d 6.LGEYGFQNALIVR.+2y8.light	740.40	960.56	85.1	35.5
Bos d 6.LGEYGFQNALIVR.+2y7.light	740.40	813.49	85.1	35.5
Bos d 6.LGEYGFQNALIVR.+2y6.light	740.40	685.44	85.1	35.5
Bos d 6.ATEEQLK.+2y6.light	409.72	747.39	61	23.6
Bos d 6.ATEEQLK.+2y5.light	409.72	646.34	61	23.6
Bos d 6.ATEEQLK.+2y4.light	409.72	517.30	61	23.6
Bos d 6.ATEEQLK.+2y3.light	409.72	388.26	61	23.6
Bos d 6.EAC[CAM]FAVEGPK.+2y8.light	554.26	907.43	71.5	28.8
Bos d 6.EAC[CAM]FAVEGPK.+2y7.light	554.26	747.40	71.5	28.8
Bos d 6.EAC[CAM]FAVEGPK.+2y6.light	554.26	600.34	71.5	28.8
Bos d 6.EAC[CAM]FAVEGPK.+2y5.light	554.26	529.30	71.5	28.8
Bos d 6.LVVSTQTALA.+2y7.light	501.80	691.36	67.7	26.9
Bos d 6.LVVSTQTALA.+2y6.light	501.80	604.33	67.7	26.9
Bos d 6.LVVSTQTALA.+2y5.light	501.80	503.28	67.7	26.9
Bos d 6.LVVSTQTALA.+2y4.light	501.80	375.22	67.7	26.9
Bos d 9.FFVAPFPEVFGK.+2y9.light	692.87	991.52	81.6	30.8
Bos d 9.FFVAPFPEVFGK.+2y8.light	692.87	920.49	81.6	30.8
Bos d 9.FFVAPFPEVFGK.+2y6.light	692.87	676.37	81.6	33.8
Bos d 9.EDVPSER.+2y6.light	416.20	702.34	61.5	23.8
Bos d 9.EDVPSER.+2y5.light	416.20	587.31	61.5	23.8
Bos d 9.EDVPSER.+2y4.light	416.20	488.25	61.5	23.8
Bos d 9.EDVPSER.+2y3.light	416.20	391.19	61.5	23.8

ID	Q1 Mass	Q3 Mass	DP	CE
Bos d 9.YLGYLEQLLR.+2y8.light	634.36	991.56	77.4	31.7
Bos d 9.YLGYLEQLLR.+2y6.light	634.36	771.47	77.4	31.7
Bos d 9.YLGYLEQLLR.+2y5.light	634.36	658.39	77.4	31.7
Bos d 9.VPQLEIVPNSAEER.+2y8.light	790.92	901.44	88.8	37.3
Bos d 9.VPQLEIVPNSAEER.+2y7.light	790.92	802.37	88.8	37.3
Bos d 9.VPQLEIVPNSAEER.+2y6.light	790.92	705.32	88.8	37.3
Bos d 9.VPQLEIVPNSAEER.+2y5.light	790.92	591.27	88.8	37.3
Bos d 10.ALNEINQFYQK.+2y6.light	684.35	827.40	81	33.5
Bos d 10.ALNEINQFYQK.+2y4.light	684.35	585.30	81	33.5
Bos d 10.ALNEINQFYQK.+2y3.light	684.35	438.23	81	33.5
Bos d 10.LTEEEK.+2y5.light	374.69	635.29	58.4	22.3
Bos d 10.LTEEEK.+2y4.light	374.69	534.24	58.4	22.3
Bos d 10.LTEEEK.+2y3.light	374.69	405.20	58.4	22.3
Bos d 10.FALPQYLK.+2y7.light	490.28	832.49	66.9	23.5
Bos d 10.FALPQYLK.+2y6.light	490.28	761.46	66.9	20.5
Bos d 10.FALPQYLK.+2y5.light	490.28	648.37	66.9	20.5
Bos d 10.FALPQYLK.+2y3.light	490.28	423.26	66.9	26.5
Bos d 10.PWIQPK.+2y5.light	384.72	671.39	59.2	22.7
Bos d 10.PWIQPK.+2y4.light	384.72	485.31	59.2	22.7
Bos d 10.PWIQPK.+2y3.light	384.72	372.22	59.2	22.7
Bos d 10.VIPYVR.+2y5.light	373.73	647.39	58.4	22.3
Bos d 10.VIPYVR.+2y4.light	373.73	534.30	58.4	22.3
Bos d 10.VIPYVR.+2y3.light	373.73	437.25	58.4	22.3
Bos d 11.FQSEEQQQTEDELQDK.+2y8.light	991.43	977.44	103.4	44.5
Bos d 11.FQSEEQQQTEDELQDK.+2y7.light	991.43	876.39	103.4	44.5
Bos d 11.FQSEEQQQTEDELQDK.+2y6.light	991.43	747.35	103.4	44.5
Bos d 11.FQSEEQQQTEDELQDK.+2y5.light	991.43	632.32	103.4	44.5
Bos d 11.VLPVPQK.+2y5.light	390.75	568.35	59.6	13.9
Bos d 11.VLPVPQK.+2y4.light	390.75	471.29	59.6	22.9
Bos d 11.VLPVPQK.+2y3.light	390.75	372.22	59.6	25.9
Bos d 11.AVPYPQR.+2y5.light	415.73	660.35	61.4	17.8
Bos d 11.AVPYPQR.+2y4.light	415.73	563.29	61.4	26.8
Bos d 11.AVPYPQR.+2y3.light	415.73	400.23	61.4	26.8
Bos d 11.GPFPIIV.+2y6.light	371.73	685.43	58.2	22.2
Bos d 11.GPFPIIV.+2y5.light	371.73	588.38	58.2	22.2
Bos d 11.GPFPIIV.+2y4.light	371.73	441.31	58.2	22.2
Bos d 12.YIPIQYVLSR.+2y8.light	626.36	975.56	76.8	31.4
Bos d 12.YIPIQYVLSR.+2y7.light	626.36	878.51	76.8	31.4
Bos d 12.YIPIQYVLSR.+2y6.light	626.36	765.43	76.8	31.4
Bos d 12.YIPIQYVLSR.+2y5.light	626.36	637.37	76.8	31.4
Bos d 12.YPSYGLNYYQQK.+2y8.light	762.36	1013.51	86.7	36.3
Bos d 12.YPSYGLNYYQQK.+2y7.light	762.36	956.48	86.7	36.3
Bos d 12.YPSYGLNYYQQK.+2y6.light	762.36	843.40	86.7	36.3
Bos d 12.YPSYGLNYYQQK.+2y5.light	762.36	729.36	86.7	36.3

ID	Q1 Mass	Q3 Mass	DP	CE
Bos d 12.SPAQILQWQVLSNTVPAK.+2y9.light	990.55	928.55	103.3	44.5
Bos d 12.SPAQILQWQVLSNTVPAK.+2y8.light	990.55	829.48	103.3	44.5
Bos d 12.SPAQILQWQVLSNTVPAK.+2y7.light	990.55	716.39	103.3	44.5
Bos d 12.SPAQILQWQVLSNTVPAK.+2y6.light	990.55	629.36	103.3	44.5
Ara h 1 lit.VLLEENAGGEQEER.+2y9.light	786.88	989.43	88.5	37.2
Ara h 1 lit.VLLEENAGGEQEER.+2y8.light	786.88	875.39	88.5	37.2
Ara h 1 lit.VLLEENAGGEQEER.+2y7.light	786.88	804.35	88.5	37.2
Ara h 1 lit.VLLEENAGGEQEER.+2y6.light	786.88	747.33	88.5	37.2
Ara h 1 lit.GTGNLELVAVR.+2y8.light	564.82	913.55	72.3	29.2
Ara h 1 lit.GTGNLELVAVR.+2y7.light	564.82	799.50	72.3	29.2
Ara h 1 lit.GTGNLELVAVR.+2y6.light	564.82	686.42	72.3	29.2
Ara h 1 lit.GTGNLELVAVR.+2y5.light	564.82	557.38	72.3	29.2
Ara h 1 lit.QPGDYDDDRR.+2y7.light	618.77	954.39	76.2	31.1
Ara h 1 lit.QPGDYDDDRR.+2y6.light	618.77	839.36	76.2	31.1
Ara h 1 lit.QPGDYDDDRR.+2y5.light	618.77	676.30	76.2	31.1
Ara h 1 lit.QPGDYDDDRR.+2y4.light	618.77	561.27	76.2	31.1
Ara h 2 lit.QQWELQGDR.+2y7.light	580.28	903.43	73.4	29.7
Ara h 2 lit.QQWELQGDR.+2y6.light	580.28	717.35	73.4	29.7
Ara h 2 lit.QQWELQGDR.+2y5.light	580.28	588.31	73.4	29.7
Ara h 2 lit.QQWELQGDR.+2y4.light	580.28	475.23	73.4	29.7
Ara h 2 lit.NLPQQC[CAM]GLR.+2y7.light	543.28	858.43	70.7	28.4
Ara h 2 lit.NLPQQC[CAM]GLR.+2y6.light	543.28	761.37	70.7	28.4
Ara h 2 lit.NLPQQC[CAM]GLR.+2y5.light	543.28	633.31	70.7	28.4
Ara h 2 lit.NLPQQC[CAM]GLR.+2y4.light	543.28	505.26	70.7	28.4
Ara h 2 lit.CMCEALQQIMENQSDR.+2y8.light	1006.92	992.45	104.5	45.1
Ara h 2 lit.CMCEALQQIMENQSDR.+2y7.light	1006.92	879.36	104.5	45.1
Ara h 2 lit.CMCEALQQIMENQSDR.+2y6.light	1006.92	748.32	104.5	45.1
Ara h 2 lit.CMC]EALQQIMENQSDR.+2y5.light	1006.92	619.28	104.5	45.1
Ara h 2 lit.CC[CAM]NELNETENNQR.+2y8.light	840.84	1004.44	92.4	39.1
Ara h 2 lit.CC[CAM]NELNETENNQR.+2y7.light	840.84	890.40	92.4	39.1
Ara h 2 lit.CC[CAM]NELNETENNQR.+2y6.light	840.84	761.35	92.4	39.1
Ara h 2 lit.CC[CAM]NELNETENNQR.+2y5.light	840.84	660.31	92.4	39.1
Ara h 3_4 lit.SLPYSPYSPQSQPR.+2y9.light	803.90	1059.52	89.7	37.8
Ara h 3_4 lit.SLPYSPYSPQSQPR.+2y8.light	803.90	962.47	89.7	37.8
Ara h 3_4 lit.SLPYSPYSPQSQPR.+2y7.light	803.90	799.41	89.7	37.8
Ara h 3_4 lit.AHYQVVDSNGDR.+2y9.light	680.82	989.46	80.7	33.4
Ara h 3_4 lit.AHYQVVDSNGDR.+2y8.light	680.82	861.41	80.7	33.4
Ara h 3_4 lit.AHYQVVDSNGDR.+2y7.light	680.82	762.34	80.7	33.4
Ara h 3_4 lit.AHYQVVDSNGDR.+2y6.light	680.82	663.27	80.7	33.4
Ara h 3_4 lit.YQQQSR.+2y5.light	405.20	646.33	60.7	23.4
Ara h 3_4 lit.YQQQSR.+2y4.light	405.20	518.27	60.7	23.4
Ara h 3_4 lit.YQQQSR.+2y3.light	405.20	390.21	60.7	23.4
Cor a 9 lit.QGQVLTIPQNFAVAK.+2y10.light	807.45	1088.61	90	37.9
Cor a 9 lit.QGQVLTIPQNFAVAK.+2y9.light	807.45	987.56	90	37.9

ID	Q1 Mass	Q3 Mass	DP	CE
Cor a 9 lit.QGQVLTIPQNFAVAK.+2y8.light	807.45	874.48	90	37.9
Cor a 9 lit.QGQVLTIPQNFAVAK.+2y7.light	807.45	777.43	90	37.9
Jug r 1 lit.DLPNEC[CAM]GISSQR.+2y9.light	688.32	1050.46	81.3	33.6
Jug r 1 lit.DLPNEC[CAM]GISSQR.+2y8.light	688.32	936.42	81.3	33.6
Jug r 1 lit.DLPNEC[CAM]GISSQR.+2y7.light	688.32	807.38	81.3	33.6
Jug r 1 lit.DLPNEC[CAM]GISSQR.+2y6.light	688.32	647.35	81.3	33.6
Jug r 1 lit.GEEMEEMVQSAR.+2y9.light	698.30	1080.48	82	34
Jug r 1 lit.GEEMEEMVQSAR.+2y8.light	698.30	949.44	82	34
Jug r 1 lit.GEEMEEMVQSAR.+2y7.light	698.30	820.40	82	34
Jug r 1 lit.GEEMEEMVQSAR.+2y6.light	698.30	691.36	82	34
Oval lit.HIATNAVLFFGR.+2y8.light	673.37	923.51	80.2	33.7
Oval lit.HIATNAVLFFGR.+2y7.light	673.37	809.47	80.2	33.1
Oval lit.HIATNAVLFFGR.+2y6.light	673.37	738.43	80.2	33.1
Oval lit.HIATNAVLFFGR.+2y5.light	673.37	639.36	80.2	33.1
Oval lit.YPILPEYLQC[CAM]VK.+2y8.light	761.90	1036.51	86.7	36.3
Oval lit.YPILPEYLQC[CAM]VK.+2y7.light	761.90	939.46	86.7	36.3
Oval lit.YPILPEYLQC[CAM]VK.+2y6.light	761.90	810.42	86.7	36.3
Oval lit.YPILPEYLQC[CAM]VK.+2y5.light	761.90	647.35	86.7	36.3
Oval lit.LTEWTSSNVMEER.+2y9.light	791.36	1052.47	88.8	37.3
Oval lit.LTEWTSSNVMEER.+2y8.light	791.36	951.42	88.8	37.3
Oval lit.LTEWTSSNVMEER.+2y7.light	791.36	864.39	88.8	37.3
Oval lit.LTEWTSSNVMEER.+2y6.light	791.36	777.36	88.8	37.3
Oval lit.EDTQAMPFRV.+2y7.light	597.28	848.44	74.7	30.4
Oval lit.EDTQAMPFRV.+2y6.light	597.28	720.39	74.7	30.4
Oval lit.EDTQAMPFRV.+2y5.light	597.28	649.35	74.7	30.4
Oval lit.EDTQAMPFRV.+2y4.light	597.28	518.31	74.7	30.4
alpha s2 cas lit.NAVPITPTLNR.+2y7.light	598.34	814.48	74.7	30.4
alpha s2 cas lit.NAVPITPTLNR.+2y6.light	598.34	701.39	74.7	36.4
alpha s2 cas lit.NAVPITPTLNR.+2y5.light	598.34	600.35	74.7	36.4
alpha s2 cas lit.NAVPITPTLNR.+2y4.light	598.34	503.29	74.7	30.4
beta_lactoglob lit.LIVTQTMK.+2y6.light	467.28	707.38	65.2	25.7
beta_lactoglob lit.LIVTQTMK.+2y5.light	467.28	608.31	65.2	25.7
beta_lactoglob lit.LIVTQTMK.+2y4.light	467.28	507.26	65.2	25.7
beta_lactoglob lit.LIVTQTMK.+2y3.light	467.28	379.20	65.2	25.7
beta_lactoglob lit.VLVLDTDYK.+2y7.light	533.29	853.43	70	28.1
beta_lactoglob lit.VLVLDTDYK.+2y6.light	533.29	754.36	70	28.1
beta_lactoglob lit.VLVLDTDYK.+2y5.light	533.29	641.28	70	28.1
beta_lactoglob lit.VLVLDTDYK.+2y4.light	533.29	526.25	70	28.1
alpha lactalb lit.VGNYWLAHK.+2y7.light	544.29	931.48	70.8	28.5
alpha lactalb lit.VGNYWLAHK.+2y6.light	544.29	817.44	70.8	28.5
alpha lactalb lit.VGNYWLAHK.+2y5.light	544.29	654.37	70.8	28.5
alpha lactalb lit.VGNYWLAHK.+2y4.light	544.29	468.29	70.8	28.5

2.8.1.2 UHPLC-Settings

Eluent A was milliQ water with 0.1 % of formic acid and Eluent B was methanol with 0.1 % formic acid. The analytical column was an Aeris PEPTIDE 3.6u XB-C18 150*2.10 mm (Phenomenex) which was supported with a Security Guard ULTRA Cartridge UHPLC C18-Peptide for 2.1 mm ID Columns (Phenomenex). The separation gradient is displayed in Figure 11.

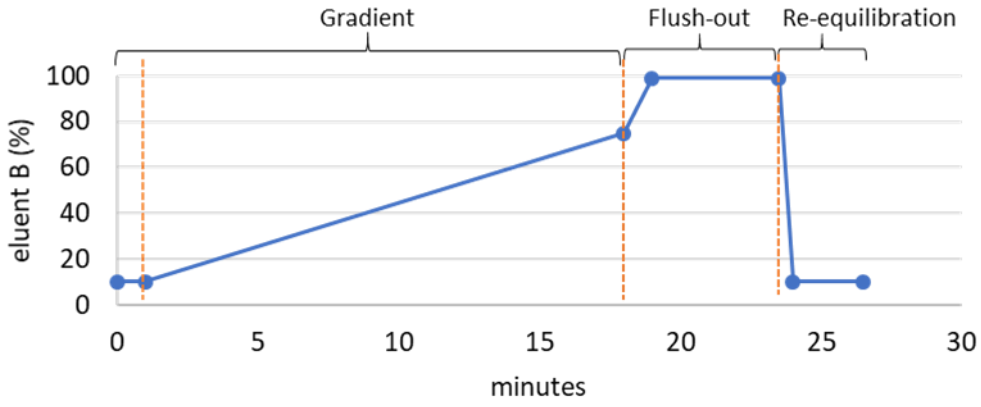


Figure 11: UHPLC gradient used in the preliminary targeted LC-MS/MS method for optimisation evaluation.

The separation of the injected sample happened within a 17 minutes time window running a gradient from 10 % Eluent B to 75 % Eluent B as shown in Table 21. Total time of the UHPLC method is 26.5 minutes including flush out of sample matrix and re-equilibration of the analytical column. The flow was forwarded to the mass spectrometer between minute 2 – 18.5.

Table 21: Settings of the UHPLC pump.

Step	Total Time(min)	Flow Rate(µL/min)	A (%)	B (%)
0	0	300	90	10
1	1	300	90	10
2	18	300	25	75
3	19	300	1	99
4	23.5	300	1	99
5	24	300	90	10
6	26.5	300	90	10

Injection volume was 15 µL for all samples with a draw speed of 100 µL/min and an eject speed of 200 µL/min. The needle was washed for 5 seconds with Eluent B after injection. The column oven was set at 30 °C.

2.8.1.3 MS Parameters

Source temperature of the Turbo Spray IonDrive was set to 350 °C and was used in ESI-mode. Ion Source Gas 1 (GS1) and Ion Source Gas 2 (GS2) were set to 50 and the IonSpray Voltage (IS) was set to 4500. The Curtain Gas (CUR) was set to 40. DP and CE for the different transitions are listed in Table 19 and Table 20.

2.9 Possible Marker peptide – Refinement 1

The targeted MRM-measurements of the tryptic optimization under the final chosen conditions were consulted to make a choice which peptides seem to be promising for further investigation and might serve as potential allergen marker peptides.

The three biological replicate MRM-spectra of the chocolate dessert matrix containing 100 ppm protein of each allergenic ingredient extracted with the optimized extraction protocol and digested with the optimized tryptic digestion protocol were loaded into the data analysis software Skyline. The MRM-method and HPLC-MS/MS set up is described above in the section Evaluation procedure for the tryptic digest optimization.

Sensitivity – peak area

Peptides which were represented by all y-ions were kept in the list and can be found in Table 33. Afterwards the datafile was refined with Skyline keeping only the 3 transitions per peptide which gave the highest signals. Ion chromatograms including the 3 most intense y-ions per peptide from the 3 biological replicates were extracted. Peptides were ordered according to their peak area counts.

Uniqueness check

In respect to the uniqueness of the peptides for the allergenic proteins, a Uniprot-peptide-BLAST Search was performed for all of the peptides. This is essential for proper quantification of the correct protein, as peptides need to be unique for the respective allergen.

Specificity testing

Not only the sensitivity is an important parameter for the selection of suitable peptides and transitions, but also their specificity. Therefore, specificity was checked against blank chocolate matrix.

At least 4 peptides per allergen were kept if possible for further investigation. For some allergens the number of 4 peptides could not be met, for those less peptides were chosen. The chosen peptides are listed in Table 26.

2.10 MS-parameter Optimisation

To find the most suitable marker peptides and transitions to serve as quantifier and qualifier, 44 synthetic peptides were analysed. 2 mixes containing each 22 peptides of the 44 candidate peptides were prepared and carbamidomethylated using DTT and IAA prior to chromatographic separation as carbamethylation is included in the tryptic digest protocol. Per peptide 24 pmol were loaded onto the column for analysis. MRM methods and data analysis were performed using the Software Skyline.

Choice of +2 or +3 precursor ions

In a first measurement +2 and +3 precursor ions and their single charged y-ions were measured with the CE and the DP values predicted by Skyline. For each peptide the precursor giving the highest peak area (TIC of all measured y-ions) was assumed to be also the one providing best transitions for quantification and therefore was the one chosen for the further optimization of CE values and DP.

Optimisation of Declustering Potential

For each mix a scheduled MRM-method containing the pre-chosen +2 or +3 charged precursors and all of their +1 charged y-ions was established, which measured at maximum 200 concurrent transitions. Each transition was measured at 7 different Declustering Potential values (-9, -6, -3, 0, +3, +6, +9 relative to the DP predicted by Skyline).

Optimisation of Collision Energy

Optimum DP for each transition was chosen by Skyline and for Collision Energy optimization each transition was measured at 7 different CE values (-9, -6, -3, 0, +3, +6, +9 relative to the DP predicted by Skyline).

2.10.1 LC-MS settings

Experiments were performed on a QTRAP® 6500 System (Sciex) equipped with an IonDrive™ Turbo V source directly coupled with an Agilent 1290 Infinity Binary LC System in MRM-mode.

Eluent A was milliQ water with 0.1 % of formic acid and Eluent B was methanol with 0.1 % formic acid. The analytical column was an Aeris PEPTIDE 3.6u XB-C18 150*2.10 mm (Phenomenex) which was supported with a Security Guard ULTRA Cartridge UHPLC C18-Peptide for 2.1 mm ID Columns (Phenomenex). The LC separation started with a flow rate of 0.3 mL/min, 90 % solvent A for 1 min. Elution of the peptides started then with a linear gradient to 25 % A within 10 minutes and a linear gradient for a further minute to 1 % A, which was then kept for 1 minute. Equilibration was performed for 3 minutes with 90% solvent A. Gradient is displayed in Table 22.

Table 22: UHPLC – separation method for the optimisation of MS-parameter for peptide determination and for the final validation method

Step	Total Time(min)	Flow Rate(µL/min)	A (%)	B (%)
0	0	300	90	10
1	1	300	90	10
2	11	300	25	75
3	12	300	1	99
4	15	300	1	99
5	15.5	300	90	10
6	19	300	90	10

Injection volume was 15 µL for all samples with a draw speed of 100 µL/min and an eject speed of 200 µL/min. The needle was washed for 5 seconds with Eluent B after injection. The column oven was set at 30 °C.

2.10.2 Preparation of the peptide mixes

Synthetic peptides (see Table 23) were ordered by a project partner (IRMM) at the company JPT as lyophilised peptides. About 51 nmol of peptide were solubilised in 200 µL of 80 % 0.1 M ammonium-bicarbonate and 20 % acetonitrile. The resulting solutions were gently agitated for 30 min at room temperature. Of each peptide, 40 µL (containing about 10 nmol) was placed in a separate pre-labelled low volume glass screw top auto-sampler vial which was capped and frozen at -20 °C. The peptides were shipped on dry ice.

Peptide mixes and volumes of the single peptide solution can be found in Table 23. Those volumes were evaluated in a pre-experiment to find out which amount gives a respond in the LC-MS/MS measurement that lies in a range of $1*10^3$ and $1*10^5$.

Table 23: volumes of the single peptide solution combined for the mixes which were used for MS-parameter optimisation.

Mix 1	pipetting volume (µL)	Mix 2	Pipetting volume (µL)
DLAFPGSGEQVE	2.5	NAVPITPTLN	2.5
SQSENFEYVAF	2.5	AVPYPQ	5
LLSGIENF	2.5	GGLEPINFQTAADQA	2.5
QGQVLTIPQNFAVA	2.5	VLPVPQ	2.5
FALPQYL	2.5	TPEVDDEALE	2.5
IPAVF	2.5	GTDVQAWI	5
IDALNEN	2.5	LYAEE	2.5

FFVAPFPEVFG	2.5	ATYLDCI	7.5
ADIYTEQVG	2.5	YLGYLEQLL	2.5
ALPDDVLANAFQIS	2.5	FYTVISSL	2.5
TNDNAQISPLAG	2.5	VLLEENAGGEQEE	2.5
DQSSYLQGFS	2.5	NLPQQCGL	2.5
ALNEINQFYQ	2.5	FFVPPSEQSL	2.5
GTGNLELVAV	5	WLGLSAEYGNLY	2.5
GNIVNEFE	5	DLPNECGISSQ	5
INTVNSNTLPVL	2.5	ATLTLVSQET	2.5
YIPIQYVLS	2.5	SPDQSYL	2.5
EGVII	2.5	EDVPSE	5
ALPEEVLATAFQIP	2.5	LTEWTSSNVMEE	2.5
LDQWLCE	5	NTDGSTDYGILQINS	2.5
AQSDFGVDT	2.5	ALNEINQFYQ	2.5
TANELNLLIL	2.5	FALPQYL	2.5
Total volume	62.5	Total volume	70

Peptide mixes were reduced with 10 µL of solution of 0.64 nMol DTT/µL in 50 mM ABC for 30 min at 37 °C on a Thermocycler, then alkylated with 10 µL of a solution 3.52 nMol IAA/µL in 50 mM ABC at room temperature in the dark. The mix was then filled up with 80 % 0.1 M ABC and 20 % ACN mix to 260 µL, transferred in a glass vial with micro-insert and measured with the in Skyline generated targeted method.

2.11 Preliminary determination of LOD and LOQ with optimized DP and CE values:

For the first preliminary estimation of LOD and LOQ of the chosen crude peptides, the chocolate dessert (100 ppm material and blank) was extracted according to the final extraction protocol. Then extracts containing 0, 3, 10, 30, 50 and 100 ppm of total allergenic ingredient protein/chocolate dessert were prepared by mixing the extract of the 100 ppm material and the blank material. Tryptic digestion was performed according to the final digestion protocol.

Scheduled MRM-methods including the y-ions of the +2 or +3 charged precursor chosen due to the optimisation experiment before were set up, using the previously optimized CE and DP values. Data was then analysed with Skyline. Peak areas were integrated manually. LOD and LOQ were calculated using the formulas below. A linear regression over the 6 mentioned concentration levels was used to calculate the ppm values (total allergenic ingredient protein/ total food) for each tryptic peptide. In

the Table 34 the best performing peptides are listed with the according MRM-parameters. (The experiment was performed in triplicates)

LOD = average of the blank + 3*standard-deviation of the blank

LOQ = average of the blank + 10*standard-deviation of the blank

It has to be mentioned, that this data was used to select the most suitable peptide candidates to be ordered as labelled peptides for final assay development.

2.12 Validation of the targeted LC-MS/MS method for allergens

Reagents

- MilliQ water
- Formic acid (LC-MS grade), Fa. Merck
- Methanol (hypergrade for LC-MS LiChrosolv®), Fa. Merck

Materials

- Glass vials for LC-MS/MS
- Caps for glass vails incl. Septum

Equipment

- **LC system:** Agilent 1290 Infinity Binary LC System (Agilent); column oven (Agilent 1290 G1316C), binary pump (Agilent 1290 G4220A), autosampler (Agilent 1290 G4226A)
- **LC guard column:** SecurityGuard ULTRA Cartridge UHPLC C18-Peptide for 2.1 mm ID Columns (Phenomenex)
- **LC column:** Aeris PEPTIDE 3.6u XB – C18 column 150*2.1 mm (Phenomenex)
- **MS:** QTRAP® 6500+ System (AB Sciex) equipped with an IonDrive™ Turbo V source
- **Software:** Skyline (MacCoss Lab Software)

Experiments were performed on a QTRAP® 6500+ System (Sciex) equipped with an IonDrive™ Turbo V source directly coupled with an Agilent 1290 Infinity Binary LC System.

2.12.1 General set-up

According to "AOAC Official Methods of Analysis (2002) Appendix D", minimum requirements for samples and calibration are a minimum of 5 samples running in duplicate. Therefore, 5 levels of allergen contaminated chocolate dessert were incorporated in the method validation. 0, 3, 10, 30 and 100 ppm materials (mg protein of allergenic ingredient/kg matrix) were analysed. The hydrated chocolate dessert was provided in levels containing 0, 3, 10, 30, 100 ppm of allergenic protein per ingredient in hydrated chocolate dessert. These chocolate desserts were the basis for all experiments done for the matrix-assisted quantification of the allergenic ingredients.

Heavy labelled peptides (SpikeTides_TQL) having uniformly 13C- and 15N- labelled C-terminal arginine or lysine residues, were added as internal standard at the same level in all samples, to compensate for variations occurring during sample tryptic digestion. A single extract of each level on three separate days was digested three times and each digest was measured 3 times, as visualised in Figure 12.

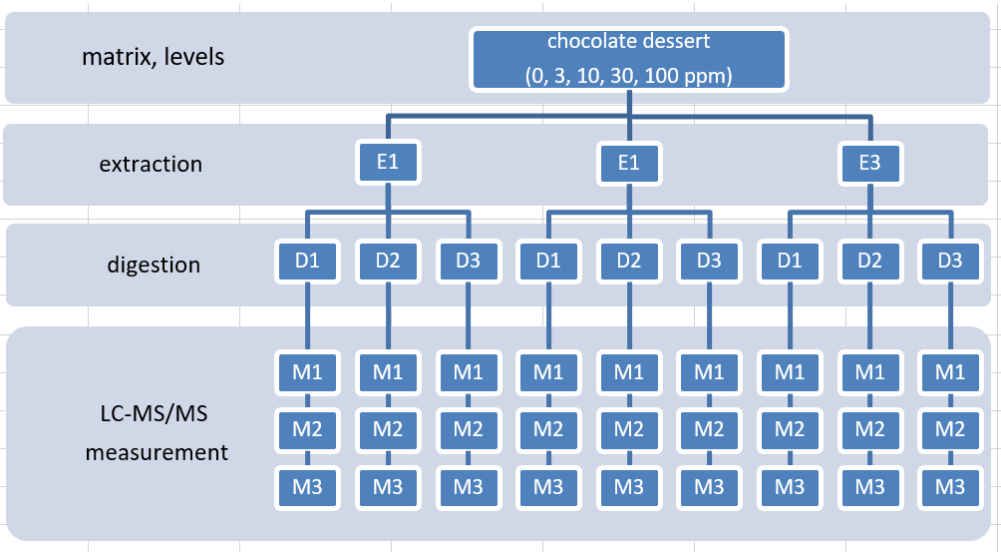


Figure 12: Validation scheme

2.12.2 Heavy labelled peptides – Heavy peptide working Mix

Heavy labelled peptides (SpikeTides_TQL) of the final chosen target marker peptides from Table 34 have been prepared by JPT Technologies Gmbh (Berlin, Germany) and have uniformly 13C- and 15N-labelled C-terminal arginine or lysine residues. These amino acids were selected for labelling as all proteotryptic peptides that result from tryptic digestion of proteins contain C-terminal arginine or lysine. Additionally, the stable isotope labelled quantified peptide standards have a small chemical Qtag attached, which is cleavable by trypsin. This assures that the internal labelled standard peptide undergoes the same digestion reaction as the natural peptides. All target peptides listed before are used as heavy labelled SpikeTides_TQL, care has to be taken at the peptide NLPQQCGLR (Ara h 2) as the cysteine is incorporated in the heavy labelled peptide already as carbamidomethylated form (C[+57 Da]). Spiking heavy-labelled SpikeTides_TQL into the matrix assisted-calibration samples permits a calibration to be developed which can be used for relative quantification using a heavy:light peptide ratio which helps to correct for instrumental day to day variation.

Solubilisation of peptides

The JPT recommendation was to solubilize the peptides in a solution consisting of 80 % (v/v) of 0.1 M ammonium bicarbonate and 20 % (v/v) acetonitrile. Lyophilised peptides (1 nmol confirmed by AAA) were re-solubilised under gentle agitation (30 min, room temperature) using 100 µL of the ammonium bicarbonate-acetonitrile buffer, resulting in peptide solutions containing 10 pmol peptide/µL.

Preparation of Heavy Peptide Working Mix (HPWM)

An aliquot of either 50 µL or 25 µL per peptide as listed in the table below of each heavy synthetic peptide solution (10 pmol/µL) is combined and completed with the addition of 75 µL of 80% (v/v) of 0.1 M ammonium bicarbonate and 20 % (v/v) acetonitrile to a final volume of 1000 µL. HPWM was aliquoted in 100 µL portions and stored at -20°C.

Finally amount of peptide which is applied onto the column is listed in Table 24.

Table 24: Composition of the heavy peptide working mix (HPWM) which was added during the validation of the final LC-MS/MS method to the samples as internal standard

Peptide	µL per heavy peptide combined	pmol/µL of peptides in the HPWM	fmol on column (15 µL injection volume)
DLAFPGSGEQVEK	50	0.5	600
NLPQQC[+57]GLR	25	0.25	300
TANELNLLILR	25	0.25	300
WLGLSAEYGNLYR	50	0.5	600
FFVPPSEQSLR	25	0.25	300
ATLTLVSQETR	25	0.25	300
EGVIIR	25	0.25	300
ALPEEVLATAFQIPR	25	0.25	300
INTVNSNTLPVLR	50	0.5	600
ALPDDVLANAFQISR	25	0.25	300
LLSGIENFR	25	0.25	300
DILNQITKPNDVYSFSLASR	50	0.5	600
LYAEER	25	0.25	300
GGLEPINFQTAADQAR	50	0.5	600
HIATNAVLFFGR	50	0.5	600
AQSDFGVDTK	25	0.25	300
YFGYTGALR	25	0.25	300
IPAVFK	50	0.5	600
IDALNENK	25	0.25	300
FFVAPFPEVFGK	50	0.5	600
YLGYLEQLLR	50	0.5	600
NAVPITPTLNR	25	0.25	300
FALPQYLK	25	0.25	300
VLPVPQK	50	0.5	600
AVPYPQR	50	0.5	600
TIEPNGLLLPQYSNAPELIYIER	25	0.25	300

2.12.3 LC-separation

Eluent A was milliQ water with 0.1 % of formic acid and Eluent B was methanol with 0.1 % formic acid. The analytical column was an Aeris PEPTIDE 3.6u XB-C18 150*2.10 mm (Phenomenex) which was supported with a Security Guard ULTRA Cartridge UHPLC C18-Peptide for 2.1 mm ID Columns (Phenomenex). The LC separation started with a flow rate of 0.3 mL/min, 90 % solvent A for 1 min. Elution of the peptides started then with a linear gradient to 25 % A within 10 minutes and a linear gradient for a further minute to 1 % A, which was then kept for 1 minute. Equilibration was performed for 3 minutes with 90 % solvent A. Gradient is displayed in Table 22.

Injection volume was 15 µL for all samples with a draw speed of 100 µL/min and an eject speed of 200 µL/min. The needle was washed for 5 seconds with Eluent B after injection. The column oven was set at 30 °C.

2.12.4 MS/MS measurement

A QTRAP® 6500+ Low Mass from Sciex equipped with an IonDriveTM Turbo V source was used in positive mode. A scheduled, targeted MRM method was set up with a MRM detection window of 90 sec resulting in a target scan time of 0.9 sec, with the following MS parameters in Table 25.

Table 25: MS-settings used for the validation method

Curtain Gas (CUR)	40.0
Collision Gas (CAD)	Low
Ion Spray Voltage (IS)	4500
Source Temperature (TEM)	350
Ion Source Gas 1/Gas 2	50.0/50.0
Entrance Potential (EP)	10

For a quantitative method at least 10 datapoints per peak should be measured, to assure well characterisation of the peak. The generated method included 156 transition that were measured. The targeted method with the scheduled detection window of 90 sec resulted in at least a dwell time of 20 ms per transition, where the highest number of transitions at the same time was measured. For the other datapoints, dwell time was even up to 30 ms.

In Table 26 the list of peptides inclusively retention times, mass of the precursor ions of the light and the heavy peptide, declustering potential, collision energy and the y-ions measured in the method can

be found. Those y-ions chosen as quantifiers and therefore serving for quantitative data analysis are marked with a (Q).

Table 26: Marker peptides included in the final validation method for the detection of PN, WN, HN, milk and egg allergens and the respective precursor, RT (retention time) DP (declustering potential), CE (collision energy) and fragment ions

Protein	Peptide Sequence	RT	Precursor	DP	CE	Fragment
Ara h 1	DLAFPGSGEQVEK	7.2	688.8383++	75.3	30.7	y10
					30.7	y9(Q)
			692.8454++ (heavy)		24.7	y6
Ara h 2	NLPQQCGLR	5.0	543.2797++	64.7	25.4	y7(Q)
					34.4	y6
			548.2838++ (heavy)		34.4	y5
Ara h 3/4	TANELNLLILR	9.3	635.3799++	68.4	31.7	y9
					34.7	y7
			640.3840++ (heavy)		34.7	y6(Q)
Ara h 3/4	WLGLSAEYGNLYR	8.7	771.3910++	78.4	36.6	y9(Q)
					36.6	y8
			776.3951++ (heavy)		36.6	y7
Ara h 3/4	FFVPPSEQSLR	7.4	653.8431++	72.8	32.4	y9
					32.4	y8(Q)
			658.8473++ (heavy)		41.4	y7
Jug r 2	ATLTLVSQETR	6.6	609.8381++	69.6	30.8	y7
					30.8	y6(Q)
			614.8422++ (heavy)		30.8	y5
Jug r 2	EGVIIR	5.2	343.7134++	50.2	21.2	y5
					18.2	y4

			348.7175++ (heavy)		18.2	y3(Q)
Jug r 4	ALPEEVLATAFQIPR	10.4	827.9618++	85.8	44.7	y9
					44.7	y8(Q)
			832.9659++ (heavy)		41.7	y7
Cor a 9	INTVNSNTLPVLR	7.6	720.9121++	80.7	37.8	y9(Q)
					40.8	y8
			725.9162++ (heavy)		37.8	y4
Cor a 9	ALPDDVLANAFQISR	10.0	815.4334++	90.6	44.2	y9
					47.2	y8(Q)
			820.4375++ (heavy)		44.2	y7
Cor a 11	LLSGIENFR	7.6	524.7929++	63.4	24.7	y7(Q)
					27.7	y6
			529.7971++ (heavy)		24.7	y4
Gal d 2	DILNQITKPNDVYSF SLASR	9.8	761.0656+++	86.6	36	y8
					39	y7(Q)
			764.4017+++ (heavy)		39	y6
Gal d 2	LYAEER	3.6	390.6980++	53.6	22.9	y5
					16.9	y4(Q)
			395.7021++ (heavy)		19.9	y3
Gal d 2	GGLEPINFQTAADQ AR	8.1	844.4235++	86.7	48.3	y10(Q)
					48.3	y9
			849.4277++ (heavy)		48.3	y8
Gal d 2	HIATNAVLFFGR	8.4	673.3724++	74.2	33.7	y10(Q)
					33.7	y9
			678.3765++ (heavy)		33.7	y8

Gal d 3	AQSDFGVDTK	5.1	534.2538++ 538.2609++ (heavy)	64.1	22.1	y8(Q)
					22.1	y7
					28.1	y6
Gal d 3	YFGYTGALR	7.2	524.2665++ 529.2707++ (heavy)	63.3	27.7	y8
					24.7	y7(Q)
					24.7	y5
Bos d 5	IPAVFK	6.4	337.7154++ 341.7225++ (heavy)	49.7	15	y5(Q)
					18	y4
					24	y3
Bos d 5	IDALNENK	4.4	458.7404++ 462.7475++ (heavy)	58.6	22.4	y7
					22.4	y6(Q)
					22.4	y4
Bos d 9	FFVAPFPEVFGK	10.0	692.8686++ 696.8757++ (heavy)	72.6	30.8	y10
					30.8	y9
					30.8	y8(Q)
Bos d 9	YLGYLEQLLR	9.6	634.3559++ 639.3600++ (heavy)	77.4	31.7	y8(Q)
					31.7	y6
					31.7	y5
Bos d 10	NAVPITPTLNR	6.6	598.3433++ 603.3474++ (heavy)	68.7	24.4	y8(Q)
					36.4	y6
					36.4	y5
Bos d 10	FALPQYLK	8.3	490.2842++ 494.2913++ (heavy)	60.9	23.5	y7
					20.5	y6
					20.5	y5(Q)
Bos d 11	VLPVPQK	5.2	390.7525++	53.6	13.9	y5(Q)

					22.9	y4
			394.7596++ (heavy)		25.9	y3
Bos d 11	AVPYPQR	4.5	415.7296++	55.4	17.8	y5(Q)
					26.8	y4
			420.7337++ (heavy)		26.8	y3
Cor a 9	TIEPNGLLLPQYSNA PELIYIER	10.2	881.8058+++	95.4	45.6	y10
					39.6	y8(Q)
			885.1419+++ (heavy)		39.6	y4
Enolase	VNQIGTLSESIK	6.2	644.8590++	78.1	32.1	y10
					32.1	y9(Q)
					32.1	y8

2.12.5 Determination of LOD and LOQ of validated method

To determine the LOD and LOQ of the assay synthetic isotopically labelled peptides (SpikeTides™ TQL, JPT Peptide Technologies GmbH, Berlin, Germany) were spiked into the samples at a constant level as standards. This allowed two sets of fragment ions to be detected: non-labelled (from the endogenous digested protein) and heavy-isotope labelled (from the heavy labelled SpikeTides™ TQL, mass difference +8 Lys, +10 Arg). The ratio of heavy:light peptides in the samples were used to build calibration curves for each target peptide. The LOD and LOQ of the 26 target peptides were calculated by analysing the blank chocolate dessert matrix and bases on 27 data sets (3 extractions x 3 digestions x 3 analysis). For the determination of the LOD and LOQ the following formulas were used.

LOD = average of the blank matrix + 3 x standard deviation

LOQ = average of the blank matrix + 10 x standard deviation

By using the slope and interception of the respective calibration curve of each peptide the corresponding ppm value for the LOD and the LOQ was calculated. LOD and LOQ and r-squared value, as well as slope and interception of each peptide are listed in Table 35.

3 Results

3.1 In-Silico proteolytic digestion of allergenic ingredients

Using Trypsin as enzyme for the digestion of the protein samples before application to the LC-MS/MS measurement is state-of-the art in proteomics. However. the sole use of trypsin may exclude some possible stable marker peptides especially for proteins where less Arginine and Lysine are present. Therefore, some alternative proteases were used to proteolytically digest the allergen-set of Table 18 on a theoretical level, to check if applying a different protease with these proteins might generate relevant peptides for a LC-MS/MS method. To touch criteria for relevant marker peptides for a targeted LC-MS approach, settings for the in-silico digest were chosen as listed in chapter 2.3. These criteria were listed in a publication of Johnson et. al (2011) (88).

Cor a 1 (Bet v 1 family) and Cor a 2 (profilins) were not included in Table 28 because for those allergens multiple isoform entries could be found in the NCBI database. Between the isoforms there were no resulting identical peptides, which makes these peptides irrelevant for a quantitative LC-MS/MS method that should be universally applicable.

Table 27: Resulting number of relevant peptides for quantitative targeted LC- MS/MS method after the in-silico digest

	Trypsin	Pepsin	Chymotrypsin	GluC	AspN
Hazelnut	32	22	33	30	30
Peanut	18	13	15	17	18
Walnut	23	13	18	13	13
Cow´s milk	10	15	10	12	12
Hen´s egg	4	4	4	3	3
Total	87	67	80	75	76

The potential peptide markers resulting from the in-silico digest are presented in Table 28-Table 32. The in-silico digestion with trypsin resulted in a total of 87 relevant marker peptides for all allergens as shown in Table 27. Especially in the case of walnut allergens, trypsin seemed to be most promising as the biggest pool of possible peptide markers was achieved. No advantage in higher number of relevant peptides was seen for the other proteases in the in-silico digest. Additionally, trypsin is less expensive and has its pH optimum (7.5-8.5) in a range that fits to the pH range of the extraction buffers that will be used. These reasons combined resulted in the decision to stay with trypsin in this work.

Table 28: in-silico digest of peanut allergens – resulting peptides given as fragment sequence [position of the fragment on protein sequence]

	Trypsin	Pepsin	Chymotrypsin	GluC	AspN
Ara h 1	K.PNTLVLPK.H [220. 227]	F.STRYGNQNGRIRVL.Q [182. 195]	Y.DDDRRQPRREEGGRW.G [100. 114]	E.RTRGRQPGD.Y [90. 98]	G.ERTRGRQPG.D [89. 97]
	R.IPSGFISYILNR.H [265. 276]	F.PGSGEQVEKL.I [563. 572]	W.GPAGPREREREEDW.R [115. 128]	D.QSSYLQGFSRNTLE.A [308. 321]	R.DQSSYLQGFSRNTL.E [307. 320]
	R.DQSSYLQGFSR.N [307. 317]		Y.GNQNGRIRVL.Q [186. 195]	E.GVIVKVSKE.H [362. 370]	N.EGVIVKVSK.E [361. 369]
	R.SSENNEGVIVK.V [356. 366]		F.PGSGEQVEKL.I [563. 572]	D.ITNPINLRE.G [393. 401]	G.DITNPINLR.E [392. 400]
	R.EGEPDLSNNFGK.L [401. 412]			D.LSNNFGKLFE.V [406. 415]	P.DLSNNFGKLF.E [405. 414]
	K.DNVIDQIEK.Q [547. 555]			D.KKNPQLQD.L [420. 427]	P.DKKNPQLQ.D [419. 426]
	K.DLAFPGSGEQVEK.L [559. 571]			E.VRRYTARLKE.G [496. 505]	R.EVRRYTARLK.E [495. 504]
	R.PQSQSQSPSSPEK.E [586. 598]			D.LAFPGSGE.Q [560. 567]	K.DLAFPGSG.E [559. 566]
	K.EDQEEENQGGK.G [604. 614]			E.KLIKNQKE.S [571. 578]	V.EKLIKNQK.E [570. 577]
	K.GPLLSILK.A [615. 622]				
Ara h 2	no peptides	L.QGRQQEQQF.K [132. 140]	Y.SPSQDPDRRDPY.S [72. 83]	D.PYSPSPYD.R [82. 89]	R.DPYSPSPY.D [81. 88]
		L.EVESGGRDRY.- [162. 171]	L.QGRQQEQQF.K [132. 140]	D.RLQGRQQE.Q [130. 137]	S.DRLQGRQQ.E [129. 136]
			L.EVESGGRDRY.- [162. 171]		
		F.YSNAPQEIF.I [84. 92]	L.TDTNNNDNQL.D [179. 188]		
Ara h 3/4	R.SPDIYNPQAGSLK.T [370. 382]	F.IQQGRGYF.G [93. 100]	Y.QQQSRRRSL.P [209. 217]	E.TWNPNNQE.F [54. 61]	I.ETWNPNNQ.E [53. 60]
	K.TANELQLNLLILR.W [383. 395]	L.IAVPTGVAF.W [155. 163]	Y.SPQTQPKQEDREF.S [223. 235]	D.VVAVSLTD.T [173. 180]	T.DVVAVSLT.D [172. 179]
	R.WLGLSAEYGNLYR.N [396. 408]	L.TDTNNNDNQL.D [179. 188]	F.QVDDRQIL.Q [272. 279]	E.GGNIFSGFTPE.F [255. 265]	N.EGGNIFSGFTP.E [254. 264]
	R.AHVQVVDSNGDR.V [431. 442]	F.QVDDRQIL.Q [272. 279]	L.SPDRKRRQQY.E [305. 314]	D.RKRRQQYE.R [308. 315]	P.DRKRRQQY.E [307. 314]
	K.SQSENFEYVAFK.T [466. 477]	F.AVAGKSQSENF.E [461. 471]	F.AVAGKSQSENF.E [461. 471]	D.IYNPQAGSLKTANE.L [373. 386]	P.DIYNPQAGSLKTAN.E [372. 385]
	K.FFVPPSEQSLR.A [524. 534]	L.PEEVVANSYGL.P [498. 508]	L.PEEVVANSY.G [498. 506]		S.EQSLRAVA.- [530. 537]
		L.PREQARQL.K [509. 516]	L.PREQARQL.K [509. 516]		
Ara h 5	R.LGDYLIDTGL.- [121. 130]	L.GQDGSVWAQSSNF.P [26. 38]	no peptides	no peptides	no peptides
Ara h 6	no peptides	no peptides	no peptides	D.IRSTRSSD.Q [66. 73]	Y.DIRSTRSS.D [65. 72]

Table 29: in-silico digest of hazelnut allergens – resulting peptides given as fragment sequence [position of the fragment on protein sequence]

	Trypsin	Pepsin	Chymotrypsin	GluC	AspN
Cor a 8	no fragments	L.KDTAKGIAGL.N [75. 84]	L.KDTAKGIAGL.N [75. 84]	no fragments	no fragments
Cor a 9	R.LNALEPTNR.I [42. 50]	L.RRQQQRYF.G [27. 34]	F.REGDIIAL.P [150. 157]	E.PNGLLLPQYSNAPE.L [82. 95]	I.EPNGLLLPQYSNAP.E [81. 94]
	R.QGQGQSQR.S [130. 137]	F.REGDIIAL.P [150. 157]	F.NVDVDTARRL.Q [253. 262]	D.RHQKIRHFRE.G [142. 151]	Q.DRHQKIRHFR.E [141. 150]
	R.HFYLAGNPDDEHQR.Q [194. 207]	F.NVDVDTARRL.Q [253. 262]	L.QVVRPERSRQEW.E [280. 291]	E.GNNVFSGFD.A [236. 244]	G.EGNNVFSGF.D [235. 243]
	R.ADIYTEQVGR.I [340. 349]	F.AVAKRAESEGF.E [428. 438]	Y.TEQVGRINTVNSNTL.P [344. 358]	D.TARRLQSNQD.K [258. 267]	V.DTARRLQSNQ.D [257. 266]
	R.INTVNSNTLPVLR.W [350. 362]	F.KTNDNAQISPL.A [444. 454]	F.AVAKRAESEGF.E [428. 438]	D.KRRNIVKVE.G [268. 276]	Q.DKRRNIVKV.E [267. 275]
	R.WLQLSAER.G [363. 370]	L.AGRTSAIRAL.P [455. 464]	F.KTNDNAQISPL.A [444. 454]	E.GRLQVVRPE.R [277. 285]	V.EGRLQVVRP.E [276. 284]
	K.TNDNAQISPLAGR.T [445. 457]	F.QISREEARRL.K [474. 483]	L.AGRTSAIRAL.P [455. 464]	E.RQRRQGGRGRD.V [307. 317]	R.ERQRRQGGRGR.D [306. 316]
	R.ALPDDVLANAFQISR.E [463. 477]	L.KYNRQETTL.V [484. 492]	F.QISREEARRL.K [474. 483]	E.WVAFKTND.N [440. 447]	F.EWVAFKTN.D [439. 446]
Cor a 10	K.NGHVEIIANDQGNR.I [54. 67]	L.GTVIGIDL.G [35. 42]	L.GTVIGIDL.G [35. 42]	D.QGNRITPSWVGFTD.G [64. 77]	N.DQGNRITPSWVGFT.D [63. 76]
	R.ITPSWVGFTDGER.L [68. 80]	L.DKKGGEKNIL.V [218. 227]	Y.KIVNKDGKPY.I [124. 133]	E.AAKNQAAVNPE.R [85. 95]	G.EAAKNQAAVNP.E [84. 94]
	K.NQAAVNPER.T [88. 96]	L.RREAERAKRAL.S [295. 305]	Y.IQVKIKDGETKVF.S [134. 146]	D.GKPYIQVKIKD.G [130. 140]	K.DGKPYIQVKIK.D [129. 139]
	K.DAVVTVPAYFNDAQR.Q [173. 187]	L.AKNQIDEIVL.V [358. 367]	L.DKKGGEKNIL.V [218. 227]	E.AFLGKKIKD.A [165. 173]	A.EAFLGKKIK.D [164. 172]
	K.DAGIIAGLNVAR.I [192. 203]	L.IPRNTVIPTKKSQVF.T [445. 459]	L.STNGDTHL.G [252. 259]	D.AVVTVPAYFND.A [174. 184]	K.DAVVTVPAYFN.D [173. 183]
	R.FEELNNDFVQK.D [333. 343]	F.TTYQDQQTTVSIQVF.E [460. 474]	L.RREAERAKRAL.S [295. 305]	D.AQRQATKD.A [185. 192]	N.DAQRQATK.D [184. 191]
	K.NQIDEIVLVGGSTR.I [360. 373]	F.EVDANGIL.N [510. 517]	L.AKNQIDEIVL.V [358. 367]	D.AGIIAGLNVARIINE.P [193. 207]	K.DAGIIAGLNVARIIN.E [192. 206]
	K.FDLTGVPPAPR.G [490. 500]		Y.GAAVQGSIL.S [403. 411]	E.PTAAAIAYGLD.K [208. 218]	N.EPTAAAIAYGL.D [207. 217]
	R.LSQEEIDR.M [541. 548]		L.IPRNTVIPTKKSQVF.T [445. 459]	E.YFIKLIKKKHGKD.I [271. 283]	M.EYFIKLIKKKHGK.D [270. 282]
			Y.QDQQTTVSIQVF.E [463. 474]	D.NRAIGKLRRE.A [288. 297]	K.DNRAIGKLRR.E [287. 296]
			F.EVDANGIL.N [510. 517]	E.RAKRALSSQHQVRVE.I [300. 314]	A.ERAKRALSSQHQVRV.E [299. 313]
				D.VAPLTLGIE.T [427. 435]	L.DVAPLTLGI.E [426. 434]
				D.QQTTVSIQVFE.G [465. 475]	Q.DQQTTVSIQVF.E [464. 474]
				D.LTGVPPAPRGTPQIE.V [492. 506]	F.DLTGVPPAPRGTPQI.E [491. 505]
Cor a 11	R.LLSGIENFR.L [91. 99]	F.ESRVKTEEGRVQVL.E [69. 82]	Y.GKEQEENPY.V [53. 61]	E.NFRLAILE.A [97. 104]	I.ENFRLAIL.E [96. 103]
	R.AFSWEVLEAALK.V [201. 212]	F.YGAGGEDPESF.Y [188. 198]	F.ESRVKTEEGRVQVL.E [69. 82]	E.ANPHTFISPAHFD.A [105. 117]	L.EANPHTFISPAHF.D [104. 116]
	R.ALSQHEEGPPR.I [240. 250]	L.SSSSGSYQKISARL.R [336. 349]	L.QPVSAPGHF.E [176. 184]	E.SFYRAFSWE.V [197. 205]	P.ESFYRAFSW.E [196. 204]

	K.HPSQSNQFGR.L [268. 277]	L.AGKGNIVNEF.E [391. 400]	L.SQHEEGPPRIW.P [242. 252]	E.AALKVRRE.Q [209. 216]	L.EAALKVRR.E [208. 215]
	R.LYEAHPDDHK.Q [278. 287]	L.PSREVERIF.K [412. 420]	F.EVNAHGNSRF.P [379. 388]	E.QSKGSIVKASRE.K [225. 236]	G.EQSKGSIVKASR.E [224. 235]
	K.GNIVNEFER.D [394. 402]		L.AGKGNIVNEF.E [391. 400]	E.LAFNLPSRE.V [407. 415]	K.ELAFNLPSR.E [406. 414]
	K.ELAFNLPSR.E [406. 414]		L.PSREVERIF.K [412. 420]		
	K.NQDQAFFFPGPNK.Q [422. 434]				
Cor a 12	R.PQQLQVHPQR.G [4. 13]	L.VPAAIVVGL.A [78. 86]	L.QVHPQRGHGHY.E [8. 18]	no fragments	no fragments
	R.GHGHYEGGIK.N [14. 23]		L.VPAAIVVGL.A [78. 86]		
	R.GGGPSAVK.V [27. 34]				
	R.EVGQEIQSR.A [143. 151]				
Cor a 13	R.QLQDPAHQPR.S [6. 15]	L.VPAVITVSL.I [62. 70]	L.VPAVITVSL.I [62. 70]	E.QFGQQHVTGSQGS.- [127. 139]	A.EQFGQQHVTGSQGS.- [126. 139]
	R.HPPGADQLDHAR.M [100. 111]	F.GVAAVTVL.S [82. 89]	F.GVAAVTVL.S [82. 89]		
	R.AEQFGQQHVTGSQGS.- [125. 139]		Y.VTGRHPPGADQL.D [96. 107]		
			F.GQQHVTGSQGS.- [129. 139]		
Cor a 14	no peptides	no peptides	Y.DGSNQQQQQEL.E [73. 83]	D.GSNQQQQQE.L [74. 82]	Y.DGSNQQQQQ.E [73. 81]

Table 30: in-silico digest of egg allergens – resulting peptides given as fragment sequence [position of the fragment on protein sequence]

	Trypsin	**Pepsin**	**Chymotrypsin**	**GluC**	**AspN**
Gal d 1			no peptides		
Gal d 2	R.DILNQITK.P [85. 92]	L.YAEERYPIL.P [106. 114]	F.QTAADQAREL.I [135. 144]	D.STRTQINKVVRFD.K [48. 60]	K.DSTRTQINKVVRF.D [47. 59]
	K.PNDVYSFSLASR.L [93. 104]	F.QTAADQAREL.I [135. 144]	W.VESQTNGIIRNVL.Q [149. 161]	E.PINFQTAAD.Q [131. 139]	L.EPINFQTAA.D [130. 138]
		L.SGISSAESL.K [313. 321]	L.SGISSAESL.K [313. 321]		
Gal d 4	K.FESNFNTQATNR.N [51. 62]	L.DNYRGYSL.G [35. 42]	F.NTQATNRNTDGSTDY.G [56. 70]	E.SNFNTQATNRNTD.G [53. 65]	F.ESNFNTQATNRNT.D [52. 64]
	K.GTDVQAWIR.G [134. 142]				

Table 31: in-silico digest of walnut allergens – resulting peptides given as fragment sequence [position of the fragment on protein sequence]

	Trypsin	Pepsin	Chymotrypsin	GluC	AspN
Jug r 1.0101	R.SGGYDEDNQR.Q [61. 70]	L.RQVVRRQQQQQGL.R [93. 105]	Y.DEDNQRQHF.R [65. 73]		
			L.RQVVRRQQQQQGL.R [93. 105]		
Jug r 2.0101	R.DDDDEENPR.D [3. 11]	L.VSQETRESF.N [254. 262]	L.VSQETRESF.N [254. 262]	E.NYRVVILD.A [217. 224]	I.ENYRVVIL.D [216. 223]
	R.QDPQQQYHR.C [116. 124]	L.KSESPSYSNQF.G [388. 398]	W.GRRSSGGPISL.K [377. 387]	D.QSYLRVFSND.I [317. 326]	P.DQSYLRVFSN.D [316. 325]
	R.HESEEGEVK.Y [192. 200]		Y.VVEGTGRY.E [444. 451]	E.GVIIRASQE.K [351. 359]	R.EGVIIRASQ.E [350. 358]
	R.ATLTLVSQETR.E [249. 259]			E.NLRLLGFD.I [510. 517]	N.ENLRLLGF.D [509. 516]
	R.EYYAAGAK.S [306. 313]			E.RQSRRGQGRD.H [571. 580]	T.ERQSRRGQGR.D [570. 579]
	R.VFSNDILVAALNTPR.D [322. 336]				
	R.FFDQQEQR.E [342. 349]				
	K.ATVVVYVVEGTGR.Y [438. 450]				
	R.LLGFDINGENNQR.D [513. 525]				
	R.DFLAGQNNIINQLER.E [526. 540]				
	R.DHPLASILDFAFF.- [580. 592]				
Jug r 3	K.AAATTADR.Q [64. 71]	L.KKTSGSIPGL.N [79. 88]	L.KKTSGSIPGL.N [79. 88]		
Jug r 4.0101	R.LDALEPTNR.I [40. 48]	L.AQSGGRQQQQF.G [22. 32]	L.AQSGGRQQQQF.G [22. 32]	D.RHQKIRHFRE.G [134. 143]	Q.DRHQKIRHFR.E [133. 142]
	R.QSQQGQSR.E [121. 128]	F.EESQRQSQQGQSREF.Q [116. 130]	F.EESQRQSQQGQSREF.Q [116. 130]	D.IIAFPAGVAHWSYND.G [146. 160]	G.DIIAFPAGVAHWSYN.D [145. 159]
	R.QQRPGEHGQQQR.G [215. 226]	F.REGDIIAF.P [142. 149]	F.QQDRHQKIRHF.R [131. 141]	D.GSNPVVAISLLD.T [161. 172]	N.DGSNPVVAISLL.D [160. 171]
	R.QLQVIRPR.W [272. 279]	L.AGNPDDEF.R [190. 197]	F.REGDIIAF.P [142. 149]	D.TNNNANQLD.Q [173. 181]	L.DTNNNANQL.D [172. 180]
	R.ENIGDPSR.A [327. 334]	F.NVDTETARRL.Q [246. 255]	F.RPQGQQEY.E [198. 205]	E.QHRRQQQRQQRPGE.H [207. 220]	Y.EQHRRQQQRQQRPG.E [206. 219]
	R.ADIYTEEAGR.I [335. 344]	L.RGRAEVQVVDNF.G [390. 401]	F.NVDTETARRL.Q [246. 255]	E.TARRLQSE.N [251. 258]	T.ETARRLQS.E [250. 257]
	R.ISTVNSHTLPVLR.W [345. 357]	F.AVVKRARNEGF.E [423. 433]	L.RENIGDPSRADIY.T [326. 338]	D.HRRSIVRVE.G [261. 269]	N.DHRRSIVRV.E [260. 268]
	R.WLQLSAER.G [358. 365]	L.AGRTSAIRAL.P [450. 459]	Y.TEEAGRISTVNSHTL.P [339. 353]	E.WVSFKTNE.N [435. 442]	F.EWVSFKTN.E [434. 441]
	R.NEGFEWVSFK.T [430. 439]	F.QIPREDARRL.K [469. 478]	L.RGRAEVQVVDNF.G [390. 401]		
	R.ALPEEVLATAFQIPR.E [458. 472]		F.AVVKRARNEGF.E [423. 433]		
			L.AGRTSAIRAL.P [450. 459]		
			F.QIPREDARRL.K [469. 478]		

Table 32: in-silico digest of milk allergens – resulting peptides given as fragment sequence [position of the fragment on protein sequence]

	Trypsin	Pepsin	Chymotrypsin	GluC	AspN
Bos d 9	R.FFVAPFPEVFGK.E [37. 48]	F.GKEKVNEL.S [47. 54]	F.GKEKVNEL.S [47. 54]	E.NLLRFFVAPFPE.V [33. 44]	N.ENLLRFFVAPFP.E [32. 43]
	R.YLGYLEQLLR.L [105. 114]	L.KKYKVPQL.E [116. 123]	L.EIVPNSAEERL.H [124. 134]	E.QLLRLKKYKVPQLE.I [111. 124]	L.EQLLRLKKYKVPQL.E [110. 123]
	K.VPQLEIVPNSAEER.L [120. 133]	L.EIVPNSAEERL.H [124. 134]		E.LFRQFYQLD.A [163. 171]	P.ELFRQFYQL.D [162. 170]
		L.DAYPSGAWYYVPL.G [171. 183]		D.IPNPIGSE.N [196. 203]	S.DIPNPIGS.E [195. 202]
		L.GTQYTDAPSF.S [184. 193]			
		L.TEEEKNRL.N [168. 175]	W.DQVKRNAVPITPTL.N [124. 137]	no peptides	no peptides
Bos d 10	K.ALNEINQFYQK.F [95. 105]		L.TEEEKNRL.N [168. 175]		
	K.FALPQYLK.T [188. 195]		W.IQPKTKVIPY.V [208. 217]		
Bos d 11	no peptides	L.NVPGEIVESL.S [21. 30]	L.NVPGEIVESL.S [21. 30]	E.SQSLTLTD.V [136. 143]	T.ESQSLTLT.D [135. 142]
		F.QSEEQQQTEDEL.Q [48. 59]	F.QSEEQQQTEDEL.Q [48. 59]	E.PVLGPVRGPFPIIV.- [210. 223]	Q.EPVLGPVRGPFPIIV.- [209. 223]
		F.PGPIPNSL.P [77. 84]	F.PGPIPNSL.P [77. 84]		
		F.PKYPVEPF.T [126. 133]	L.PVPQKAVPY.P [186. 194]		
Bos d 12	K.YIPIQYVLSR.Y [45. 54]	L.SRYPSYGL.N [53. 60]		E.IPTINTIASGE.P [139. 149]	T.EIPTINTIASG.E [138. 148]
	R.YPSYGLNYYQQK.P [55. 66]			E.PTSTPTTE.A [150. 157]	G.EPTSTPTT.E [149. 156]
				E.INTVQVTSTAV.- [179. 189]	P.EINTVQVTSTAV.- [178. 189]
Bos d 5	K.PTPEGDLEILLQK.W [63. 75]	L.DIQKVAGTWYSL.A [26. 37]	L.VRTPEVDDEAL.E [138. 148]	E.KTKIPAVFKID.A [90. 100]	A.EKTKIPAVFKI.D [89. 99]
	K.IDALNENK.V [99. 106]	L.VRTPEVDDEAL.E [138. 148]			
	R.TPEVDDEALEK.F [140. 150]				
Bos d 4	no peptides	L.KGYGGVSL.P [34. 41]		D.LKGYGGVSLPE.W [33. 43]	K.DLKGYGGVSLP.E [32. 42]
		L.DKVGINYWL.A [115. 123]		D.TQAIVQNND.S [56. 64]	Y.DTQAIVQNN.D [55. 63]

3.2 Extraction optimisation

In Table 33 a list of those peptides measured with the targeted LC-MS/MS method for the evaluation of the extraction optimisation and the tryptic digest optimisation is given. Information about the origin protein/allergen of the different peptides can be found. In total 151 peptides from 27 proteins were included in the measurement, but only 60 peptides of 20 proteins were found in the different stages. Already more than 50 % of the peptides generated within the in-silico digest were not fit for purpose, meaning either proteins are not extractable or peptides are not stable in digested form or ionisation properties are not suitable for LC-MS/MS measurement. Ara h 5, Ara h 6, Jug r 3, Cor a 8, Cor a 10, Cor a 12 and Cor a 13 were not represented by peptides in the results due to the above mentioned reasons.

Table 33: A list of those peptides, that have been found in the extracts during extraction optimisation and tryptic digest optimisation.

PEANUT		**EGG**		**MILK**	
Ara h 1	IPSGFISYILNR	Gal d 1	DVLVCNK	Bos d 4	LDQWLCEK
Ara h 1	DQSSYLQGFSR	Gal d 2	LYAEER	Bos d 4 *	NAVPITPTLNR
Ara h 1 *	VLLEENAGGEQEER	Gal d 2 *	GGLEPINFQTAADQAR	Bos d 5 *	LIVTQTMK
Ara h 1 *	GTGNLELVAVR	Gal d 2 *	LTEWTSSNVMEER	Bos d 5 *	VLVLDTDYK
Ara h 2 *	NLPQQCGLR	Gal d 3	DLTQQER	Bos d 5	IDALNENK
Ara h 3/4	TANELNLLILR	Gal d 3	ISLTCVQK	Bos d 5	TPEVDDEALEK
Ara h 3/4	SPDIYNPQAGSLK	Gal d 3	ATYLDCIK	Bos d 6	YICDNQDTISSK
Ara h 3/4	WLGLSAEYGNLYR	Gal d 3	DEYELLCLDGSR	Bos d 9	FFVAPFPEVFGK
Ara h 3/4	SQSENFEYVAFK	Gal d 3	AQSDFGVDTK	Bos d 9	EDVPSER
Ara h 3/4	FFVPPSEQSLR	Gal d 3	YFGYTGALR	Bos d 9	YLGYLEQLLR
HAZELNUT		Gal d 3	FYTVISSLK	Bos d 10	ALNEINQFYQK
Cor a 9 *	QGQVLTIPQNFAVAK	Gal d 4	NTDGSTDYGILQINSR	Bos d 10	FALPQYLK
Cor a 9	LNALEPTNR	Gal d 4	GTDVQAWIR	Bos d 10	VIPYVR
Cor a 9	ADIYTEQVGR	**WALNUT**		Bos d 11	VLPVPQK
Cor a 9	INTVNSNTLPVLR	Jug r 1 *	DLPNECGISSQR	Bos d 11	AVPYPQR
Cor a 9	TNDNAQISPLAGR	Jug r 2	ATLTLVSQETR	Bos d 12	YIPIQYVLSR
Cor a 9	ALPDDVLANAFQISR	Jug r 2	EGVIIR	Bos d 12	YPSYGLNYYQQK
Cor a 9	QETTLVR	Jug r 2	VFSNDILVAALNTPR		
Cor a 11	LLSGIENFR	Jug r 4	LDALEPTNR		
Cor a 11	AFSWEVLEAALK	Jug r 4	ADIYTEEAGR		
Cor a 11	GNIVNEFER	Jug r 4	ALPEEVLATAFQIPR		
Cor a 11	ELAFNLPSR				
Cor a 11	NQDQAFFFPGPNK				
Cor a 14	DLPNQCR				

3.2.1 Extraction buffers

In the first experiment within the Extraction optimisation, 4 different buffers for protein extraction commonly applied in literature were tested. These buffers are fit for LC-MS/MS measurements and cover a pH range from 5.0 – 8.5.

- ABC50 → 50 mM ammonium bicarbonate buffer, pH 7.8
- ABC100 → 100 mM ammonium bicarbonate buffer, pH 8.1
- TBB → 8 mM Tris, 10 mM Borat buffer pH 8.5
- Tris-HCl → 50 mM Tris-HCl buffer, pH 5.0

The proteins solubility is a function of many factors, such as the native or denatured state and environmental factors like pH or temperature of an extraction solution. At pH values above and below the isoelectric point (pI) more water interacts with the protein as it has negative or positive charges which leads to greater protein solubility. pIs of the relevant allergens for this work are listed in Table 18.

Proteins and number of peptides

Looking at the number of peptides found in the different extracts (see Figure 13) no big difference can be seen for the ABC100 and the ABC50 extracts, except of one peptide for Cor a 14 which is present in the ABC50 buffer but not in the ABC100 buffer. Highest number of found peptides and represented proteins is given in the TBB buffer. Especially, when looking at the peptides from peanut. In regards of the aim of developing a multi-allergen detection method, the TBB buffer was the best choice. The TRIS buffer resulted in the lowest number of found peptides, which might be due to the low pH of 5.0 in this buffer. As most of the allergens do have a pI of 5-6, the solubility of allergens in the TRIS buffer was not as effective as in the TBB buffer with a pH of 8.5. Charge state of the proteins was higher in buffers with higher pH (above the pI) which enhances solubility of the proteins and therefore availability for the further tryptic digest and the LC-MS/MS measurement.

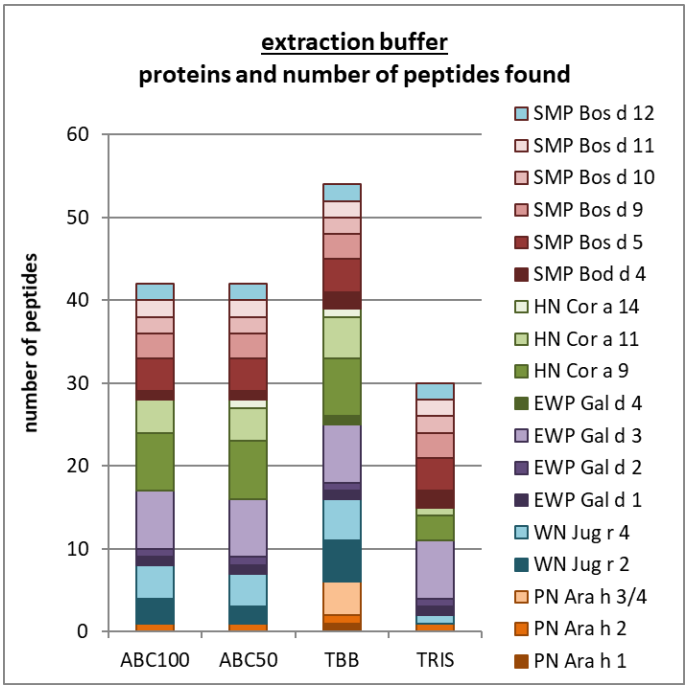


Figure 13: Comparison of the number of peptides related to its protein found in the different extraction buffers. (n=3)

Intensities

In the following Figure 14-Figure 18 the intensities (peak area of the TIC see chapter 2.8) from the MRM-spectra are plotted for the found peptides.

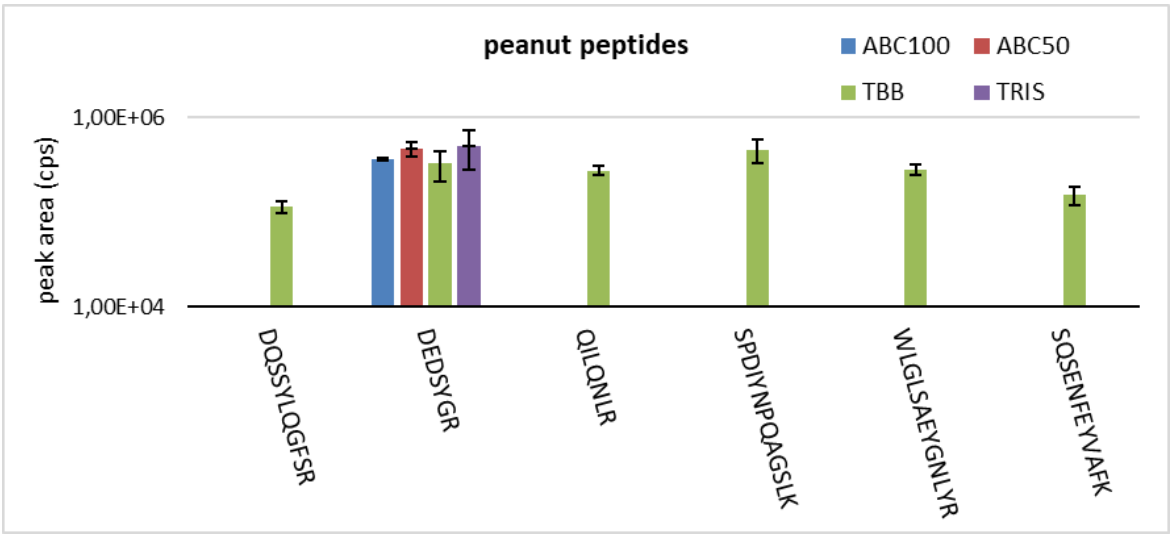


Figure 14: Comparison of the peak areas of peanut protein peptides measured in the found in the different extraction buffers. (n=3)

For PN peptide DEDSYGR derived from Ara h 2 was found in all extracts. But Ara h 1 (DQSSYLQGFSR) and Ara h 3/4 peptides were only found in the TBB extracts.

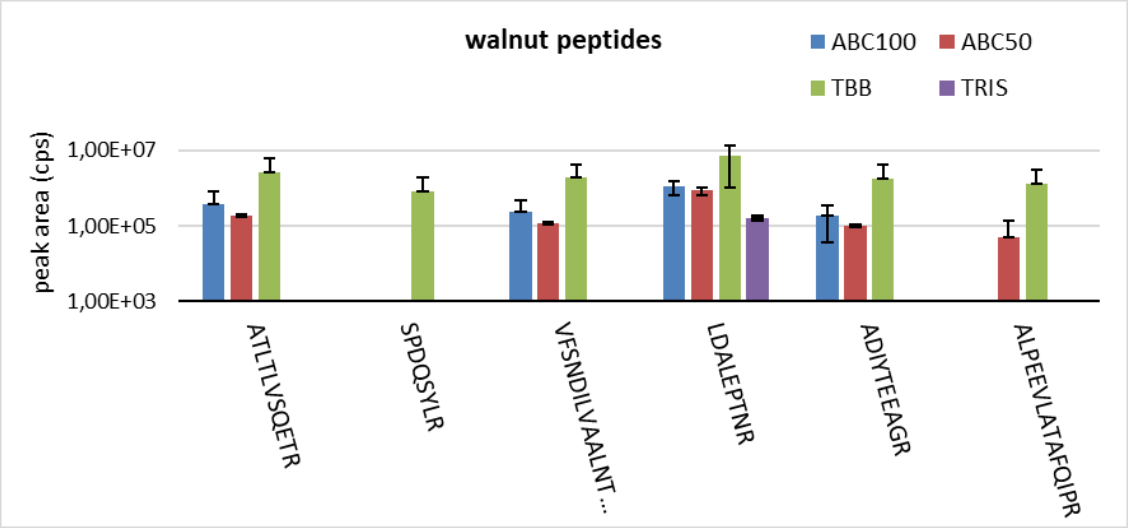


Figure 15: Comparison of the peak areas of walnut protein peptides measured in the found in the different extraction buffers. (n=3)

For HN and WN highest peak areas were also reached in the TBB-extracts. The intensities in the TBB extract were at least one order of magnitude higher than in the other extracts. An additional peptide was found for HN and for WN in the TBB buffer. TRIS buffer resulted in the lowest number of peptides found, but ABC50 and ABC100 gave similar results.

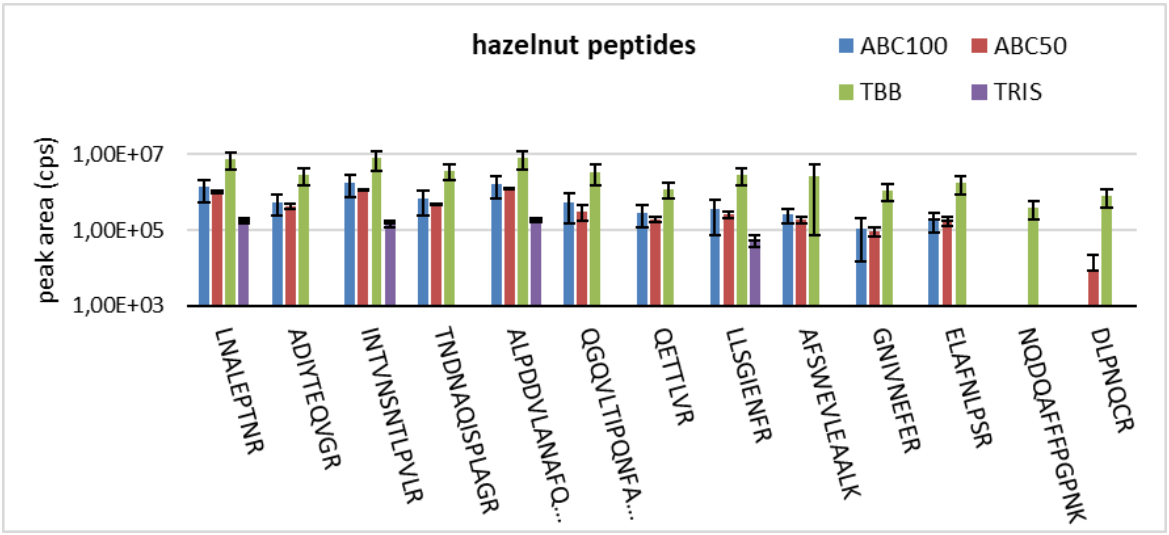


Figure 16: Comparison of the peak areas of hazelnut protein peptides measured in the found in the different extraction buffers. (n=3)

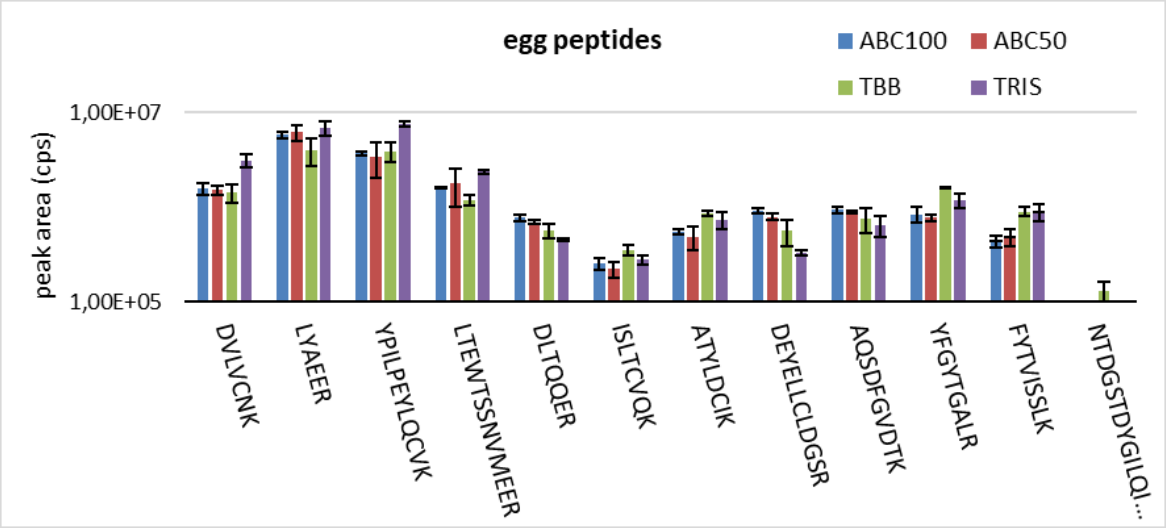


Figure 17: Comparison of the peak areas of egg protein peptides measured in the found in the different extraction buffers. (n=3)

For SMP and EWP the difference in peak areas was not as significant as for PN, HN and WN. All buffers resulted in similar intensities for the found peptides. In case of egg peptides, TRIS worked very effective for extraction. Nevertheless, aim of this work was to find a buffer for a multi-analyte method. Overall, for the 5 different allergenic ingredients in combination, the TBB buffer was most promising. Therefore, all further experiments were carried out by using TBB buffer (8 mM Tris, 10 mM Borat buffer pH 8.5).

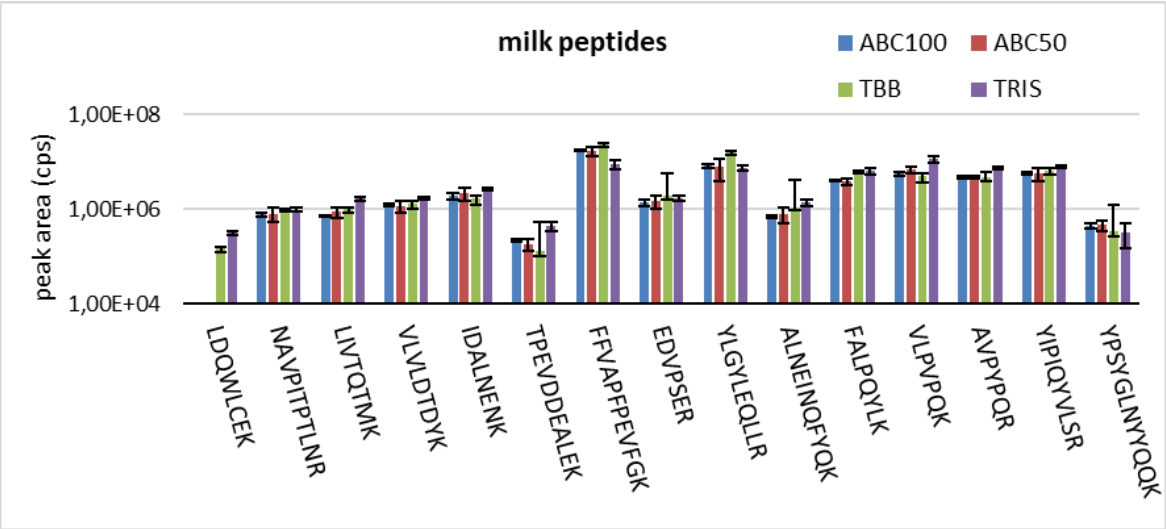


Figure 18: Comparison of the peak areas of milk protein peptides measured in the found in the different extraction buffers. (n=3)

3.2.2 Addition of Detergents

Denaturation caused by processing/backing of food decreases protein solubility compared to native protein and leads to aggregation. By using denaturating agents like urea, buried disulfide bonds (solvent-inaccessible) can be made accessible. This supports the extractability and solubility of proteins. It is well known, that additional use of reducing agents like Dithiothreitol (DTT), supports the reduction of disulfide bonds of proteins. Intramolecular and intermolecular disulfide bond formation between cysteine residues of proteins is prevented. Following combinations were tested:

I. no detergent
II. 0.6 M urea
III. 6 M urea
IV. 0.6 M urea + 50 mM DTT
V. 6 M urea + 50 mM DTT

These detergents were added to the TBB buffer, that was chosen in 3.2.1.

It should be mentioned here, that in the experiment also a buffer containing 5 % SDC was prepared and used for extraction, as SDC is known to support protein extraction. But results could not be obtained with LC-MS as there were some issues with the handling of those extracts. When extracts were cooled or frozen before tryptic digestion the extracts became viscous even gel-like after thawing. Slight heating of the extracts did not make them liquid enough to pipette them accurately to the tryptic digestion. Therefore, the SDC extracts were skipped as possible choice.

Proteins and number of peptides

Looking at the number of peptides found in the extracts with different detergents (see Figure 19) extracts containing no detergent or only 0.6 M urea were most effective. Interestingly those extracts with 6 M urea or a combination of urea plus DTT resulted in at least 8 peptides less or in case of the combination 6 M urea plus 50 mM DTT even half the number of peptides. Especially for the egg and hazelnut peptides the found number was reduced dramatically. It seemed that the addition of too much reducing and denaturation agent hindered a proper ionisation during LC-MS measurement. Due to that reason can be assumed, that the protein content of the extracts, measured with Bradford assay, was actually about 10 % higher in those extracts with 6 M urea, 0.6 M urea plus 50 mM DTT and 6 M urea plus 50 mM DTT.

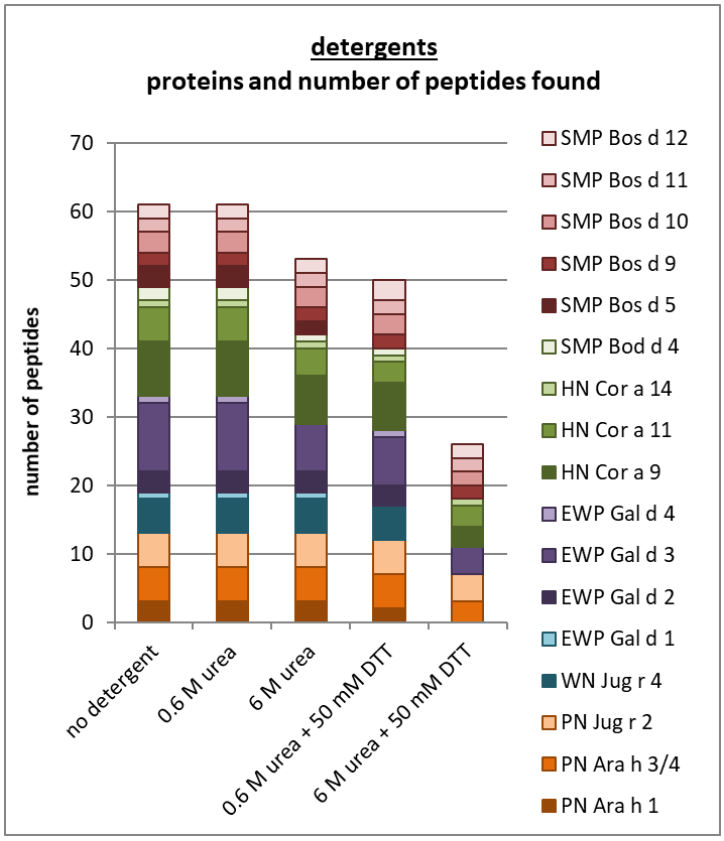


Figure 19: Comparison of the number of peptides related to its protein found in extracts with different detergents. (n=3)

Intensities

In the following Figure 20-Figure 24 the intensities (peak area of the TIC see chapter 2.8) from the MRM-spectra are plotted for the found peptides.

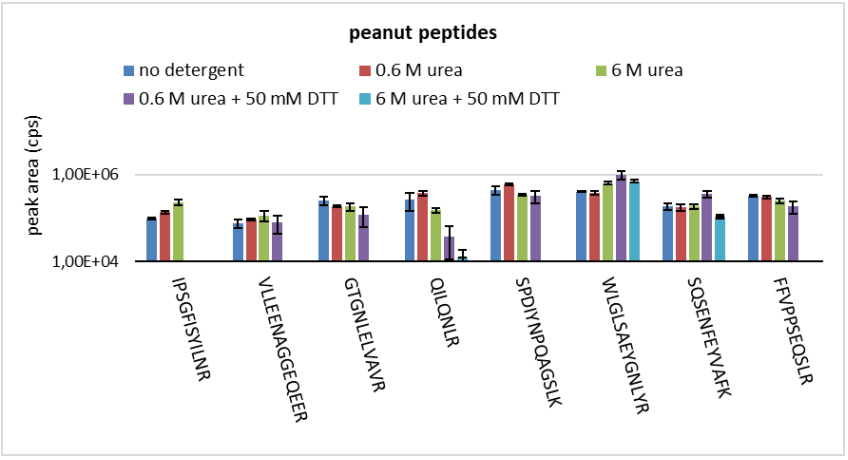


Figure 20: Comparison of the peak areas of peanut protein peptides measured in the found in extracts with different detergents. (n=3)

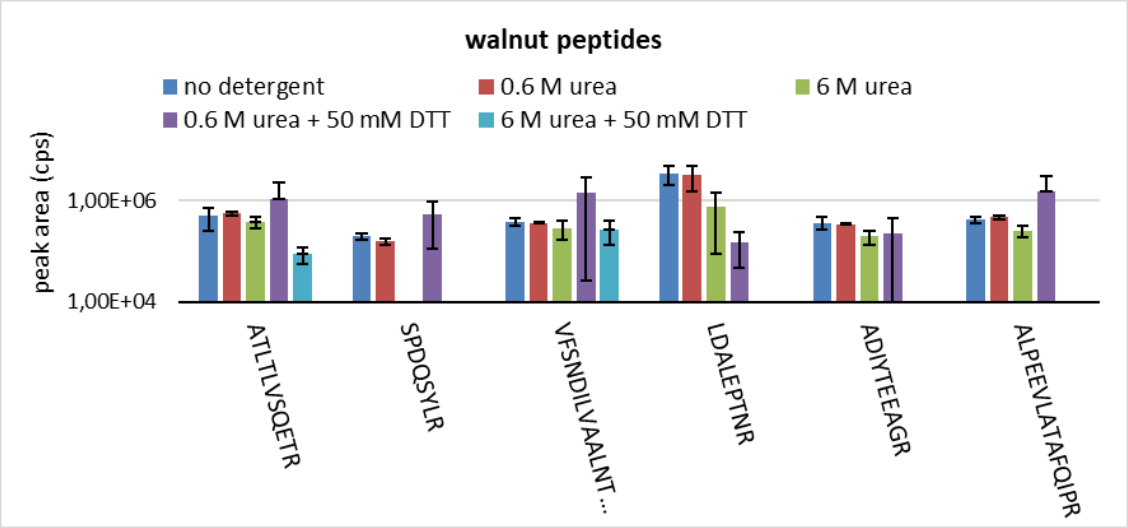


Figure 21: Comparison of the peak areas of walnut protein peptides measured in the found in extracts with different detergents. (n=3)

In the case of WN, the TBB buffer containing 0.6 M urea plus 50 mM DTT resulted in the highest peak areas for the found peptides. It is also stated in literature, that addition of reducing and denaturating agent, especially in the case of nuts, enhances protein extraction. In contrary, for the peptides found for hazelnut, the addition of urea and DTT did not result in higher peak areas for the measured peptides. The lower concentration of urea worked more effective than higher concentration.

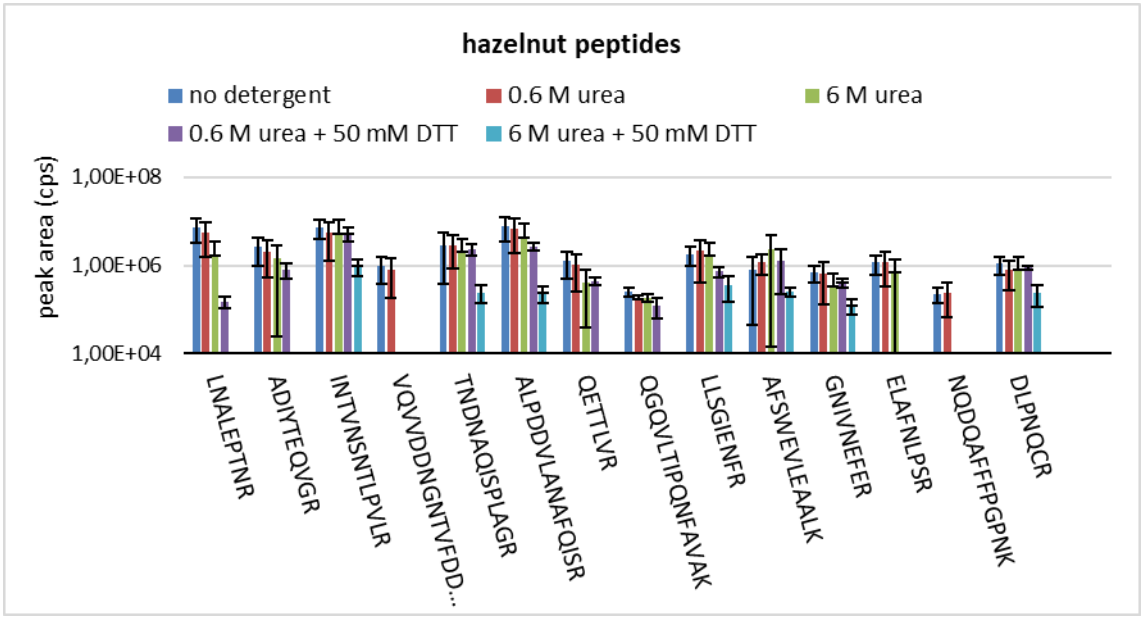


Figure 22: Comparison of the peak areas of hazelnut protein peptides measured in the found in extracts with different detergents. (n=3)

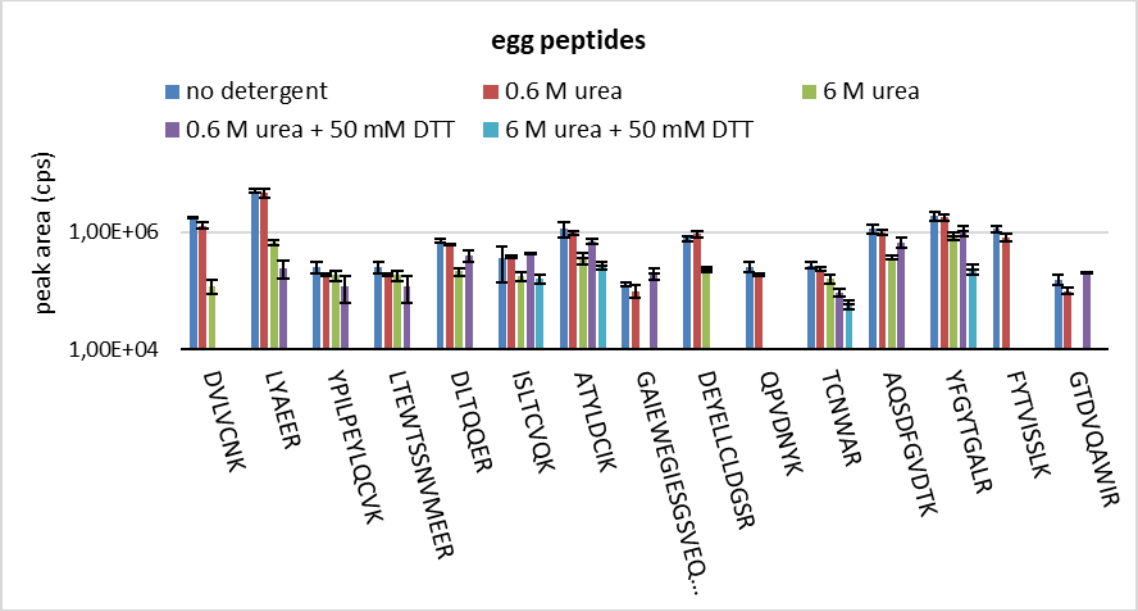


Figure 23: Comparison of the peak areas of egg protein peptides measured in the found in extracts with different detergents. (n=3)

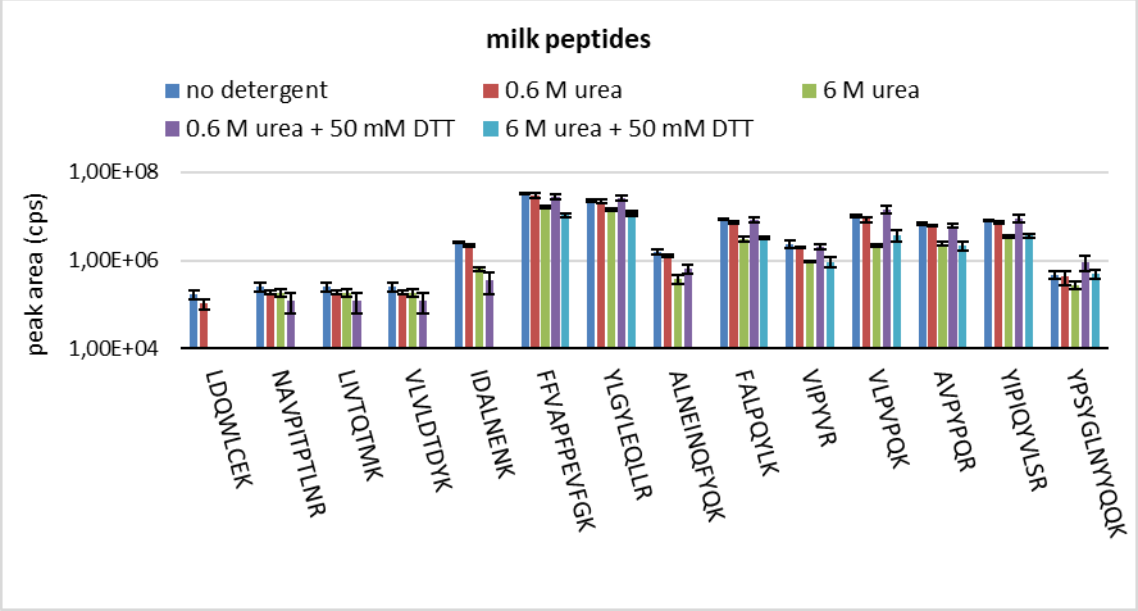


Figure 24: Comparison of the peak areas of milk protein peptides measured in the found in extracts with different detergents. (n=3)

Looking at the results for the found egg peptides in Figure 23, in extracts with 6 M urea peptide detectability was highly reduced, for some peptides even for a factor of 10. No detergent or only 0.6 M urea in the buffer gave the best results for the found peptides, and even more peptides were found in those extracts for egg. In the case of milk peptides, it was supported, that the high urea concentration was inhibitory for LC-MS analysis. Again, no detergent and only 0.6 M urea gave most

promising results. As literature supports the fact, that denaturating agents support protein extraction and results showed, that adding 0.6 M urea helped especially in the case of PN peptides but did not have an inhibitory effect for the detection of WN, HN, EWP and SMP, for the next step TBB buffer with 0.6 M urea was used.

3.2.3 Extraction time and Extraction temperature

The combinatory effect of extraction time and temperature for protein extraction was evaluated in this experiment.

Extraction times:	**Extraction temperatures:**
IV. 15 min	d) RT (22 °C)
V. 30 min	e) 37 °C
VI. 60 min	f) 60 °C

Proteins and number of peptides

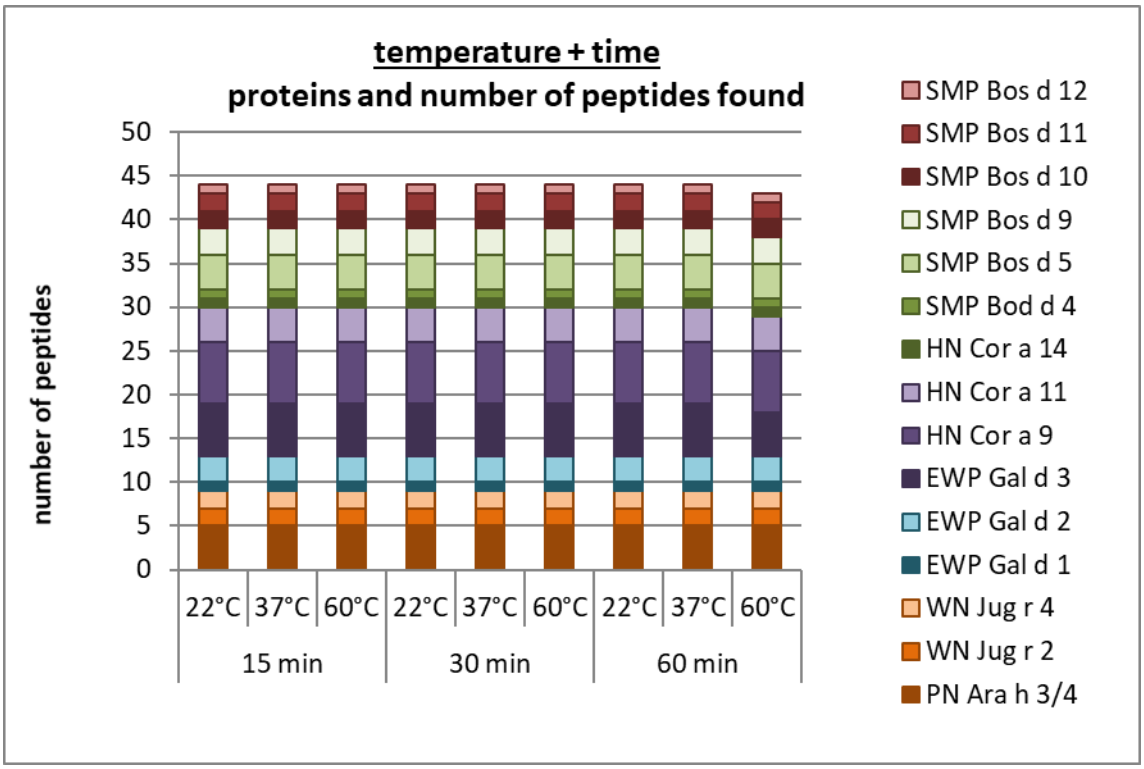


Figure 25: Comparison of the number of peptides related to its protein found in extracts of different extraction temperatures and extraction times. (n=3)

No difference was identified for the found number of peptides and proteins as shown in Figure 25.

Intensities

In the following Figure 26-Figure 30 the intensities (peak area of the TIC see chapter 2.8) from the MRM-spectra were plotted for the found peptides. All detectable peptides of PN, HN, WN, EWP and SMP were found in all combinations. In total 44 peptides were detected. The assumption was, that a longer extraction time and a higher extraction temperature will enhance the extractability of the proteins, especially in the case of HN, WN and PN.

But this assumption was not met, main reason might be, that the matrix that was used in this work, was a starch containing matrix that has not been baked or heat treated, so the proteins were better available for the extraction.

For milk and egg peptides, longer extraction duration had a negative effect, as can be seen in Figure 29 and Figure 30. Therefore, the decision was drawn for extraction of proteins for the tryptic digest optimisation for an extraction time of 30 minutes and 37 °C. For this matrix also 15 worked properly, but in regards of applying this method also to other matrices in the future, 30 minutes were chosen.

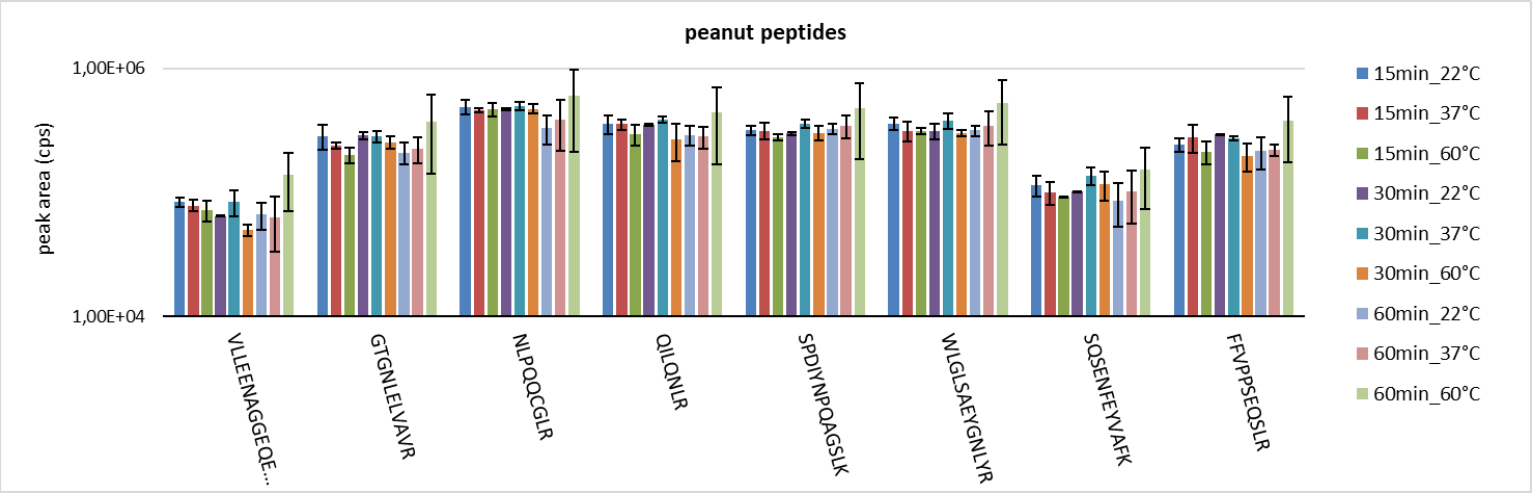


Figure 26: Comparison of the peak areas of peanut protein peptides measured in the found in extracts with extracts of different extraction temperatures and extraction times. (n=3)

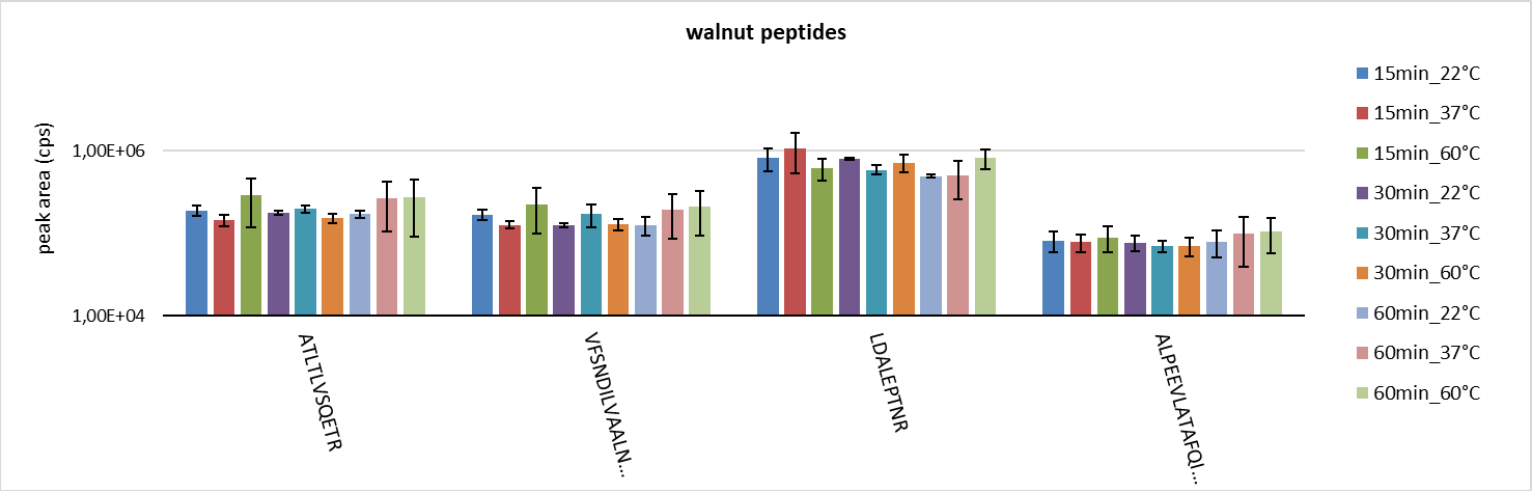


Figure 27: Comparison of the peak areas of walnut protein peptides measured in the found in extracts with extracts of different extraction temperatures and extraction times. (n=3)

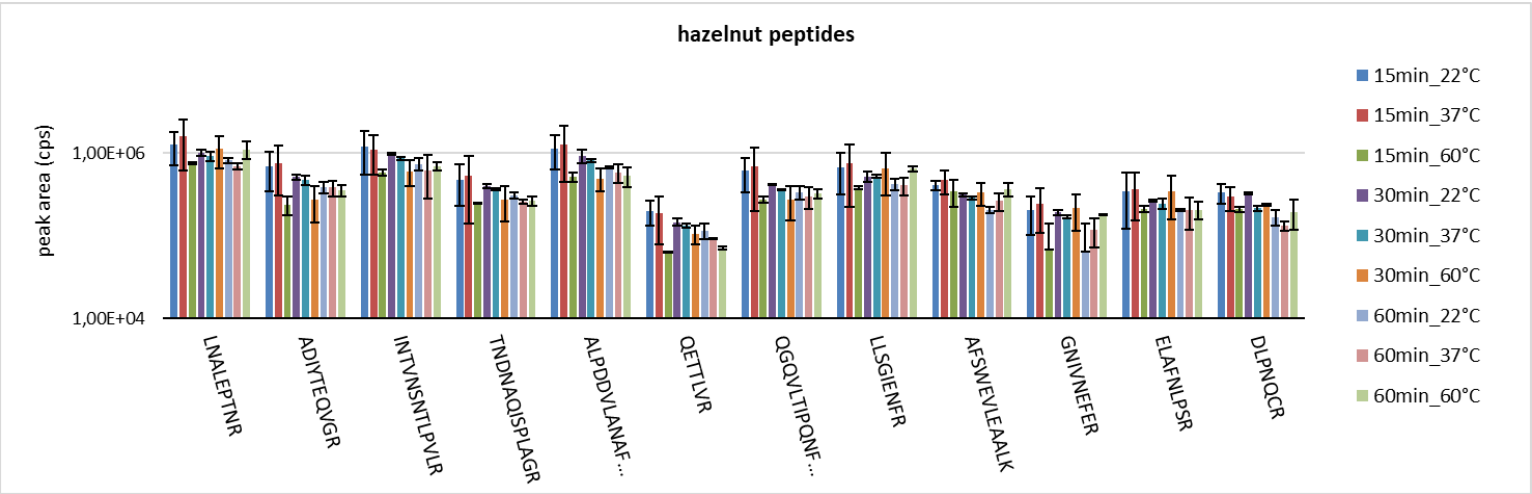


Figure 28: Comparison of the peak areas of hazelnut protein peptides measured in the found in extracts with extracts of different extraction temperatures and extraction times. (n=3)

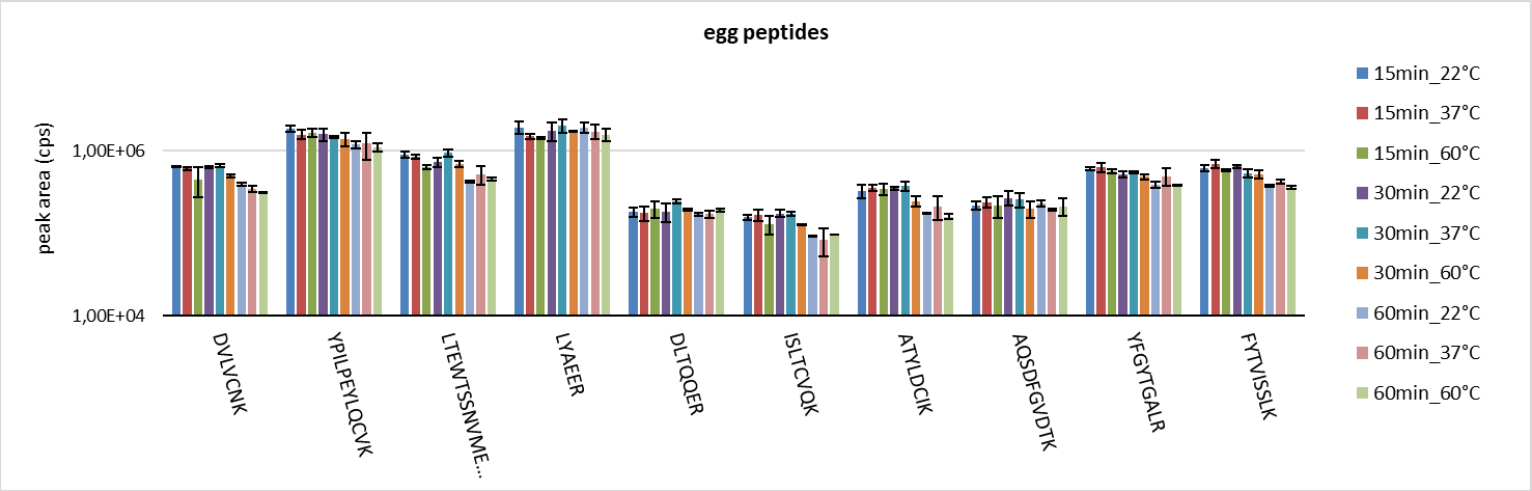


Figure 29: Comparison of the peak areas of egg protein peptides measured in the found in extracts with extracts of different extraction temperatures and extraction times. (n=3)

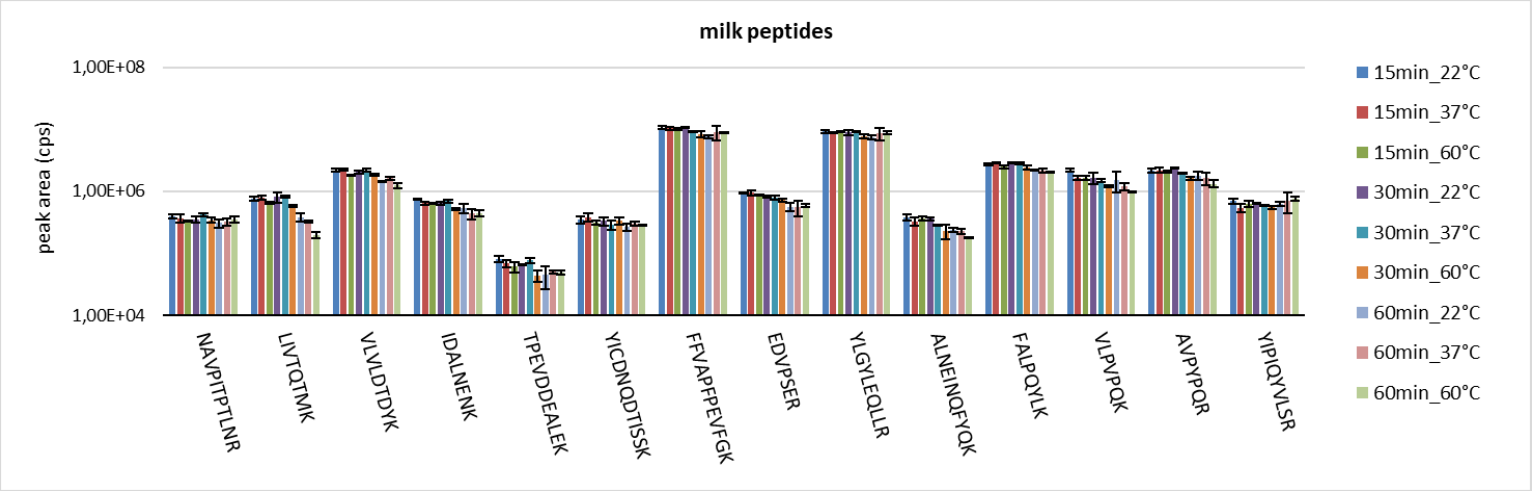


Figure 30: Comparison of the peak areas of milk protein peptides measured in the found in extracts with extracts of different extraction temperatures and extraction times. (n=3)

3.2.4 Sample-Buffer-Ratio

To determine the best sample-buffer-ratio which gives the highest peptide peak areas in the extracts, four different sample-buffer ratios were tested: 1:5, 1:10, 1:20, 1:40. TBB buffer with 0.6 M urea was used and samples were extracted for 30 minutes at a temperature of 37 °C.

Proteins and number of peptides

For the sample-buffer-ratio of 1:5 two additional peptides were found, but this did not weigh to strong, as it was only one peptide for Ara h 3/4 and one peptide for Ara h 1 in comparison to the sample-buffer-ratio of 1:10. As expected, the number of found peptides for the sample-buffer-ratio of 1:40 was the lowest. The reduction of possible matrix effects due to a higher sample-buffer-ratio did not apply, as just a very low amount of protein was extracted.

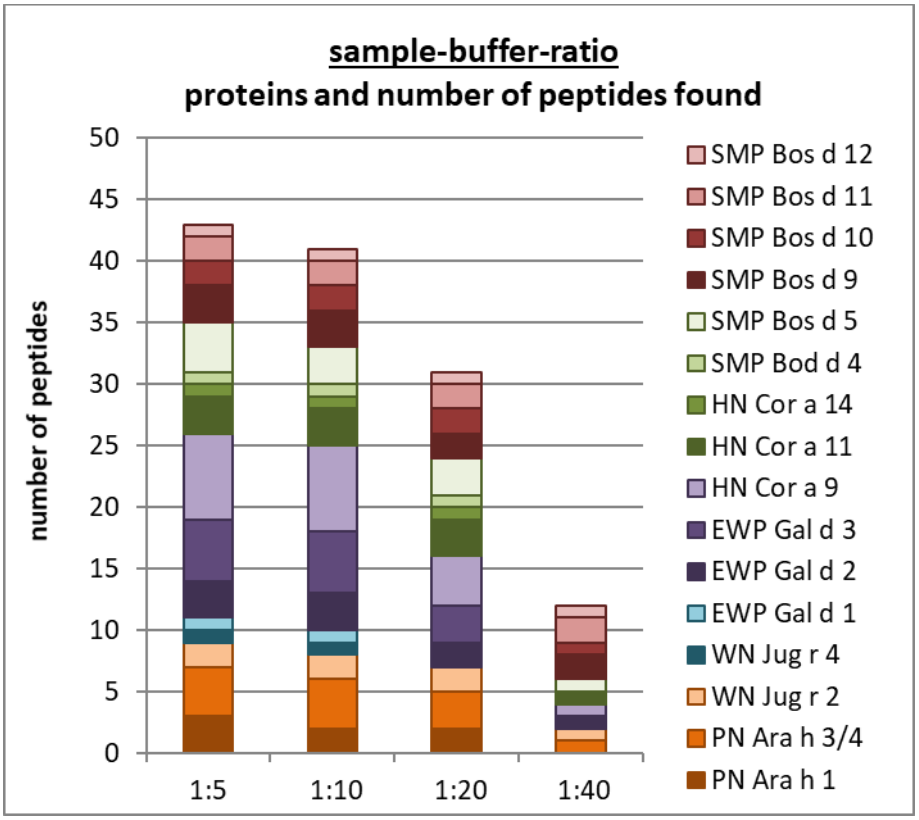


Figure 31: Comparison of the number of peptides related to its protein found in extracts of different sample-buffer-ratios. (n=3)

Intensities / Peak Areas

In the following Figure 32-Figure 36 the intensities (peak area of the TIC see chapter 2.8) from the MRM-spectra are plotted for the found peptides in the different sample-buffer-ratios.

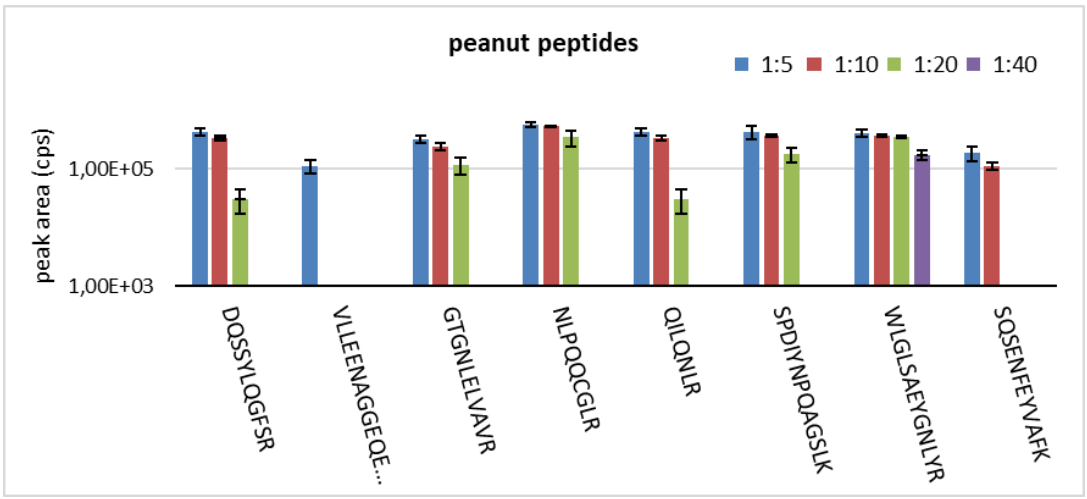


Figure 32: Comparison of the peak areas of peanut protein peptides measured in the found in extracts with extracts of different sample-buffer-ratios. (n=3)

Peak intensities in the case of PN and WN did not show any difference for the sample-buffer-ratios of 1:5 and 1:10. In case of the 1:20 sample-buffer-ratio, some peptides ATLTLVSQETR and VFSNDILVAALNTPR from walnut and WLGLSAEYGNLYR and NLPPQQCGLR from peanut gave as good results as for the lower ratios. In comparison for the other 6 peptides of peanut peak areas were significantly weaker or even not present.

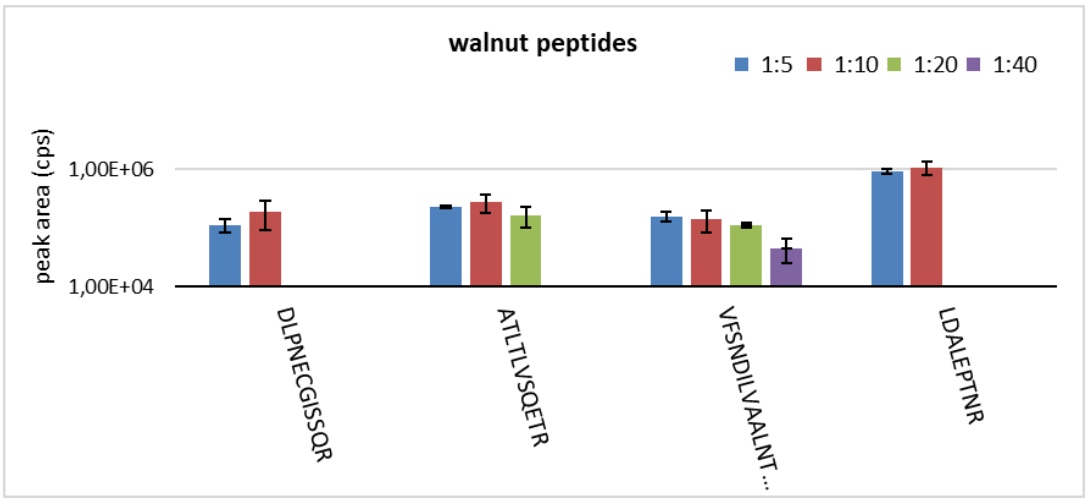


Figure 33: Comparison of the peak areas of walnut protein peptides measured in the found in extracts with extracts of different sample-buffer-ratios. (n=3)

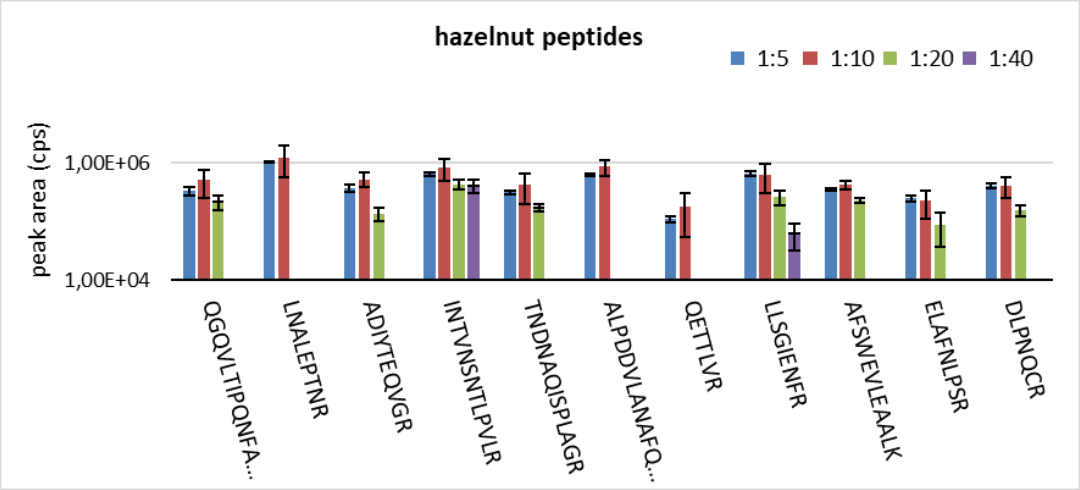


Figure 34: Comparison of the peak areas of hazelnut protein peptides measured in the found in extracts with extracts of different sample-buffer-ratios. (n=3)

For the peptides of HN, EWP and SMP sample-buffer-ratios of 1:5 and 1:10, as for WN and PN, resulted in highest peak area values. For HN (LNALEPTNR, ALPDDVLANAFQISR, QETTLVR) and EWP (DVLVCNK, LYAEER, DLTQQER, FYTVISSLK) some peptides were only found in the sample-buffer-ratio of 1:5 and 1:10 with comparable peak area values in these two ratios.

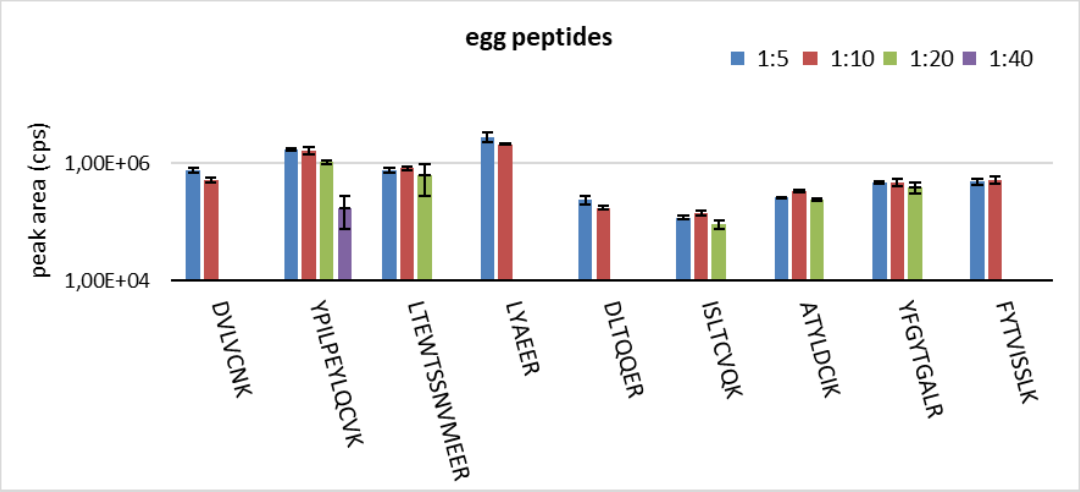


Figure 35: Comparison of the peak areas of egg protein peptides measured in the found in extracts with extracts of different sample-buffer-ratios. (n=3)

The best compromise over all five allergenic ingredients was a sample-buffer-ratio of 1:10.

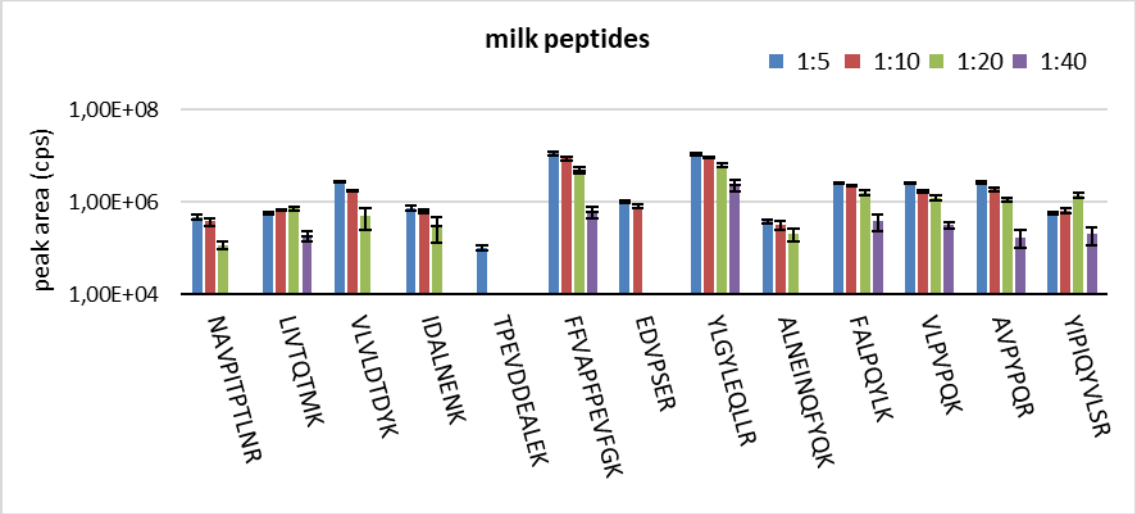


Figure 36: Comparison of the peak areas of milk protein peptides measured in the found in extracts with extracts of different sample-buffer-ratios. (n=3)

3.3 Tryptic digest optimisation

3.3.1 Trypsin brand

In the first experiment two different brands of Trypsin were tested. In a recent publication the authors concluded that Sequencing Grade Trypsin from Promega (# V5111) showed the best overall performance out of 6 brands tested (Burkhart et al. 2012) (129). Therefore, Sequencing Grade Trypsin from Promega was compared to proteomics grade trypsin from Sigma which was not part of the above-mentioned study.

Proteins and number of peptides

The total number of found proteins and peptides thereof, found in the samples digested with Trypsin, Proteomics Grade BioReagent Dimethylated (Sigma # T6567) and Trypsin Sequencing Grade Modified (Promega # V5111) are presented in Figure 37. In the digest performed with the Trypsin from Promega 2 additional peptides were found for the protein Bos d 6 and one more peptide for Cor a 10. In all samples the same proteins were found.

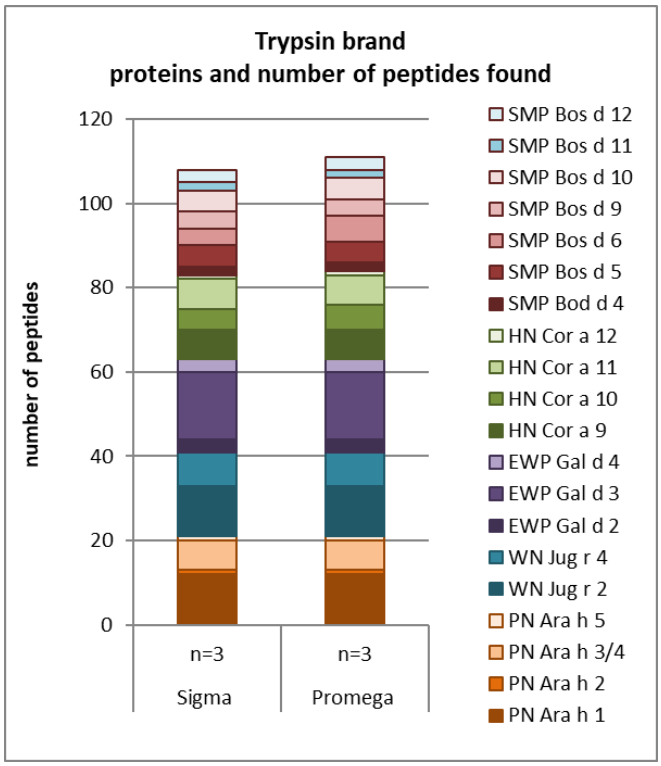


Figure 37: Comparison of the number of peptides related to its protein measured in the digests of two different Trypsin brands.

Intensities / Peak Areas

No significant trend for one or the other trypsin brand in peak area was detectable. Data is presented in Figure 38-Figure 42. As the Trypsin of Promega is also cheaper than the one of Sigma, the Trypsin of Promega was used for all further experiments.

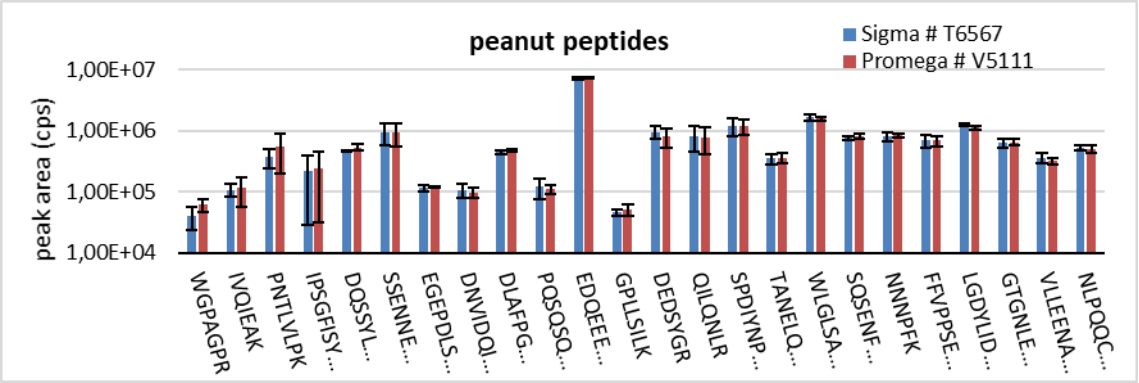


Figure 38: Comparison of the peak areas of peanut protein peptides measured in the digests of two different Trypsin brands. (n=3)

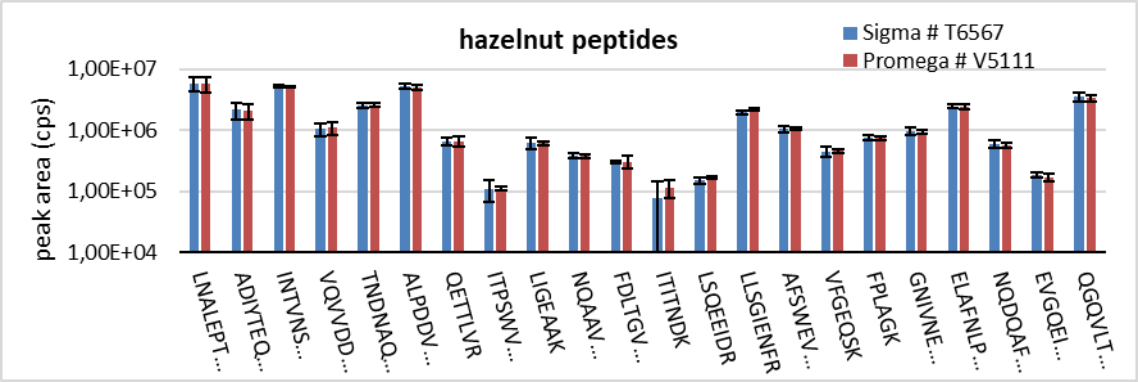


Figure 39: Comparison of the peak areas of hazelnut protein peptides measured in the digests of two different Trypsin brands. (n=3)

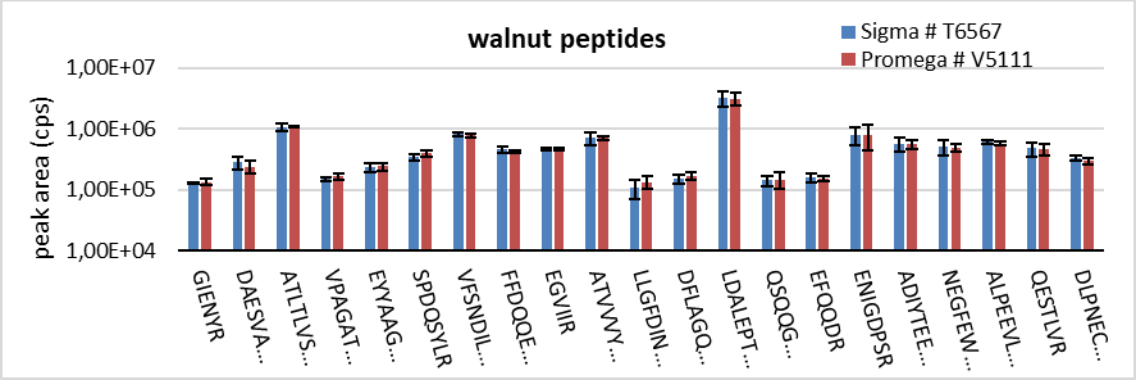


Figure 40: Comparison of the peak areas of walnut protein peptides measured in the digests of two different Trypsin brands. (n=3)

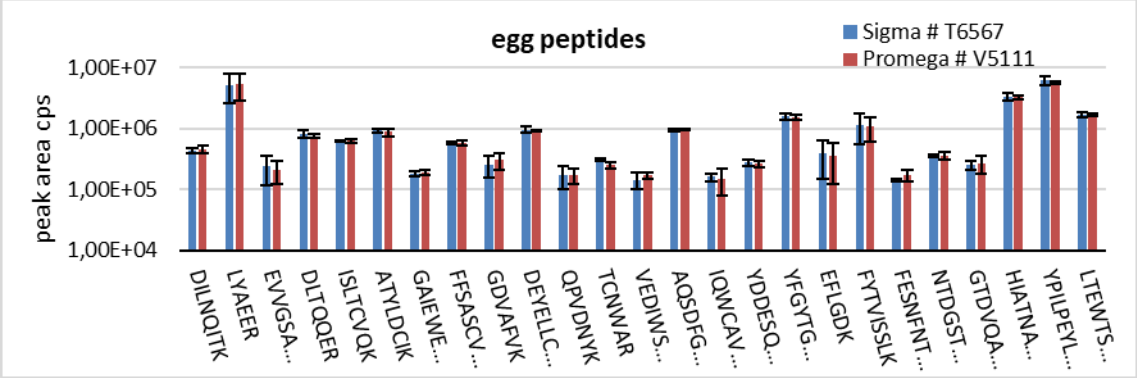


Figure 41: Comparison of the peak areas of egg protein peptides measured in the digests of two different Trypsin brands. (n=3)

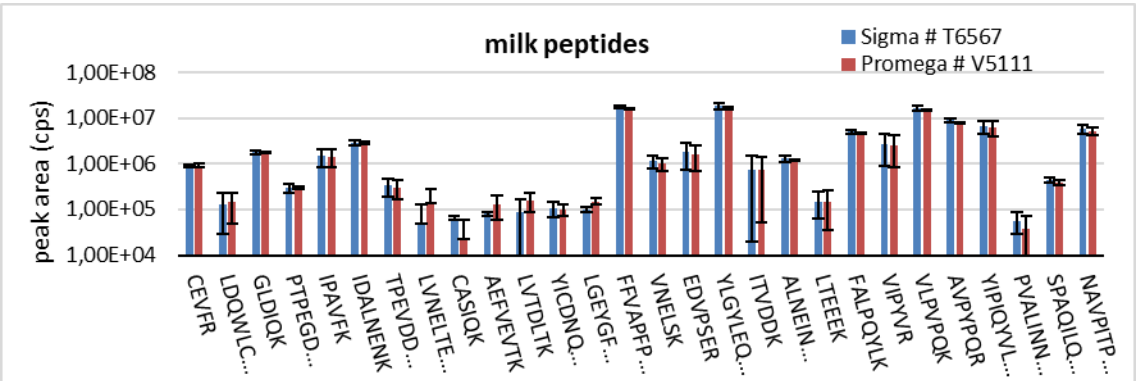


Figure 42: Comparison of the peak areas of milk protein peptides measured in the digests of two different Trypsin brands. (n=3)

3.3.2 Protease-to-protein ratio

The second series of experiments determined the appropriate protein-to-protease ratio used in the tryptic digestion protocol.

Proteins and number of peptides

The main difference in the number of found peptides related to the different protease-to-protein ratios (Figure 43) used for the tryptic digest was determined for the allergens of egg. Especially in the case of Gal d 3 the number of found peptides was reduced constantly when using less trypsin during the digestion of the sample. The trypsin concentration was important if a complete digest shall be achieved. This indicated, that peptides deriving from Gal d 3 might not be reliable as marker peptides for a later quantification approach. The same is true for Bos d 6, Bos d 4 and Bos d 10.

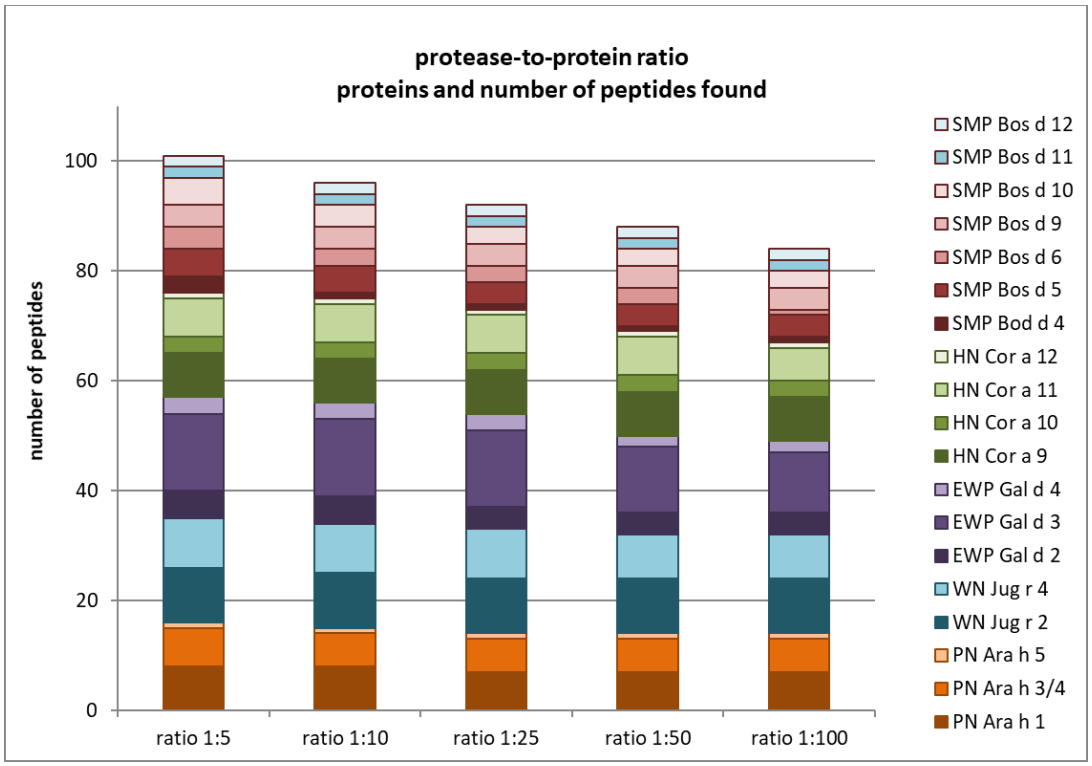


Figure 43: Comparison of the number of peptides related to its protein measured in the digests of 5 different protease-to-protein ratios. (n=3)

As number of detected peptides alone was not a single relevant factor, but also if those peptides that are found in all of the different protease-to-protein ratios differ in detected peak area. It has to be looked at the second evaluation step to be able to make a statement which of the ratios might be the best.

Intensities

The trend in Figure 44-Figure 47 shows that signal intensity raises with the higher protease concentration during the tryptic digest. For more than a third of the peptides the difference in signal intensity between a protease-to-protein ratio of 1:5 and 1:100 accounts more than 50 %, confirming that the trypsin concentration was relevant for a complete digest. A complete digest was a relevant factor when talking about quantification at minimum levels. The signal gained from using a ratio of 1:5 instead of 1:10 is overall not dominant.

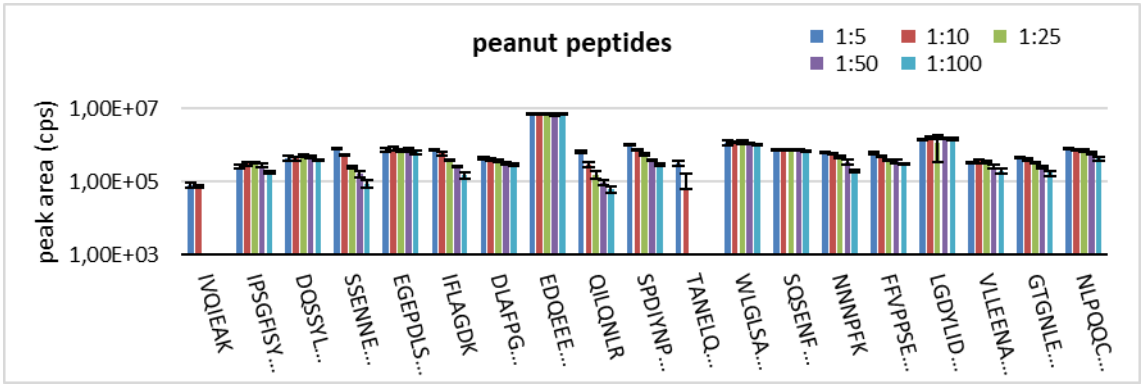


Figure 44: Comparison of the peak areas of peanut protein peptides measured in the digests of 5 different protease-to-protein ratios. (n=3)

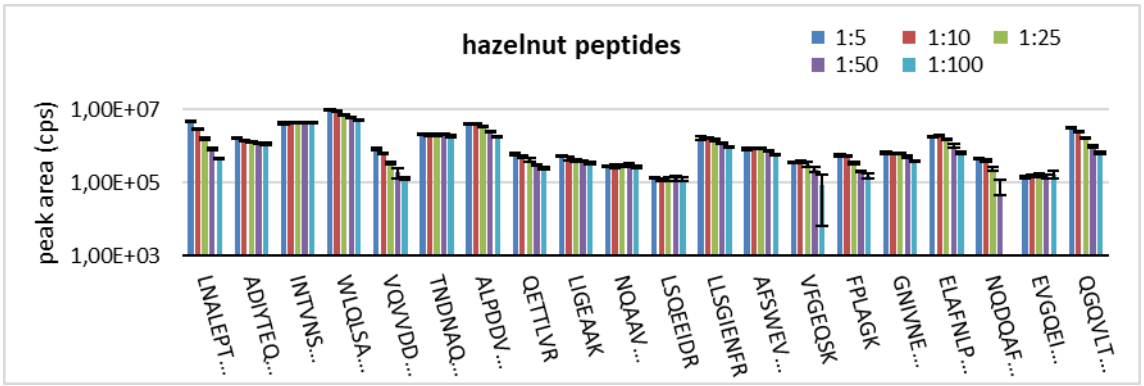


Figure 45: Comparison of the peak areas of hazelnut protein peptides measured in the digests of 5 different protease-to-protein ratios. (n=3)

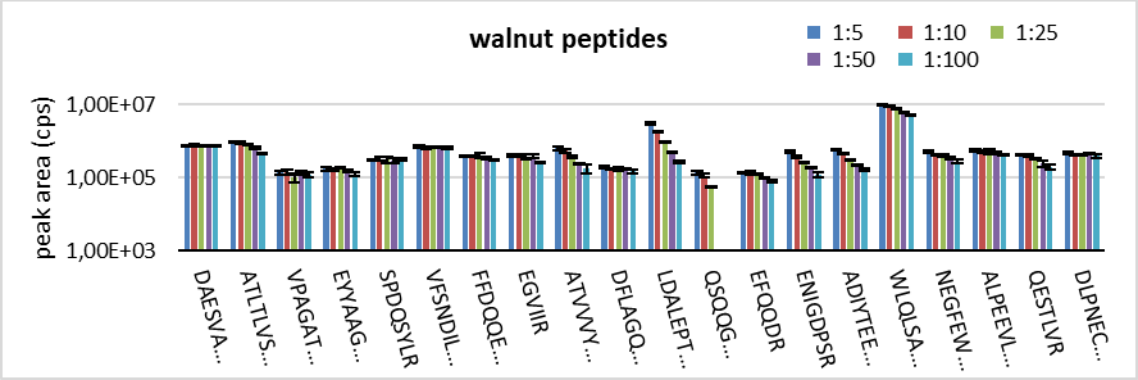


Figure 46: Comparison of the peak areas of walnut protein peptides measured in the digests of 5 different protease-to-protein ratios. (n=3)

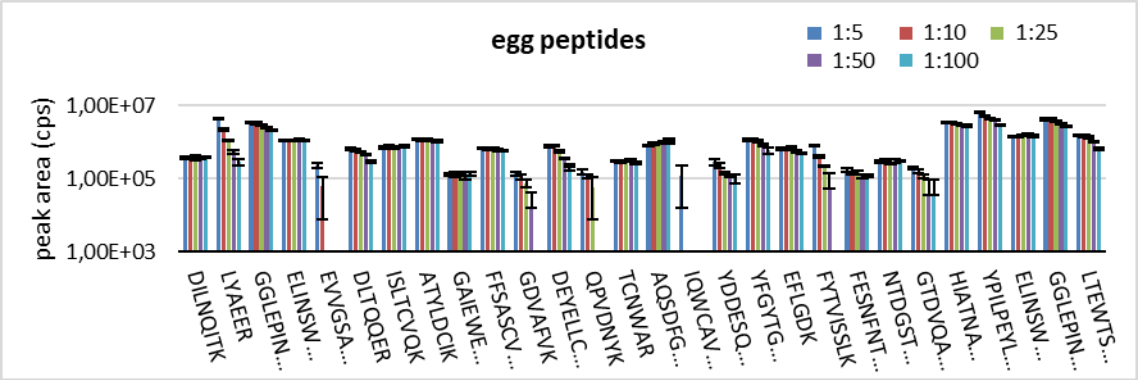


Figure 47: Comparison of the peak areas of egg protein peptides measured in the digests of 5 different protease-to-protein ratios. (n=3)

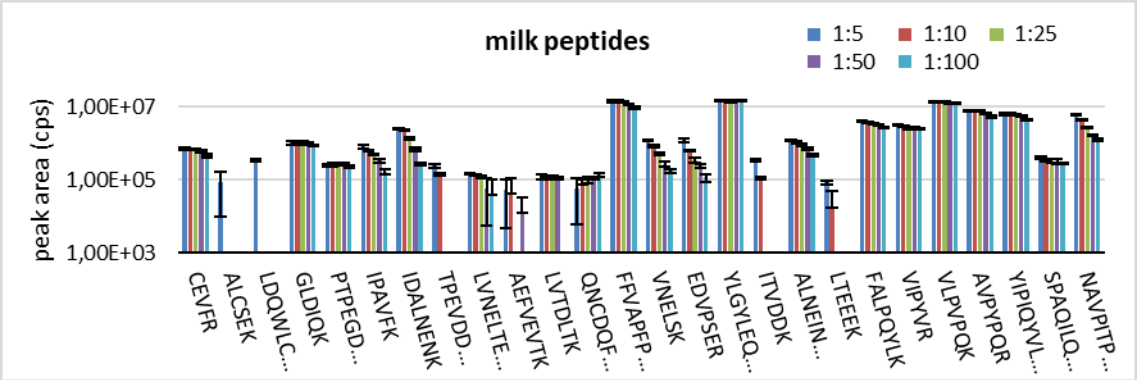


Figure 48: Comparison of the peak areas of milk protein peptides measured in the digests of 5 different protease-to-protein ratios. (n=3)

3.3.3 Incubation temperature

Two different temperatures were tested. 37 °C as this is the optimum for most standard applications and is also given as optimum temperature for the used trypsin by the provider (PROMEGA). As the trypsin activity in aqueous buffers declines sharply above 60 °C, temperatures below this value were tested. Additionally, two protease-protein-ratios (1:5 and 1:10) were analysed as peak intensities in the previous stage were quite similar and to test if a higher temperature may allow applying less trypsin. Tryptic digestion was performed as described above.

Proteins and number of peptides

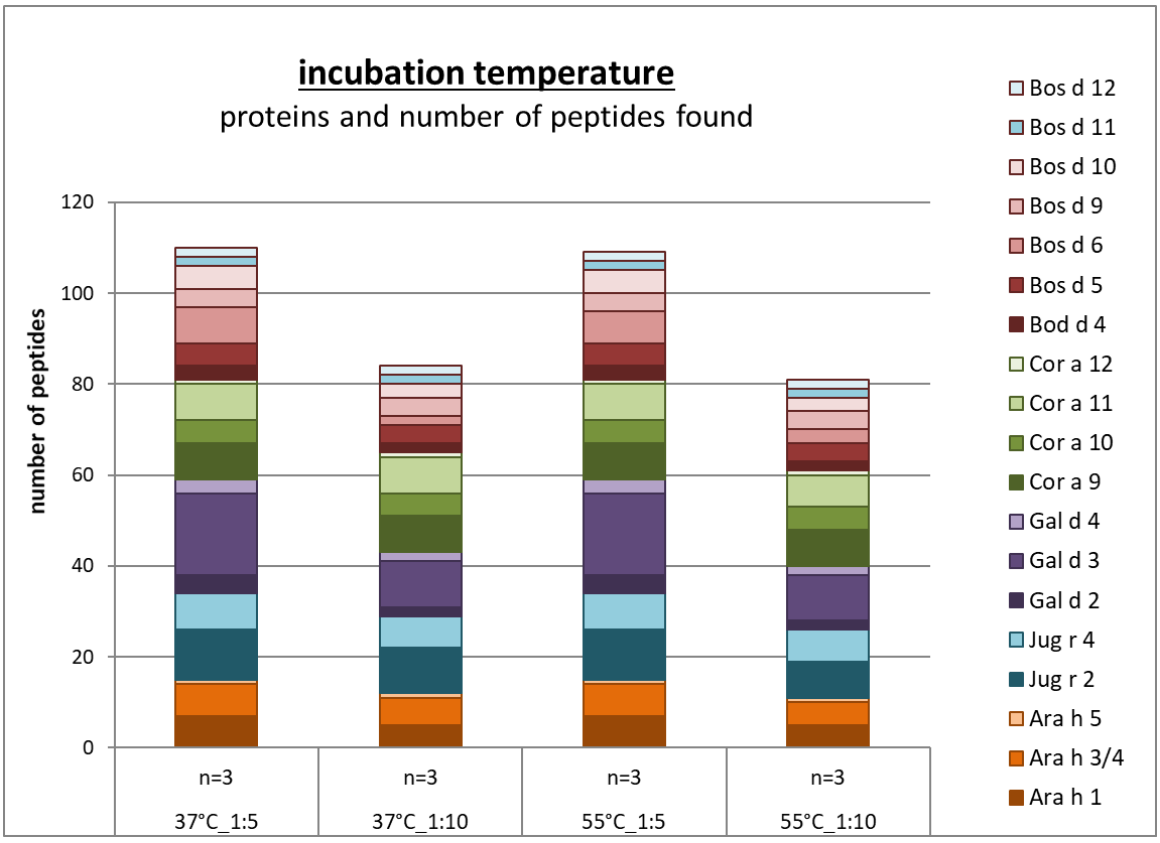


Figure 49: Comparison of the number of peptides related to its protein measured in the digests of 2 different protease-to-protein ratios and two different temperatures.

Increasing the temperature from 37 °C to 55 °C did not improve trypsin digestion. The different protease-to-protein ratio showed a clear difference, which was similar to the previous stage. At the digests with a ratio of 1:5 nearly double the number of peptides for egg white powder and approximately 25 % more peptides for skimmed milk powder could be measured.

No difference in number of found proteins was visible.

Intensities

In the digests with a protease-to-protein ratio of 1:5 a significant gain in signal intensity was observed, see Figure 50-Figure 54, for most of the peptides even more than 50 %. Therefore, the decision was made for a ratio of 1:5 for the next experiment. Temperature did not make a big difference in signal intensity. Slight advantages in signal gain were on the side of 37 °C during tryptic digestion. As this was also the proposed optimum temperature of trypsin, 37 °C was the temperature of choice.

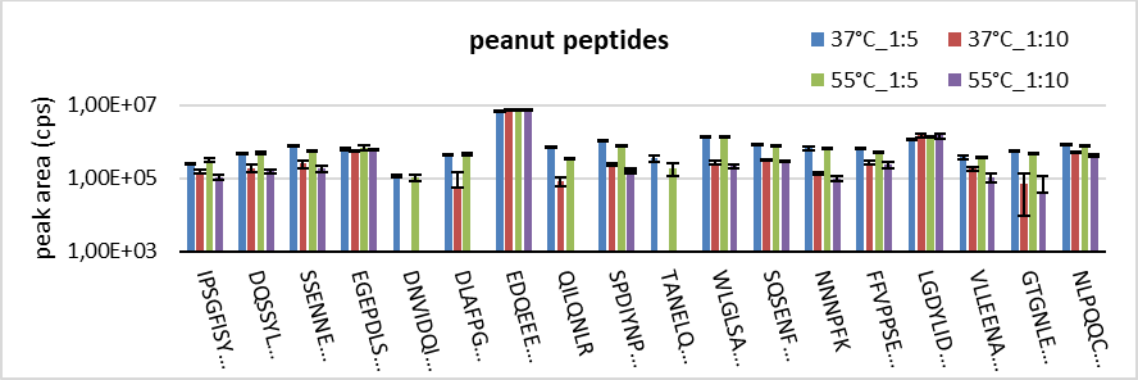


Figure 50: Comparison of the peak areas of peanut protein peptides measured in the digests of 2 different protease-to-protein ratios and two different temperatures. (n=3)

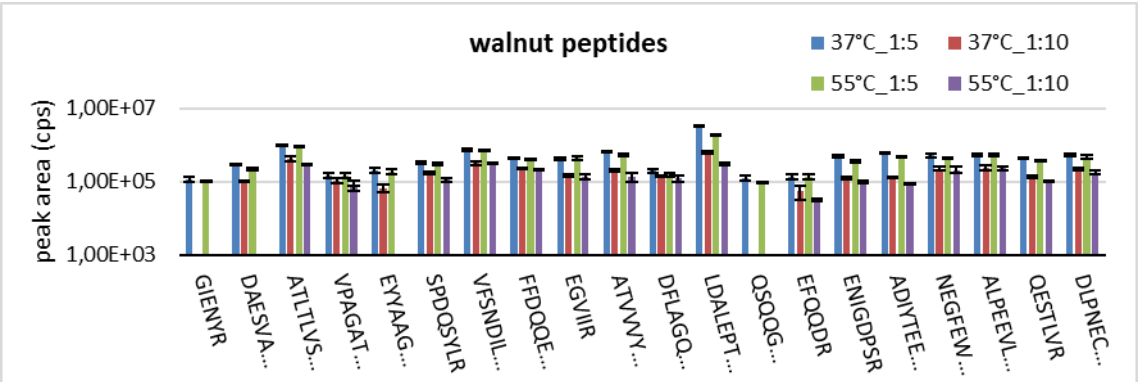


Figure 51: Comparison of the peak areas of walnut protein peptides measured in the digests of 2 different protease-to-protein ratios and two different temperatures. (n=3)

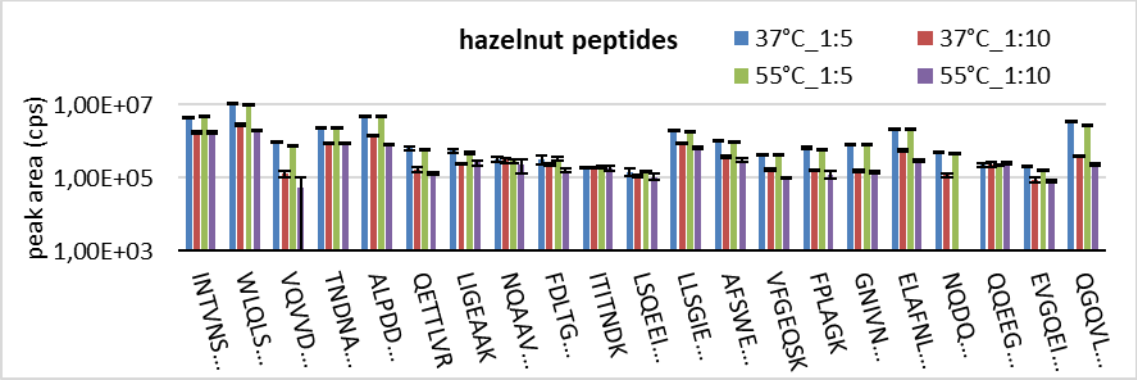


Figure 52: Comparison of the peak areas of hazelnut protein peptides measured in the digests of 2 different protease-to-protein ratios and two different temperatures. (n=3)

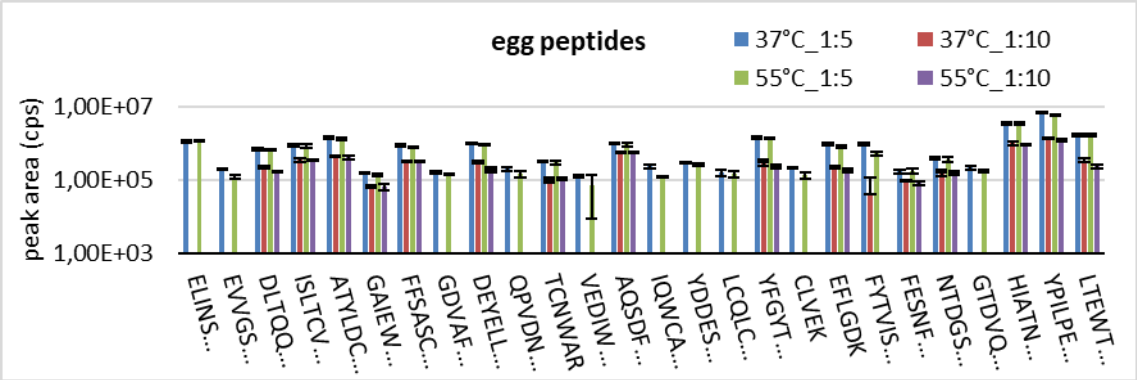


Figure 53: Comparison of the peak areas of egg protein peptides measured in the digests of 2 different protease-to-protein ratios and two different temperatures. (n=3)

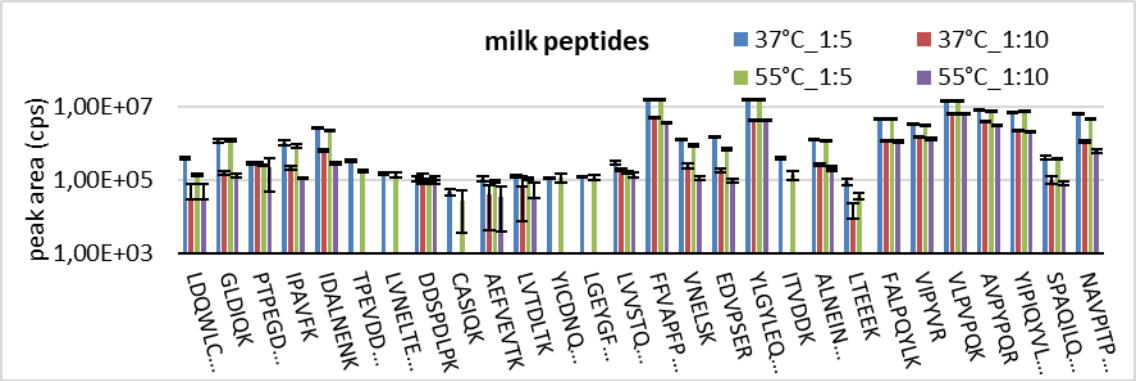


Figure 54: Comparison of the peak areas of milk protein peptides measured in the digests of 2 different protease-to-protein ratios and two different temperatures. (n=3)

3.3.4 Addition of Detergents

As described in literature there are some detergents which could have a positive effect on the protein digestion. Therefore, multiple detergents were tested.

- No detergent
- 0.1 % RapiGest SF (Waters)
- 0.04 % RapiGest SF (Waters)
- 0.5 % Sodium deoxycholate (SDC)
- 10 % Acetonitrile (ACN)

Proteins and number of peptides

The number of peptides found was very similar except for those tryptic digestions where Rapigest was added. A loss of peptides of all 5 allergenic commodities was observed resulting in around 20 peptides less in the 0.04 % Rapigest digest and even up to 40 peptides less in those digests with 0.1 % Rapigest.

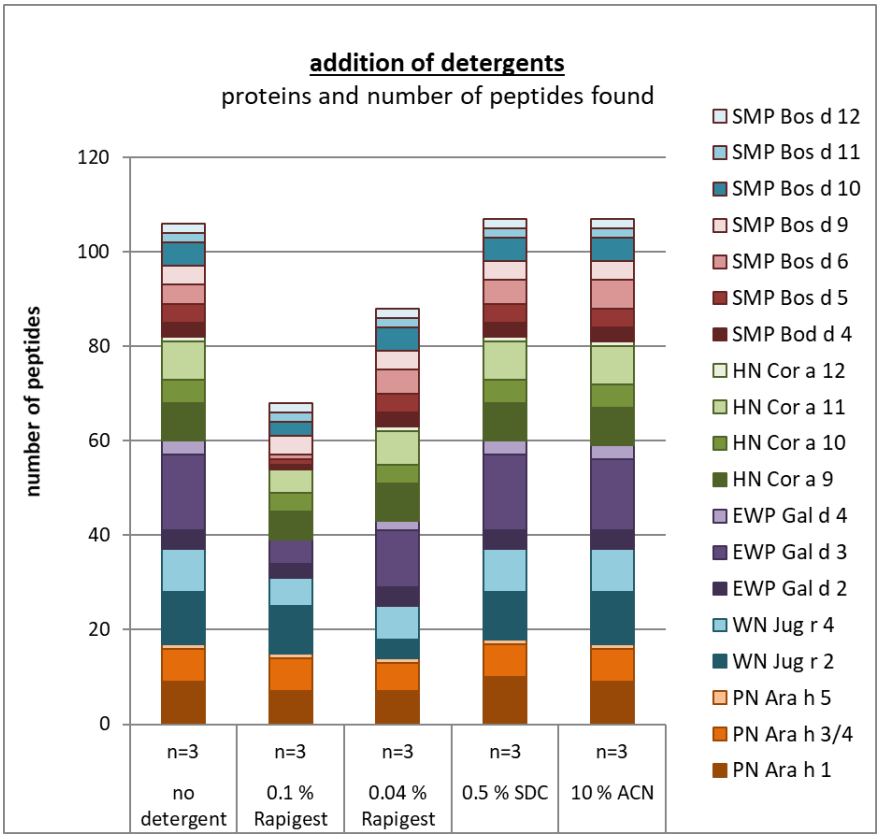


Figure 55: Comparison of the number of peptides related to its protein measured in the digests using different detergents.

Intensities

No significant trend could be observed when looking at the signal intensities for the different peptides with the different detergents added for tryptic digestion, see Figure 56-Figure 60. For peanut, hazelnut and walnut adding any detergent during tryptic digestion did not make any difference.

For the skimmed milk and egg white powder the digestions without detergent, with SDC or with ACN gave better signals and additional peptides compared to those with Rapigest.

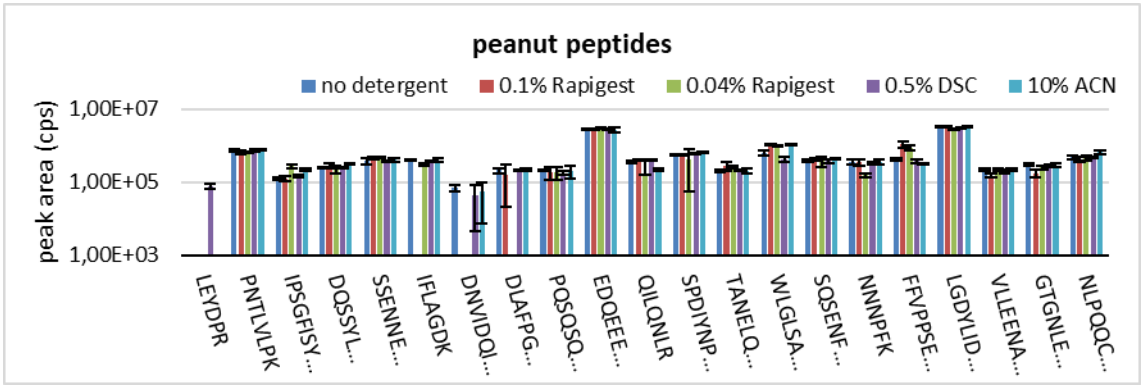


Figure 56: Comparison of the peak areas of peanut protein peptides measured in digests using different detergents. (n=3)

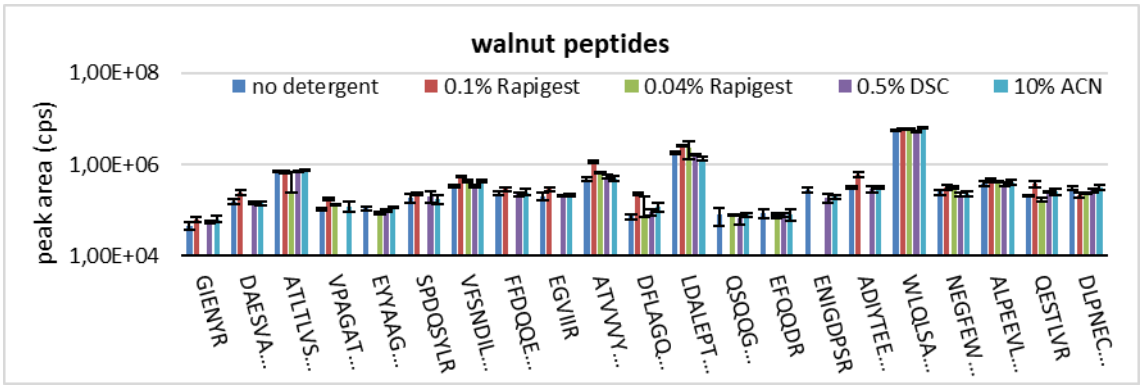


Figure 57: Comparison of the peak areas of walnut protein peptides measured in digests using different detergents. (n=3)

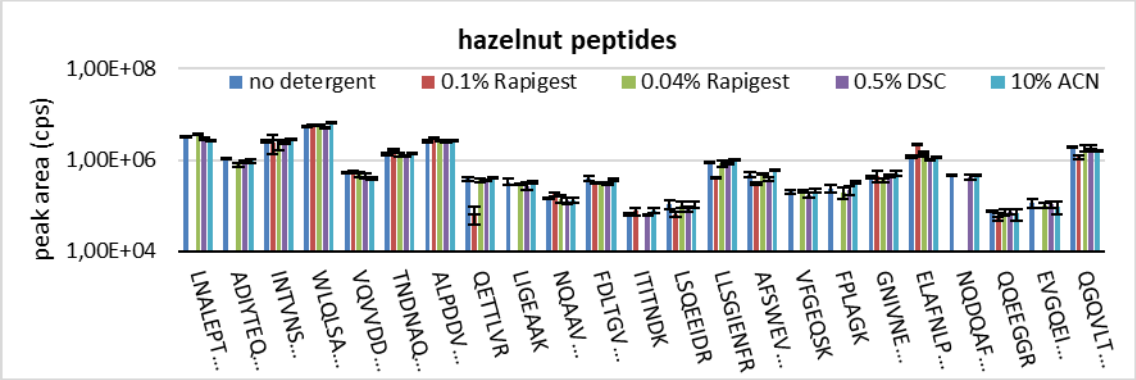


Figure 58: Comparison of the peak areas of hazelnut protein peptides measured in digests using different detergents. (n=3)

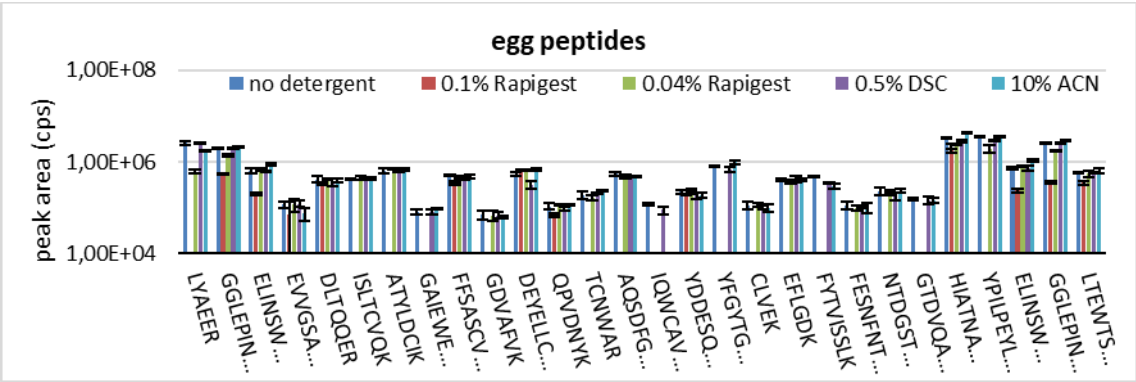


Figure 59: Comparison of the peak areas of egg protein peptides measured in digests using different detergents. (n=3)

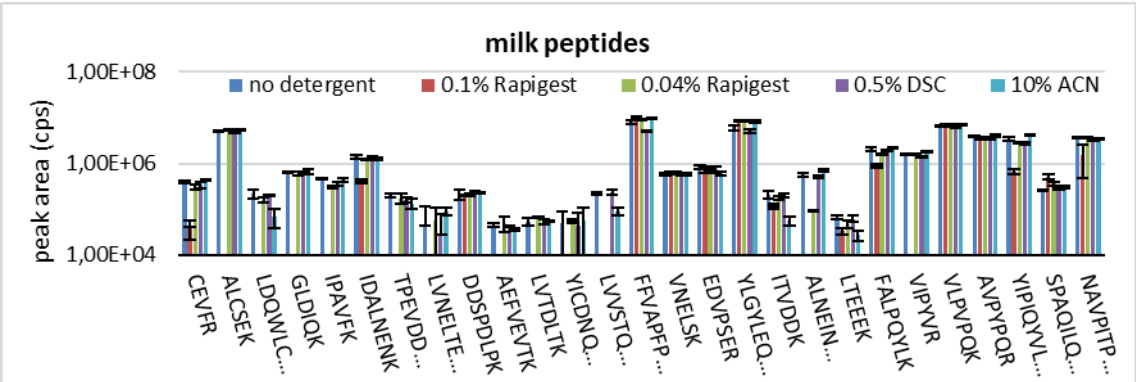


Figure 60: Comparison of the peak areas of milk protein peptides measured in digests using different detergents. (n=3)

3.4 Final extraction and tryptic digestion protocol

Extraction buffer:

- TBB: 8 mM Tris, 10 mM Borat + 0.6 M urea pH 8.5

Extraction procedure

- Weigh approximately 1.00 g of sample and add 10x volume of the extraction buffer, e.g. 1.02 g sample + 10.2 mL buffer. This was performed in 15 mL Falcons.
- Vortex to obtain a homogeneous slurry
- Incubate and rotate for 30 min at 37 °C using a rotary mixer (put rotary shaker in oven)
- Centrifuge for 30 min at 16,100 × g at 4 °C
- Collect the supernatant in 2 mL Eppis and freeze if not needed immediately

Digestion Buffers and solutions:

- ABC_{50}: 50 mM Ammonium bicarbonate
- DTT: 10 mM Dithiothreitol in ABC_{50}
- IAA: 55 mM Iodoacetamide in ABC_{50}
- Trypsin: Trypsin, Sequencing Grade Modified (Promega # V5111)
  0.25 µg/µL in 25 mM Ammonium bicarbonate
- Enolase: Massprep Enolase Digestion Standard (Fa. Waters) → 10 pmol/µL in ABC_{50}
- Fibrinopeptide: [Glu1]-Fibrinopeptide B Standard (Fa. Waters)
  2.5 µg/mL in milliQ water + 0.1 % formic acid (MS grade)

Digestion Procedure

- Apply 50 µL of extracted sample into a 500 µL Eppi
- If needed, add 10 µL of heavy peptide working mix otherwise add 10 µL of ABC_{50}
- Add 2 µL of the Enolase of a 10 pmol/µL solution
- Add 25 µL of DTT and incubate at 90 °C, 30 min in a thermocycler
- Add 25 µL of IAA and incubate at 25 °C, 30 min in the dark
- Add 8 µL of Trypsin resulting in a protease to protein ratio of about 1:10 by weight
- Incubate at 37 °C for 16 h in a thermocycler
- Add 1 µL of 50 % Formic acid in water (to stop trypsin)
- Add 4 µL of Fibrinopeptide-solution
- Transfer into a 1.5 mL glas vial equipped with a 100 µL insert for MS measurement
- Analyze samples

3.5 Possible Marker peptides – Refinement

The pool of the possible marker peptides listed in Table 33 was treated according to the criteria mentioned in the chapter 2.9 “Possible Marker peptide – Refinement 1”. Results of peptide peak areas from the LC-MS/MS measurements are presented in Figure 61-Figure 66. In respect to the uniqueness of the peptides for the allergenic proteins, a Uniprot-peptide-BLAST Search was performed for all 58 peptides. Peptides which did not meet the criteria of uniqueness were marked in blue. Not only the sensitivity is an important parameter for the selection of suitable peptides and transitions, but also their specificity. Therefore, specificity was checked against blank chocolate matrix. This was true for all the presented peptides.

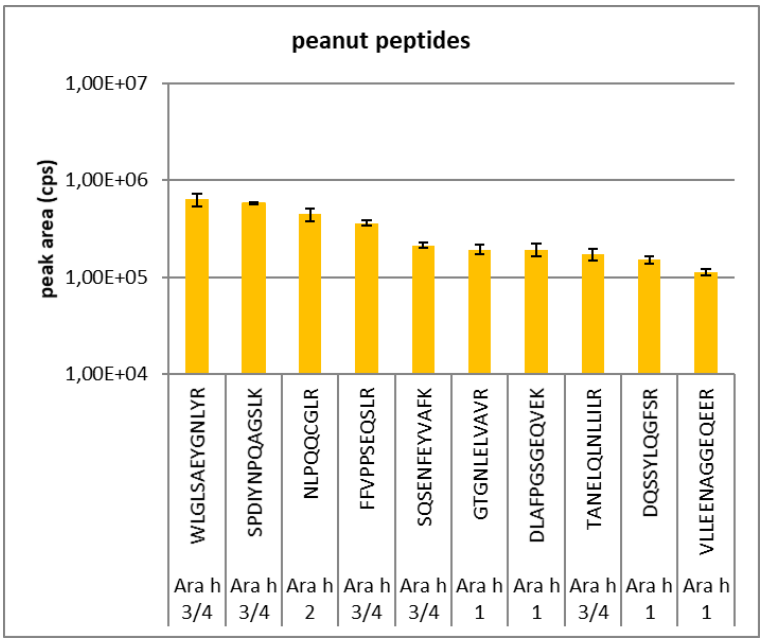


Figure 61: The yellow bars mark those peptides which were ordered as natural peptides for MS-parameter optimisation. The peptides marked in blue were not further investigated because they were not unique according to the Uniprot-peptide-BLAST.

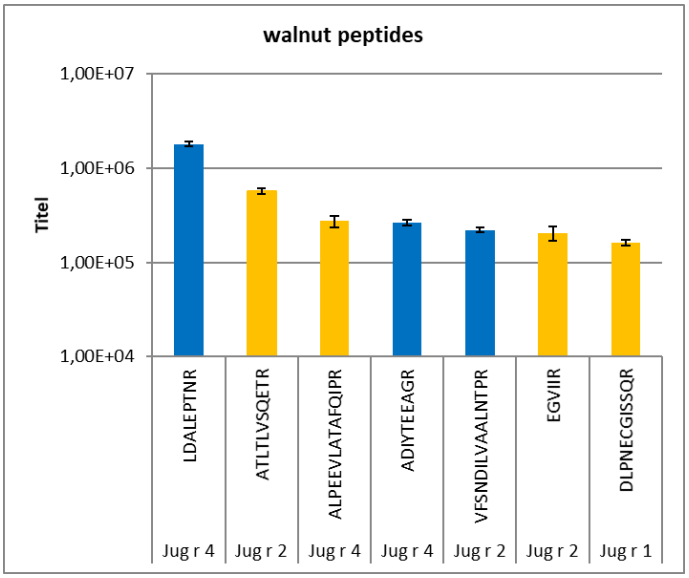


Figure 62: The yellow bars mark those peptides which were ordered as natural peptides for MS-parameter optimisation. The peptides marked in blue were not further investigated because they were not unique according to the Uniprot-peptide-BLAST.

Peptide LDALEPTNR (Jug r 4) (Figure 62) was deleted from the list, as it had only one amino acid substituted in comparison to peptide LNALEPTNR (Cor a 9), which was also refined from the list. Amino acid Aspartate (113.11 g/mol) and amino acid Asparagine (132.12 g/mol) differ on position y-8. This leads to a same set of the y-ions y1-y7. As also retention time in the extraction and tryptic digest optimisation experiments only differed by 0.4 minutes, as shown in Figure 63, partly coelution of the peptides can occur and therefore would influence quantitative results.

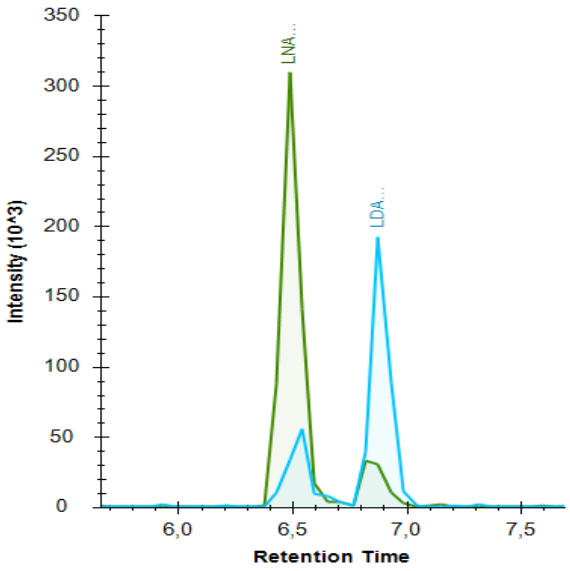


Figure 63: Retention times of the eluting peaks for peptide LDALEPTNR (Jug r 4) and peptide LNALEPTNR (Cor a 9).

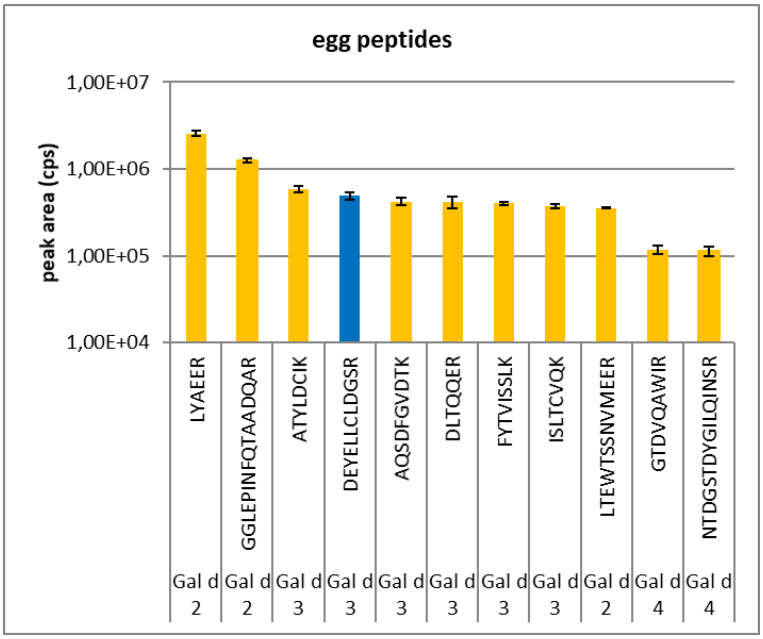


Figure 64: The yellow bars mark those peptides which were ordered as natural peptides for MS-parameter optimisation. The peptides marked in blue were not further investigated because they were not unique according to the Uniprot-peptide-BLAST.

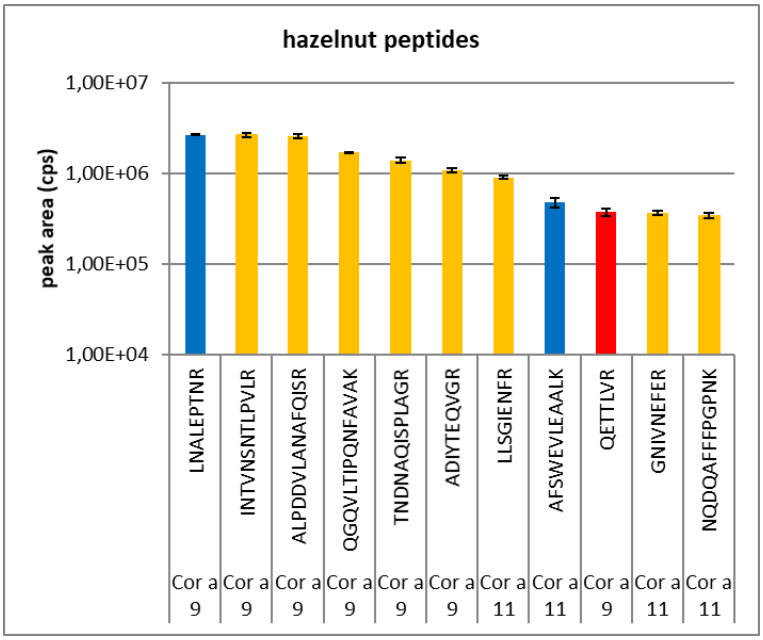


Figure 65: The yellow bars mark those peptides which were ordered as natural peptides for MS-parameter optimisation. The peptides marked in blue were not further investigated because they were not unique according to the Uniprot-peptide-BLAST. The peptide marked in red was not further investigated as already 5 peptides of Cor a 9 were in the list.

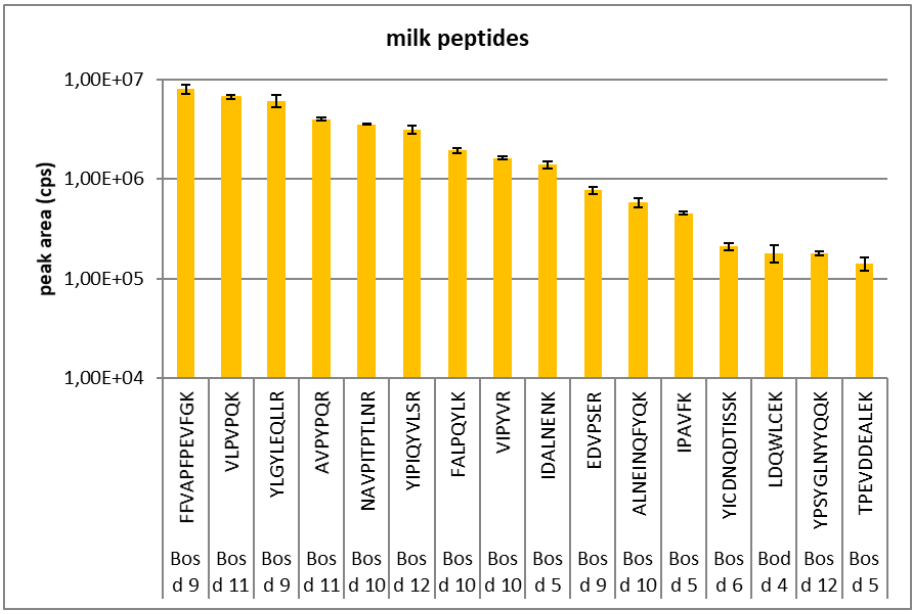


Figure 66: The yellow bars mark those peptides which were ordered as natural peptides for MS-parameter optimisation. The peptides marked in blue were not further investigated because they were not unique according to the Uniprot-peptide-BLAST.

3.6 MS-parameter Optimisation

Optimised MS-parameters were finally implemented in a first experiment to preliminary estimate LOD and LOQ reached for the method with different peptides measured in chocolate dessert. Results of the promising peptides which gave LODs and LOQs between 1 and 20 ppm are shown in Table 34. Those peptides were chosen for the final quantitative method validation.

Table 34: Preliminary determined LOD and LOD of the different peptides using optimised DP and CE values.

Tryptic peptide	LOD/LOQ	MW	Q1	Q3	Q1	Q3	DP	CE	Quantifier (Q)
	ppm	(Da)	(m/z)	(m/z)	ion	ion	(V)	(V)	Qualifier (q)
DLAFPGSGEQVEK		1375.7	688.8	930.5	$[M+2H]^{2+}$	y9	81.3	33.7	Q
LOD	< 1 ppm			833.4		y8	78.3		q
LOQ	3 ppm								
FFVPPSEQSLR		1305.7	653.8	913.5	$[M+2H]^{2+}$	y8	81.8	32.4	Q
LOD	5 ppm			816.4		y7	75.8		q
LOQ	16 ppm								
TANELNLLILR		1268.7	635.4	1097.7	$[M+2H]^{2+}$	y9	83.4	31.7	q
LOD	< 1 ppm			854.6		y7	71.4		q
LOQ	5 ppm			741.5		y6	71.4		Q
WLGLSAEYGNLYR		1540.8	771.4	1242.6	$[M+2H]^{2+}$	y11	93.4	36.6	Q
LOD	< 1 ppm			1072.5		y9	87.4		q
LOQ	1 ppm			785.4		y6	96.4		q
LLSGIENFR		1047.6	524.8	822.4	$[M+2H]^{2+}$	y7	63.4	27.7	Q
LOD	2 ppm			735.4		y6	63.4		q
LOQ	5 ppm			565.3		y4	66.4		q
ALPDDVLANAFQISR		1628.9	815.4	1019.6	$[M+2H]^{2+}$	y9	90.6	38.7	q
LOD	< 1 ppm			906.5		y8	81.6		Q
LOQ	1 ppm			835.4		y7	87.6		q
INTVNSNTLPVLR		1439.8	720.9	1013.6	$[M+2H]^{2+}$	y9	86.7	34.8	Q
LOD	< 1 ppm			899.5		y8	74.7		q
LOQ	1 ppm			484.3		y4	86.7		q
TIEPNGLLLPQYSNAPELIYIER		2642.4	881.8	1032.6	$[M+3H]^{3+}$	y8	104.4	45.6	Q
LOD	5 ppm			580.3		y4	104.1		q
LOQ	18 ppm								
ATLTLVSQETR		1217.7	609.8	832.5	$[M+2H]^{2+}$	y7	75.6	30.8	q
LOD	3 ppm			719.4		y6	69.6		Q

LOQ	13 ppm			620.3		y5	78.6		q
EGVIIR		685.4	343.7	500.4	$[M+2H]^{2+}$	y4	53.2	21.2	q
LOD	1 ppm			401.3		y3	50.2		Q
LOQ	2 ppm								
ALPDDVLATAFQIPR		1653.9	828.0	1016.6	$[M+2H]^{2+}$	y9	94.5	38.7	q
LOD	< 1 ppm			903.5		y8	97.5		Q
LOQ	1 ppm			832.5		y7	82.5		q
NAVPITPTLNR		1194.7	598.3	911.5	$[M+2H]^{2+}$	y8	68.7	30.4	Q
LOD	< 1 ppm*			701.4		y6	71.7		q
LOQ	< 1 ppm*			600.3		y5	68.7		q
VLPVPQK		779.5	390.8	568.3	$[M+2H]^{2+}$	y5	56.6	22.9	q
LOD	1 ppm*			372.2		y3	59.6		Q
LOQ	6 ppm*								
IDALNENK		915.5	458.7	803.4	$[M+2H]^{2+}$	y7	61.6	25.4	Q
LOD	11 ppm*			688.4		y6	58.6		q
LOQ	19 ppm*			504.2		y4	58.6		q
IPAVFK		673.4	337.7	561.3	$[M+2H]^{2+}$	y5	58.7	21	q
LOD	12 ppm*			464.3		y4	49.7		Q
LOQ	17 ppm*			393.2		y3	49.7		q
FFVAPFPEVFGK		1383.7	692.9	991.5	$[M+2H]^{2+}$	y9	81.6	33.8	q
LOD	< 1 ppm*			920.5		y8	72.6		Q
LOQ	< 1 ppm*			676.4		y6	78.6		q
LYAEER		779.4	390.7	667.3	$[M+2H]^{2+}$	y5	53.6	22.9	q
LOD	< 1 ppm			504.2		y4	56.6		Q
LOQ	< 1 ppm			433.2		y3	53.6		q
DILNQITKPNDVYSFSLASR		2280.2	761.1	930.5	$[M+3H]^{3+}$	y8	86.6	39	Q
LOD	< 1 ppm			767.4		y7	95.6		q
LOQ	5 ppm			680.4		y6	80.6		q
HIATNAVLFFGR		1344.7	673.4	1208.7	$[M+2H]^{2+}$	y11	86.2	33.1	q
LOD	5 ppm			1095.6		y10	71.2		Q

LOQ	15 ppm			1024.6		y9	74.2		q
YFGYTGALR		1046.5	524.3	884.5	$[M+2H]^{2+}$	y8	63.3	27.7	q
LOD	1 ppm			737.4		y7	66.3		Q
LOQ	6 ppm			517.3		y5	63.3		q
*ppm … total allergenic ingredient protein/chocolate dessert									

3.7 Validation of the final targeted LC-MS/MS method for the detection and quantification of five allergenic ingredients

Measurement files .wiff generated by the software Analyst of the QTRAP system of Sciex were loaded into Skyline and peaks were integrated manually.

3.7.1 Retention time check

In Figure 67 it can be seen, that retention times were constant over all measured runs. Retention time varied up to 1.5 % in the high concentration levels of 30 and 100 ppm and up to 2 % in the low concentration levels of 3 and 10 ppm. In Figure 68 the distribution of the single peptides in an example results file of a 100 ppm sample is shown. Average peak width on the base is 0.2 minutes.

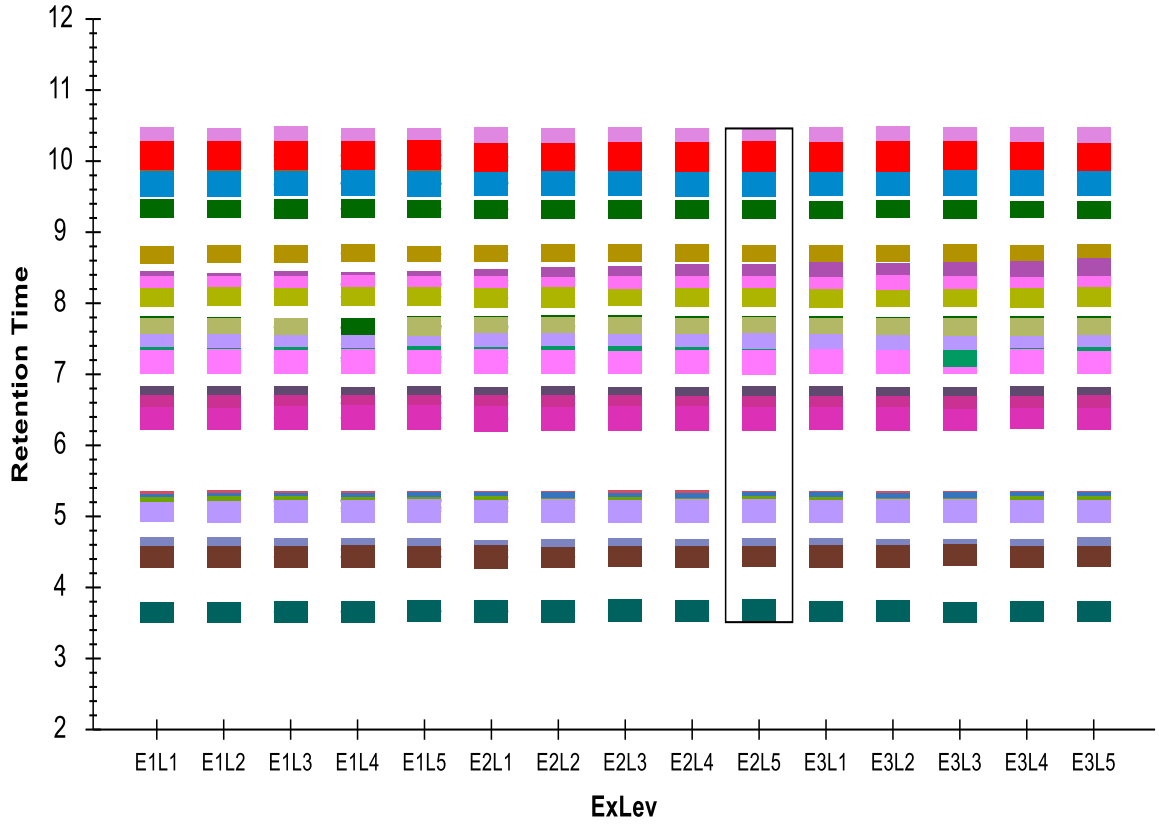


Figure 67: Retention times comparison of the different measured samples and replicates

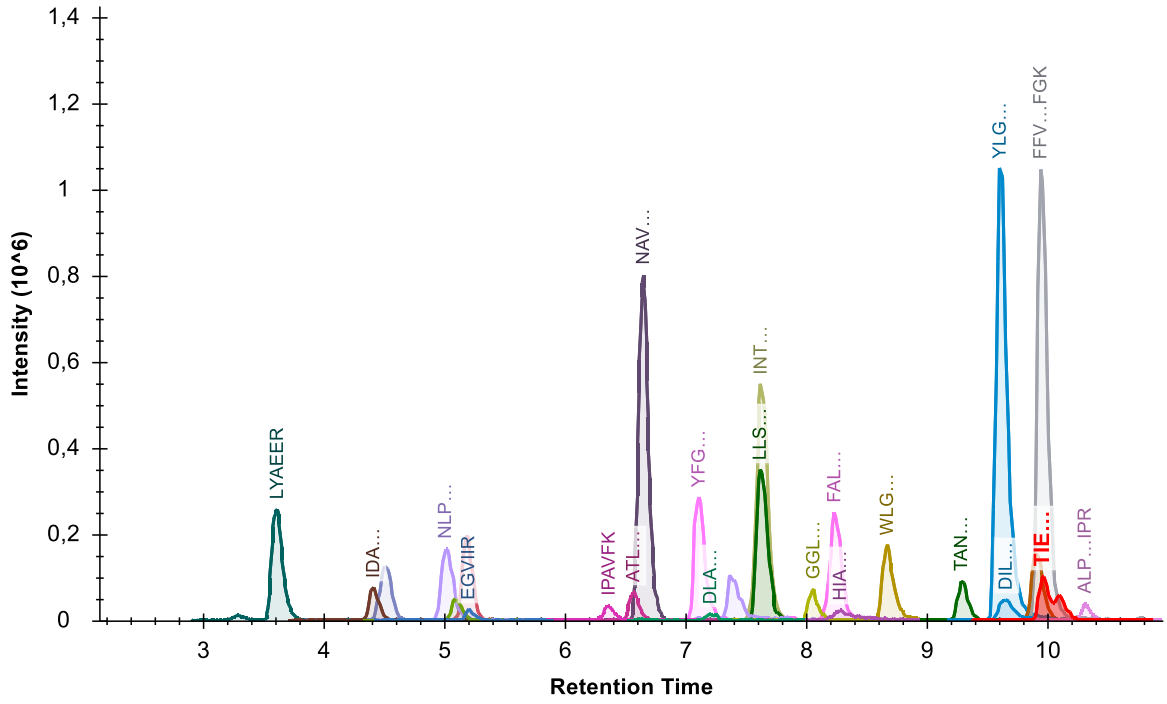


Figure 68: Distribution of the single peptides in an example results file of a 100 ppm sample

3.7.2 Calibration curves

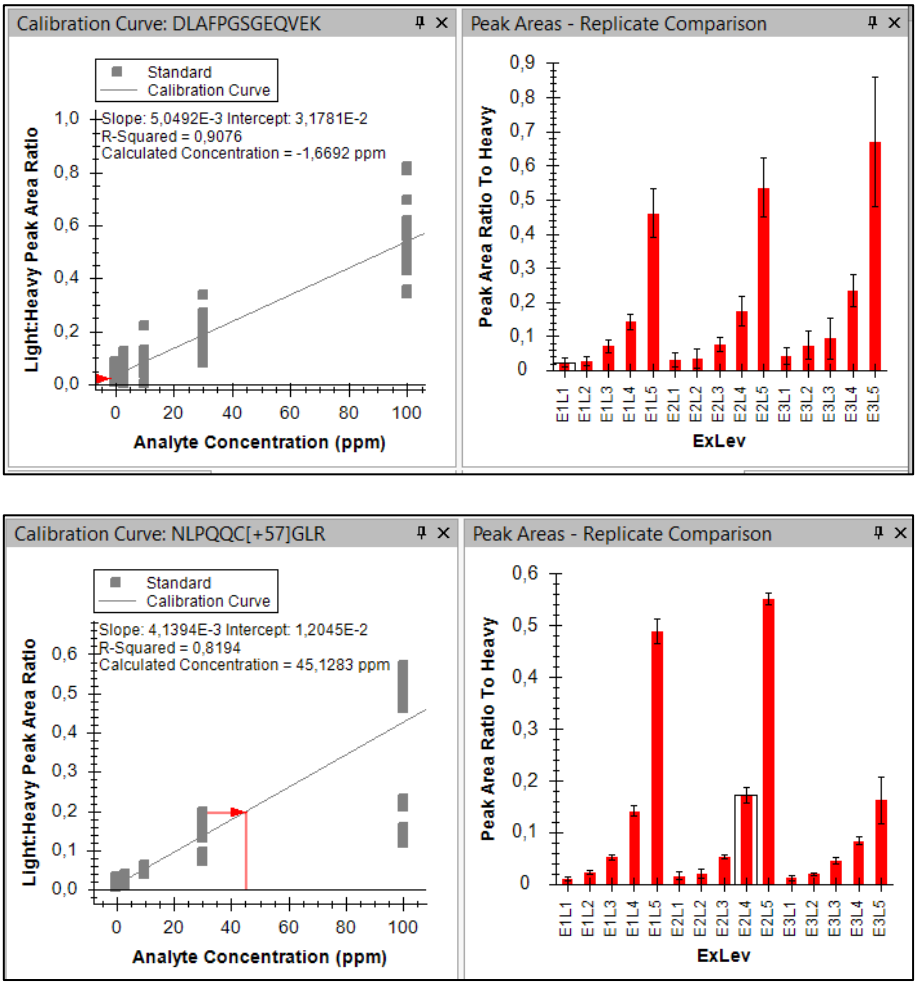
Data of the calibration curves were extracted from Skyline (see Table 35). Calibration curves were generated by calculating the mean of the respective concentration levels of all 9 replicated over the three different days of extraction.

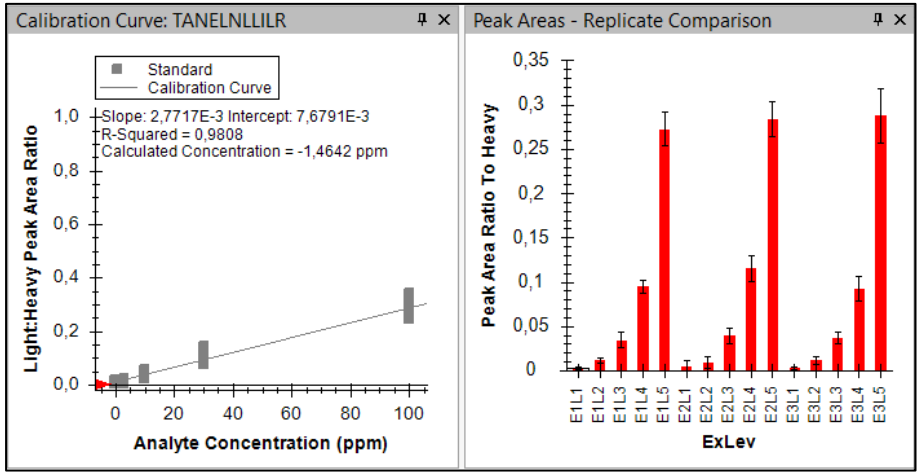
Table 35: Calibration data, linear regression and Blank mean total area ratios for the calculation of LOD and LOQ of the different marker peptides.

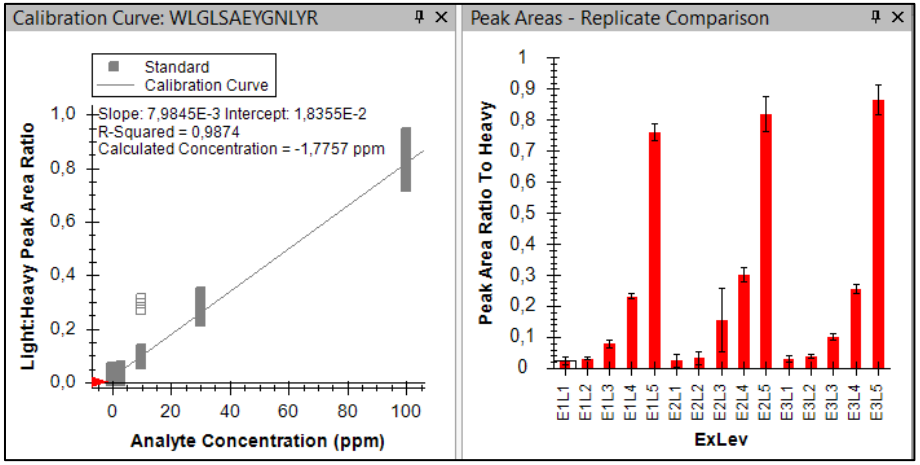
		Calibration curve (N=9)			Blank (n=27)	
Protein	**Peptide**	**Slope**	**Intercept**	**R^2**	**Mean total area ratio**	**Stdev Total area ratio**
Ara h 1	DLAFPGSGEQVEK	0.0047	0.0424	0.94	0.0305	0.0213
Ara h 2	NLPQQCGLR	0.0026	0.0473	0.61	0.0135	0.0058
Ara h 3/4	TANELNLLILR	0.0020	0.0282	0.99	0.0040	0.0035
Ara h 3/4	WLGLSAEYGNLYR	0.0072	0.0452	0.99	0.0279	0.0153
Ara h 3/4	FFVPPSEQSLR	0.0003	0.0063	0.99	0.0033	0.0019
Jug r 2	ATLTLVSQETR	0.0032	0.0151	0.98	0.0071	0.0040
Jug r 2	EGVIIR	0.0084	0.0355	0.97	0.0166	0.0143
Jug r 4	ALPEEVLATAFQIPR	0.0088	0.0435	0.99	0.0313	0.0123
Cor a 9	INTVNSNTLPVLR	0.0085	0.0372	0.98	0.0041	0.0074
Cor a 9	ALPDDVLANAFQISR	0.0377	0.1812	0.98	0.0374	0.0364

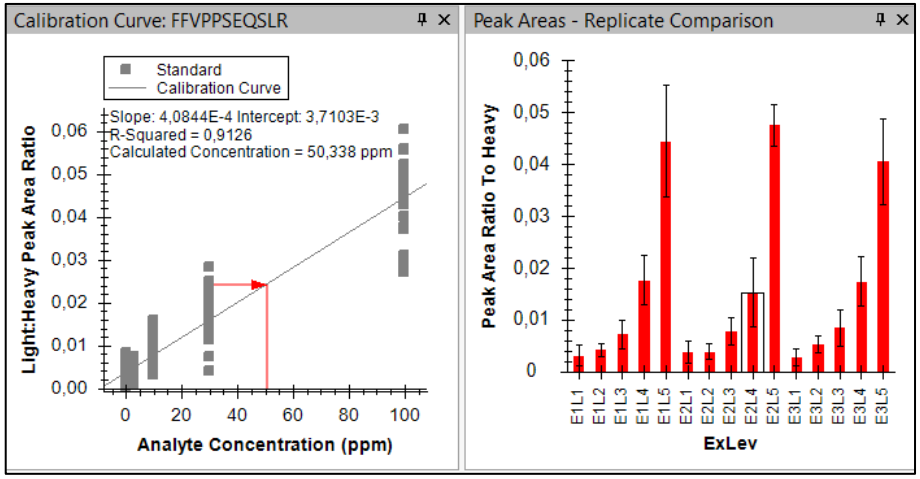
Cor a 11	LLSGIENFR	0.0030	0.0162	0.98	0.0025	0.0023
Gal d 2	DILNQITKPNDVYSFSLASR	0.3411	1.8486	0.81	2.3386	1.7917
Gal d 2	LYAEER	0.0300	0.0610	0.95	0.0120	0.0227
Gal d 2	GGLEPINFQTAADQAR	0.1452	0.3543	0.95	0.1085	0.1250
Gal d 2	HIATNAVLFFGR	0.0651	0.6761	0.96	0.6715	0.4522
Gal d 3	AQSDFGVDTK	0.0027	0.0391	0.99	0.0063	0.0041
Gal d 3	YFGYTGALR	0.0020	0.0166	0.99	0.0029	0.0028
Bos d 5	IPAVFK	0.0040	0.0152	0.99	0.0278	0.0160
Bos d 5	IDALNENK	0.0073	-0.0007	0.99	0.0085	0.0081
Bos d 9	FFVAPFPEVFGK	0.0169	0.1053	1.00	0.0196	0.0212
Bos d 9	YLGYLEQLLR	0.0221	0.0747	1.00	0.0245	0.0246
Bos d 10	NAVPITPTLNR	0.0087	0.0558	0.98	0.0055	0.0111
Bos d 10	FALPQYLK	0.0079	0.2331	0.99	0.0146	0.0152
Bos d 11	VLPVPQK	0.0019	0.0234	0.98	0.0204	0.0117
Bos d 11	AVPYPQR	0.0057	0.6444	0.98	0.6589	0.1471
Cor a 9	TIEPNGLLLPQYSNAPELIYIER	0.0111	0.0604	0.98	0.0157	0.0116

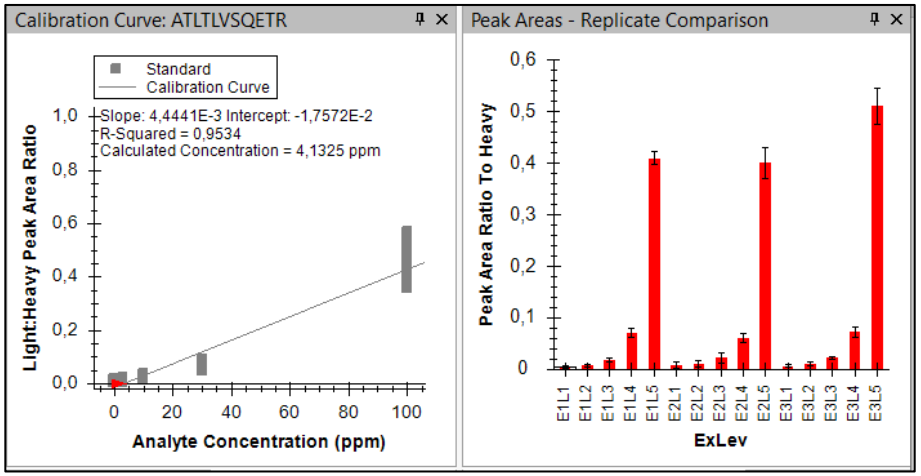
Calibration curves and peak area ratio to heavy peptide of the single levels on the different days are presented in the following diagrams (Figure 69):

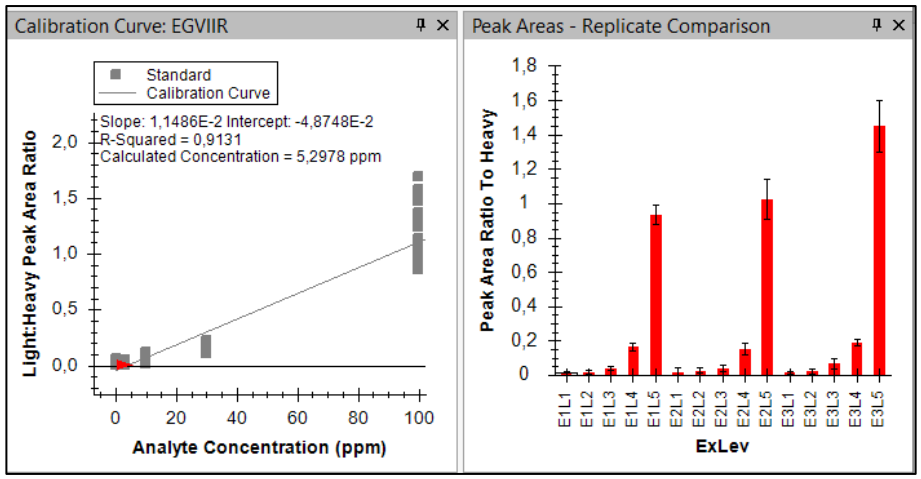


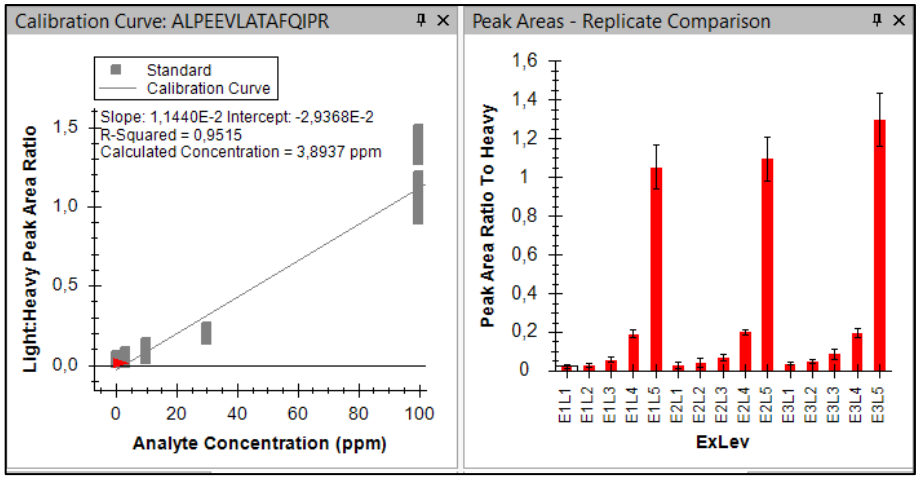

Calibration Curve: TANELNLLILR
Standard
Calibration Curve
Slope: 2,7717E-3 Intercept: 7,6791E-3
R-Squared = 0,9808
Calculated Concentration = -1,4642 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
ExLev

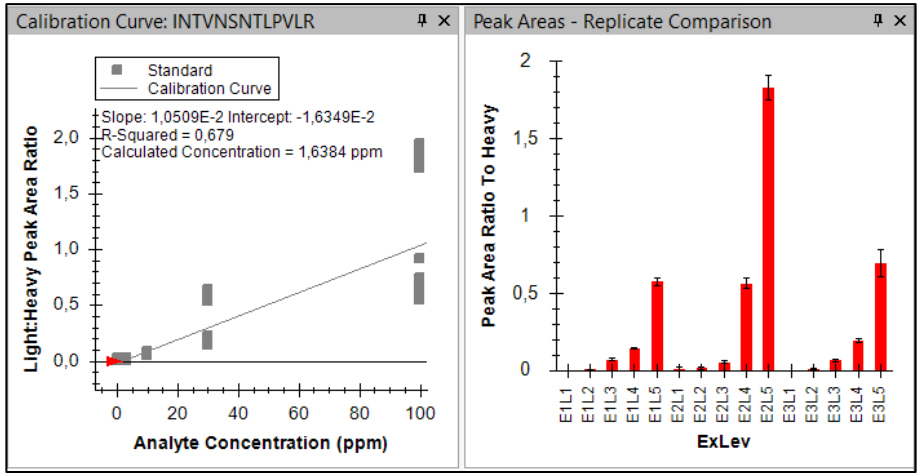

Calibration Curve: WLGLSAEYGNLYR
Standard
Calibration Curve
Slope: 7,9845E-3 Intercept: 1,8355E-2
R-Squared = 0,9874
Calculated Concentration = -1,7757 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
ExLev

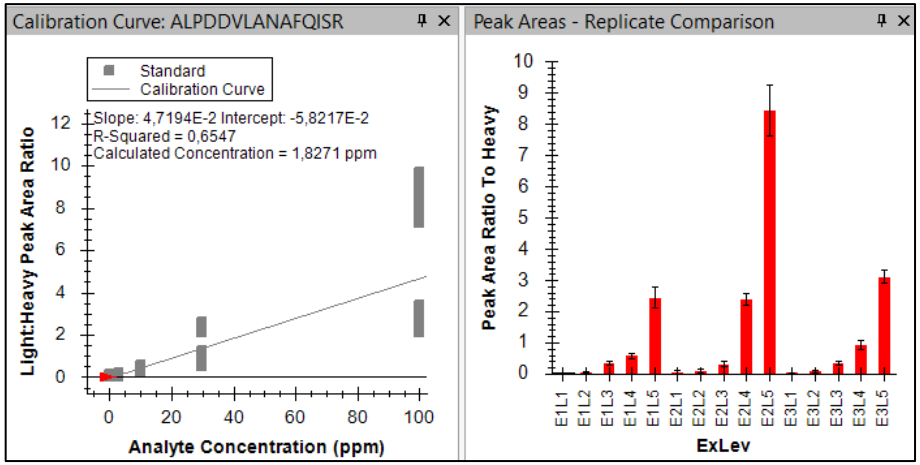

Calibration Curve: FFVPPSEQSLR
Standard
Calibration Curve
Slope: 4,0844E-4 Intercept: 3,7103E-3
R-Squared = 0,9126
Calculated Concentration = 50,338 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
ExLev

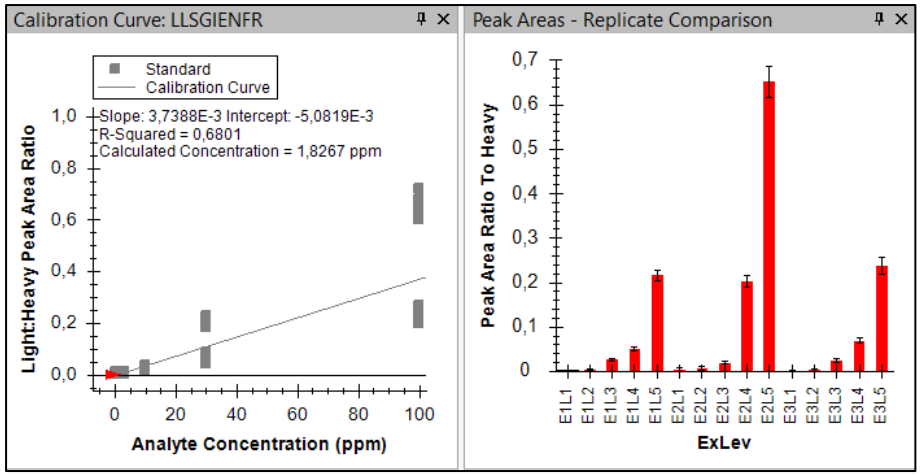

Calibration Curve: ATLTLVSQETR
Standard
Calibration Curve
Slope: 4,4441E-3 Intercept: -1,7572E-2
R-Squared = 0,9534
Calculated Concentration = 4,1325 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
ExLev

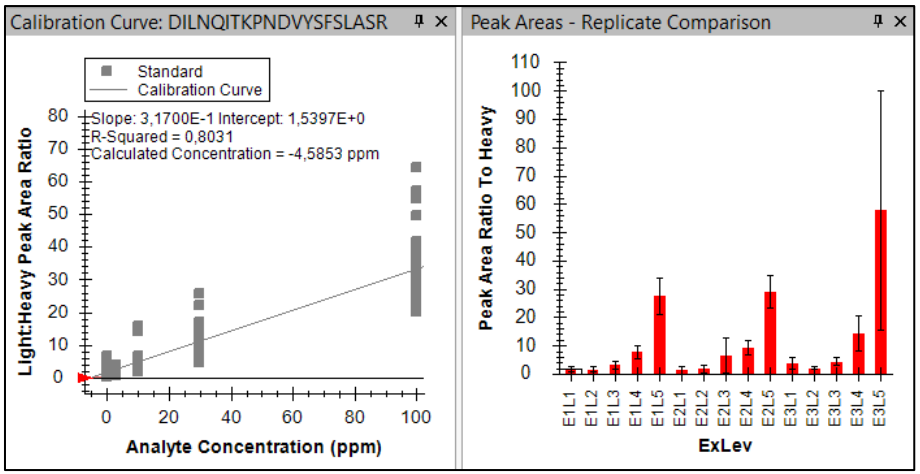

Calibration Curve: EGVIIR
Standard
Calibration Curve
Slope: 1,1486E-2 Intercept: -4,8748E-2
R-Squared = 0,9131
Calculated Concentration = 5,2978 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

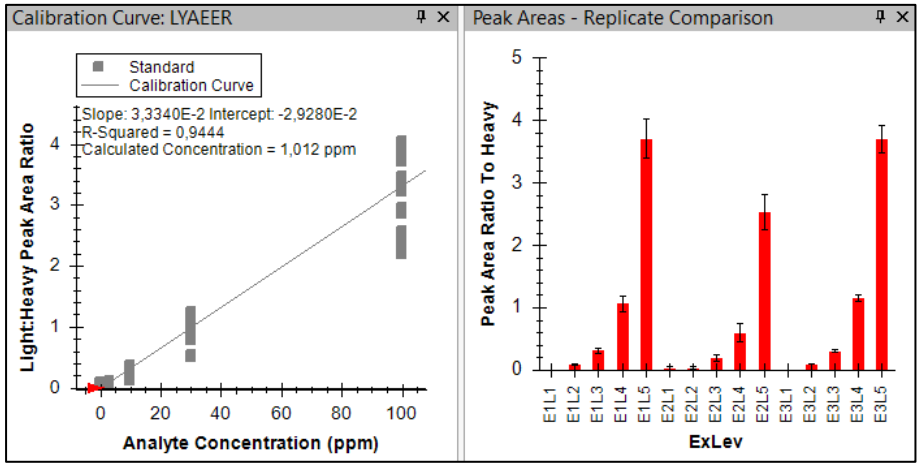

Calibration Curve: ALPEEVLATAFQIPR
Standard
Calibration Curve
Slope: 1,1440E-2 Intercept: -2,9368E-2
R-Squared = 0,9515
Calculated Concentration = 3,8937 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

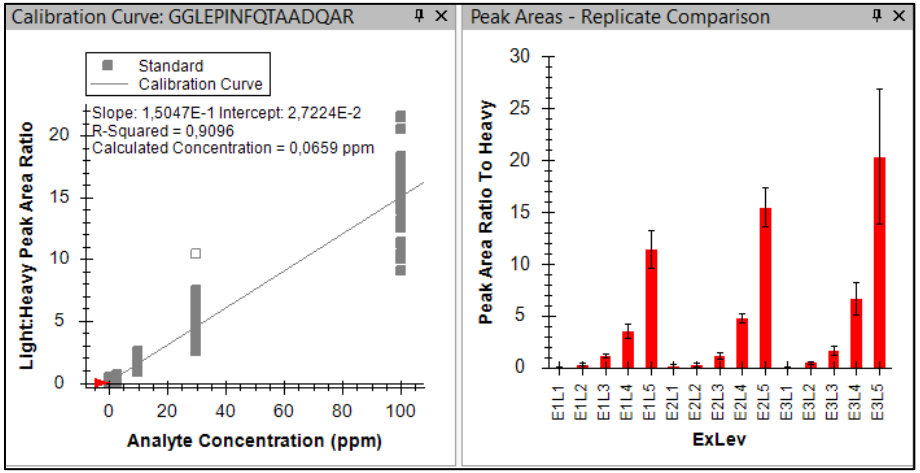

Calibration Curve: INTVNSNTLPVLR
Standard
Calibration Curve
Slope: 1,0509E-2 Intercept: -1,6349E-2
R-Squared = 0,679
Calculated Concentration = 1,6384 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

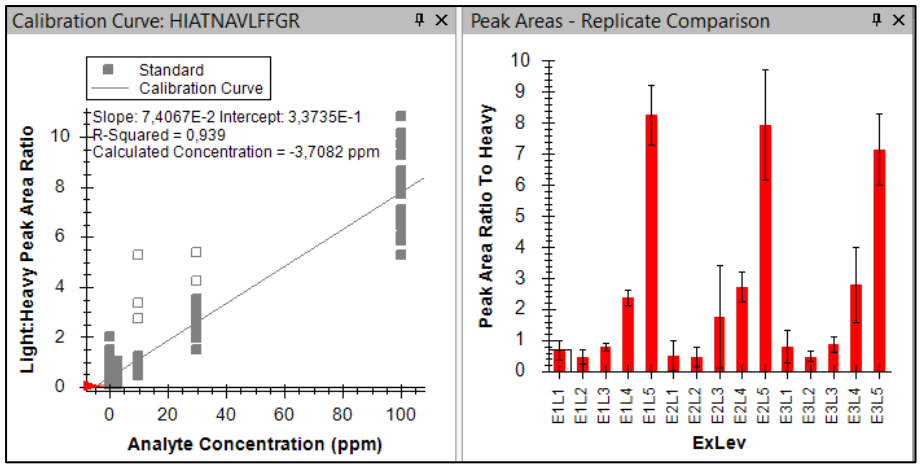

Calibration Curve: ALPDDVLANAFQISR
Standard
Calibration Curve
Slope: 4,7194E-2 Intercept: -5,8217E-2
R-Squared = 0,6547
Calculated Concentration = 1,8271 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

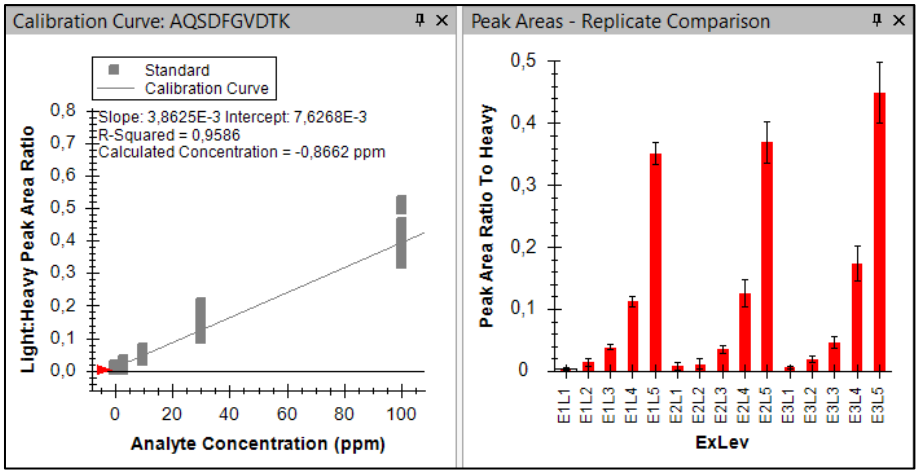

Calibration Curve: LLSGIENFR
Standard
Calibration Curve
Slope: 3,7388E-3 Intercept: -5,0819E-3
R-Squared = 0,6801
Calculated Concentration = 1,8267 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

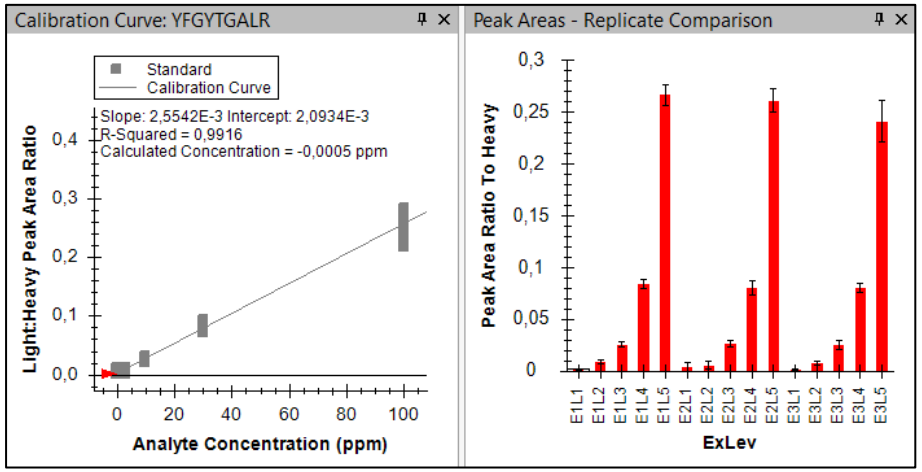

Calibration Curve: DILNQITKPNDVYSFSLASR
Standard
Calibration Curve
Slope: 3,1700E-1 Intercept: 1,5397E+0
R-Squared = 0,8031
Calculated Concentration = -4,5853 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

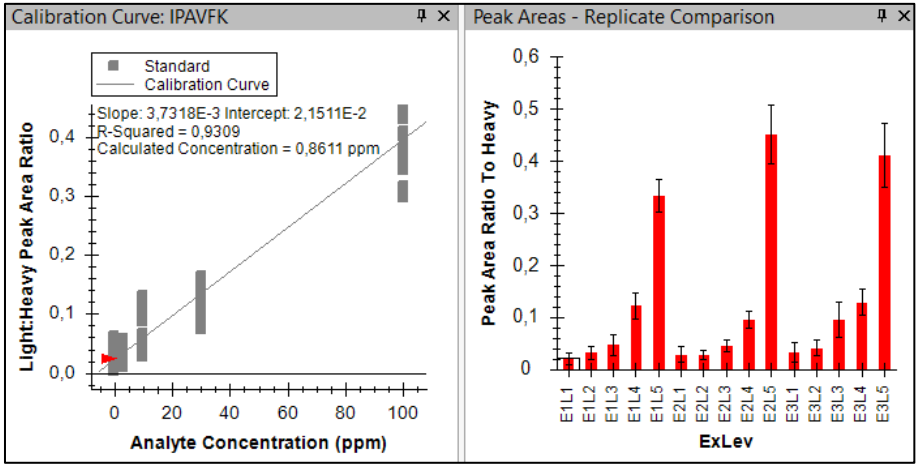

Calibration Curve: LYAEER
Standard
Calibration Curve
Slope: 3,3340E-2 Intercept: -2,9280E-2
R-Squared = 0,9444
Calculated Concentration = 1,012 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

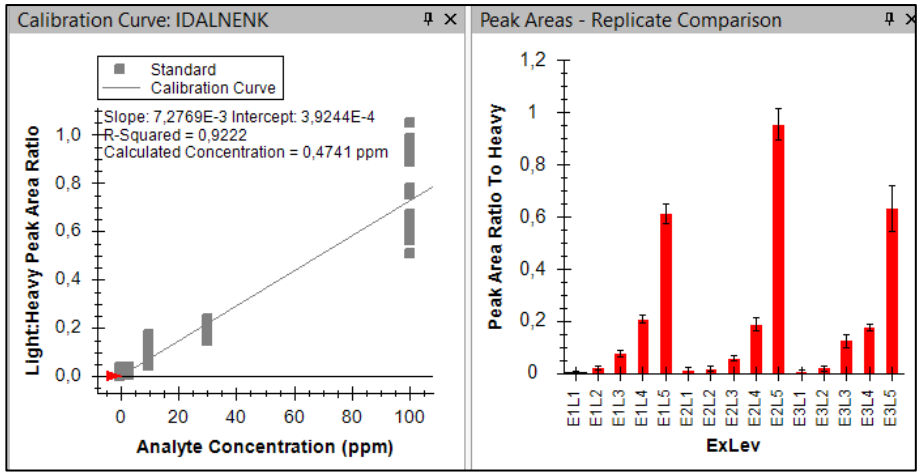

Calibration Curve: GGLEPINFQTAADQAR
Standard
Calibration Curve
Slope: 1,5047E-1 Intercept: 2,7224E-2
R-Squared = 0,9096
Calculated Concentration = 0,0659 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

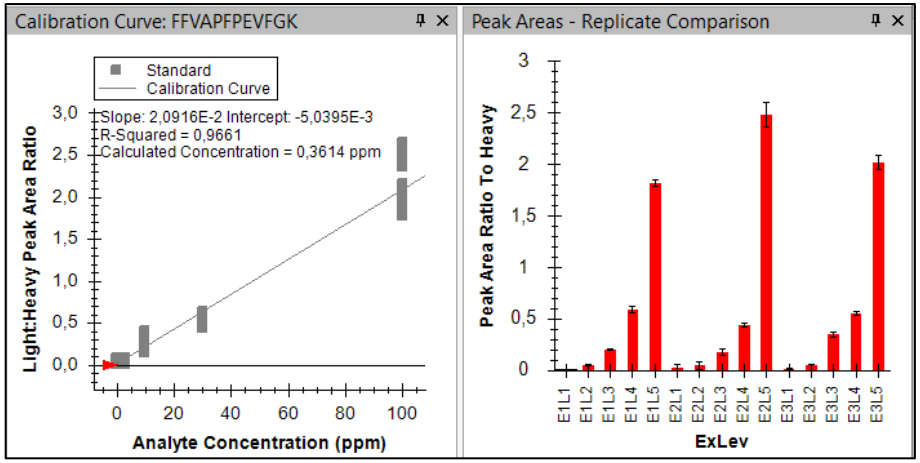

Calibration Curve: HIATNAVLFFGR
Standard
Calibration Curve
Slope: 7,4067E-2 Intercept: 3,3735E-1
R-Squared = 0,939
Calculated Concentration = -3,7082 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

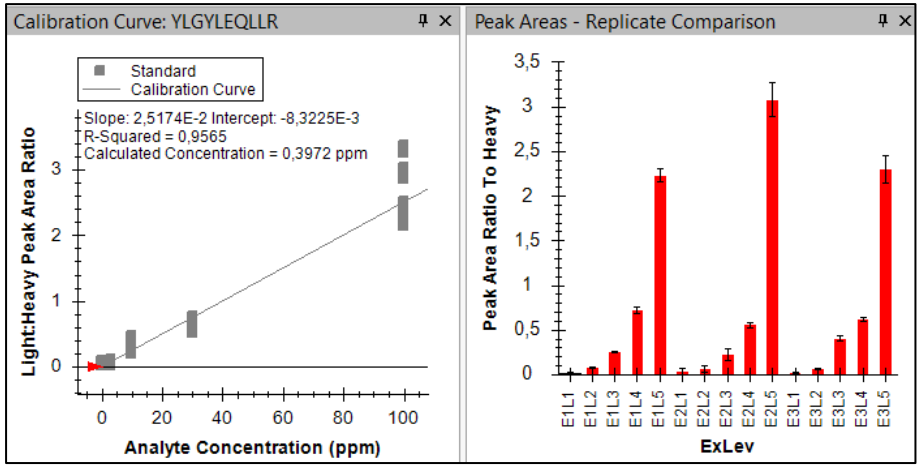

Calibration Curve: AQSDFGVDTK
Standard
Calibration Curve
Slope: 3,8625E-3 Intercept: 7,6268E-3
R-Squared = 0,9586
Calculated Concentration = -0,8662 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

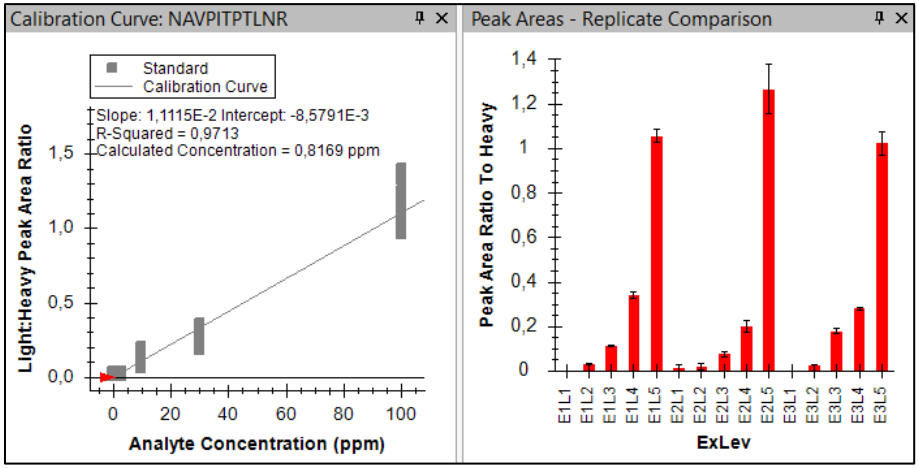

Calibration Curve: YFGYTGALR
Standard
Calibration Curve
Slope: 2,5542E-3 Intercept: 2,0934E-3
R-Squared = 0,9916
Calculated Concentration = -0,0005 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev


Calibration Curve: IPAVFK
Standard
Calibration Curve
Slope: 3,7318E-3 Intercept: 2,1511E-2
R-Squared = 0,9309
Calculated Concentration = 0,8611 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev


Calibration Curve: IDALNENK
Standard
Calibration Curve
Slope: 7,2769E-3 Intercept: 3,9244E-4
R-Squared = 0,9222
Calculated Concentration = 0,4741 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev


Calibration Curve: FFVAPFPEVFGK
Standard
Calibration Curve
Slope: 2,0916E-2 Intercept: -5,0395E-3
R-Squared = 0,9661
Calculated Concentration = 0,3614 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev


Calibration Curve: YLGYLEQLLR
Standard
Calibration Curve
Slope: 2,5174E-2 Intercept: -8,3225E-3
R-Squared = 0,9565
Calculated Concentration = 0,3972 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev


Calibration Curve: NAVPITPTLNR
Standard
Calibration Curve
Slope: 1,1115E-2 Intercept: -8,5791E-3
R-Squared = 0,9713
Calculated Concentration = 0,8169 ppm
Light:Heavy Peak Area Ratio
Analyte Concentration (ppm)
Peak Areas - Replicate Comparison
Peak Area Ratio To Heavy
E1L1 E1L2 E1L3 E1L4 E1L5 E2L1 E2L2 E2L3 E2L4 E2L5 E3L1 E3L2 E3L3 E3L4 E3L5
ExLev

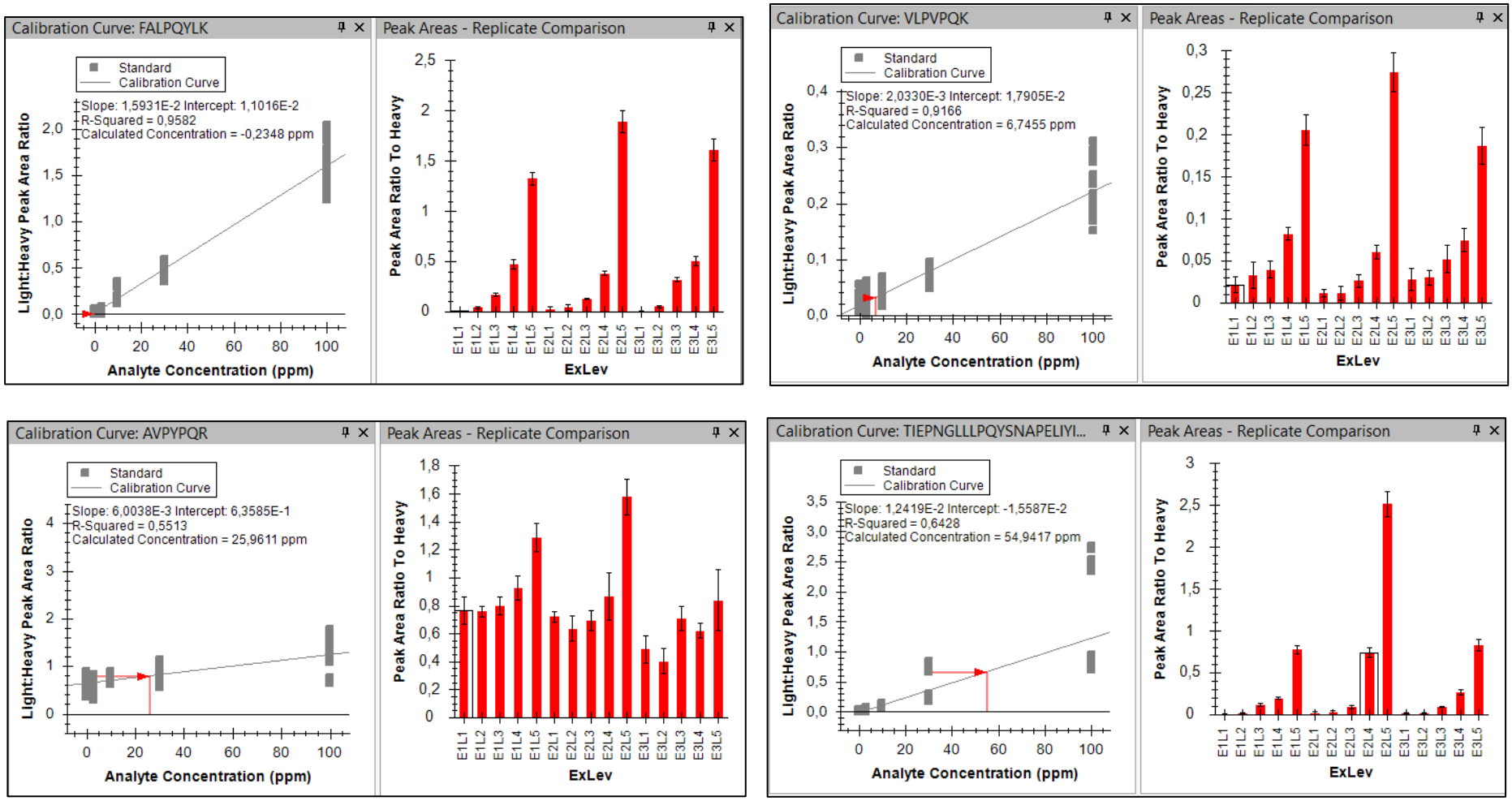


Figure 69: Calibration curves and peak area ratio to heavy peptide of the single levels on the different days

3.7.3 Calculated LOD and LOQ

In Table 36 are the LOD and LOQ for the measurements of day 1, day 2, day 3 and the complete dataset shown.

Table 36: Calculated LOD (ppm in mg allergenic protein/kg chocolate dessert) and LOQ (ppm in mg allergenic protein/kg chocolate dessert) for the datasets of the single days and the complete dataset over all 9 replicate measurements.

		day 1			day 2			day 3			complete dataset		
		LOD	LOQ	R^2	LOD	LOQ	R^2	LOD	LOQ	R^2	LOD	LOQ	R^2
Ara h 1	**DLAFPGSGEQVEK**	9	29	0.96	14	42	0.95	9	31	0.91	11	43	0.94
Ara h 2	**NLPQQCGLR**	3	7	1.00	6	16	1.00	2	19	0.89	<1	9	0.61
Ara h 3/4	**TANELNLLILR**	0	4	0.99	4	17	0.98	0	4	0.98	<1	5	0.99
Ara h 3/4	**WLGLSAEYGNLYR**	6	18	1.00	8	27	0.99	4	8	0.99	4	19	0.99
Ara h 3/4	**FFVPPSEQSLR**	14	47	0.90	17	52	0.95	7	35	0.9	9	51	0.99
Jug r 2	**ATLTLVSQETR**	7	12	0.98	10	20	0.97	8	11	0.97	1	10	0.98
Jug r 2	**EGVIIR**	9	18	0.98	12	25	0.96	8	12	0.96	3	15	0.97
Jug r 4	**ALPEEVLATAFQIPR**	8	14	0.97	9	19	0.97	8	12	0.96	3	13	0.99
Cor a 9	**INTVNSNTLPVLR**	1	2	0.99	5	9	0.99	1	2	0.98	<1	5	0.98
Cor a 9	**ALPDDVLANAFQISR**	3	7	0.96	5	9	0.98	2	6	0.99	<1	6	0.98
Cor a 9	**TIEPNGLLLPQYSNAPELIYIER**	3	7	0.99	5	10	0.99	3	8	0.99	<1	6	0.98
Cor a 11	**LLSGIENFR**	3	5	0.99	4	8	0.99	2	4	0.99	<1	3	0.98
Gal d 2	**DILNQITKPNDVYSFSLASR**	13	37	0.91	13	46	0.87	18	47	0.85	17	54	0.81
Gal d 2	**LYAEER**	1	1	0.99	7	17	0.97	0	1	0.99	1	6	0.95
Gal d 2	**GGLEPINFQTAADQAR**	1	3	0.96	5	14	0.98	1	4	0.95	1	7	0.95
Gal d 2	**HIATNAVLFFGR**	17	44	0.97	21	66	0.92	32	89	0.94	21	69	0.96
Gal d 3	**AQSDFGVDTK**	1	4	1.00	6	17	0.98	0	4	0.97	<1	3	0.99
Gal d 3	**YFGYTGALR**	1	2	1.00	6	18	1.00	1	3	0.99	<1	7	0.99
Bos d 5	**IPAVFK**	11	39	0.97	16	40	0.96	16	53	0.94	15	44	0.99
Bos d 5	**IDALNENK**	1	6	0.99	8	16	0.98	2	10	0.96	5	12	0.99

Bos d 9	**FFVAPFPEVFGK**	0	2	1.00	8	18	0.98	<1	0	0.99	<1	7	1.00
Bos d 9	**YLGYLEQLLR**	0	3	1.00	8	17	0.98	<1	0	0.98	1	9	1.00
Bos d 10	**NAVPITPTLNR**	0	0	1.00	9	18	0.97	<1	-1	0.99	<1	7	0.98
Bos d 10	**FALPQYLK**	<1	1	0.99	8	17	0.98	<1	-1	0.98	<1	-8	0.99
Bos d 11	**VLPVPQK**	14	50	0.97	9	21	0.98	16	57	0.94	17	60	0.98

4 Conclusion

The in-silico digest resulted for each used enzyme in a different number of marker peptides. As a result, trypsin came up in a total with 87 relevant marker peptides for all allergens as shown in Table 27. Especially for walnut allergens, trypsin seemed to be most promising due to the high number of possible peptide markers achieved. The other proteases resulted in most of the cases with lower numbers of relevant peptides. Due to the calculated costs for such analysis, trypsin is still less expensive. And from the chemical point of view trypsin has its pH optimum (7.5-8.5) in a range which fits to the pH range of the extraction buffers that were used. This combination of chemical and financial aspects resulted in the decision that the work was done by the use of trypsin as digestion enzyme.

Before starting the extraction optimisation a targeted LC-MS/MS method for the evaluation of the extraction optimisation and the tryptic digest optimisation was done to obtain a rough information about the origin protein/allergen of the different peptides which could be found in-silico and to apply the “rules” published by Johnson et al. (88) with the goal to exclude peptides not fit for purpose, exclusion of non-unique peptides, exclude peptides with posttranslational modifications and to generate theoretical markers which are fit for LC-MS/MS. In total 151 peptides from 27 proteins were included in the measurement, but only 60 peptides of 20 proteins (see Table 33) were found in the different stages. Already more than 50 % of the peptides generated within the in-silico digest were not fit for purpose, meaning either proteins are not extractable or peptides are not stable in digested form or ionisation properties are not suitable for LC-MS/MS measurement. Ara h 5, Ara h 6, Jug r 3, Cor a 8, Cor a 10, Cor a 12 and Cor a 13 were not represented by peptides in the results due to the above mentioned reasons.

During extraction optimisation it could be clearly shown, that the choice of buffer plays a relevant role for protein extraction and therefore peptide availability in the final tryptic digest. Buffer with a pH between 7.8 and 8.5 gave a higher peptide number response than the acidic Tris-HCL buffer with the pH of 5.0. Especially TBB buffer (pH 8.5) enhanced the number of found peptides in the LC-MS/MS measurement drastically. For peanut, peptides of proteins Ara h 1 and Ara h 3/4 were only found in TBB buffer extracts. In case of the nuts, walnut and hazelnut, found peptides and their detected intensities were significantly higher, for some even a factor of 10. For milk and egg proteins the choice of buffer would not play such a tremendous role, especially in a low processed food matrix (chocolate dessert) like the one used in this work. It is less an extraction and more a bringing the proteins in solution, which did basically work with all the tested buffer system in a very similar effective way.

The matrix used in this work is a starch containing chocolate dessert, which is only lightly processed. No heat treatment like baking was applied. This indicates, that the use of denaturating or reducing agents is not so necessary for protein extraction as it would be in heat treated samples. It was shown, that a too high concentration of urea and DTT or a combination of both tends to inhibit the number of found peptides. 6 M urea in combination with 50 mM DTT reduced the number of found peptides by half. It seems that ionisation of the peptides in the source of the mass spectrometer is inhibited here by the detergents. The addition of only 0.6 M urea to TBB buffer gave same intensities for the found peptides like the buffer without detergent, except in the case of peanut peptides, where a slight gain in peek intensity was visible. Looking at the extraction temperature and extraction time, for the chocolate dessert, no difference was found in using longer or shorter incubation times or higher or lower extraction temperatures. For that reason and in regards of applying this method in the future to other, maybe more processed samples, the golden middle of 30 minutes incubation time and 37 °C incubation temperature was chosen.

By analysing different sample-buffer-ratios it was aimed to see, if relevant suppression of the ionisation during LC-MS measurement due to matrix is visible and can be minimised by higher dilution of the sample but still have a relevant amount of protein in the extract to be measured. Extracting with 1:20 or 1:40 resulted in a massive loss of found peptides in the extracts, main reason is the less concentrated protein present in the extracts. No relevant difference was found between a sample-buffer-ratio of 1:5 and 1:10 regarding the peak intensities of the found peptides. To avoid matrix suppression during measurement the combination of 1:10 was implemented in the final extraction protocol. Both tested trypsin brands gave same results, but as the one from Promega (Trypsin Sequencing Grade Modified # V5111) is simply cheaper (about 30 %), it was used for the further experiments. Thus, the protease-protein ratio plays an important role in tryptic digest. A clear trend was shown in signal intensities, as it raised with the higher protease concentration. For more than a third of the peptides the difference in signal intensity between a protease-to-protein ratio of 1:5 and 1:10 accounts more than 50 %. The signal gained from using a ratio of 1:5 instead of 1:10 is overall not dominant. Increasing the temperature during tryptic digest did not improve trypsin digest. And the addition of detergents did not improve number of found peptides or peak intensities.

After extraction and tryptic digestion optimisation, the list of 60 peptides deriving from 20 proteins (see Table 33) was refined in regards of the uniqueness of the peptides for allergenic protein via a Uniprot-peptide-BLAST and their intensities in LC-MS/MS. For example, peptide LDALEPTNR (Jug r 4) was deleted from the list, as it had only one amino acid substituted in comparison to peptide LNALEPTNR (Cor a 9), which was also refined from the list. Amino acid Aspartate and amino acid Asparagine differ on position y-8. This leads to a same set of the y-ions y1-y7. As also retention time

in the extraction and tryptic digest optimisation experiments only differed by 0.4 minutes, as shown in Figure 63, partly coelution of the peptides can occur and therefore would influence quantitative results.

The 44 remaining peptides were optimized for their MS-parameters like choice of +2 or +3 charged precursor ion, choice of best declustering potential and choice of best collision energy.

In the finally validated scheduled MRM detection method for relevant allergens of all the 5 allergenic ingredients were incorporated. In the calculated LODs and LOQs of the complete dataset, NLPQQCGLR (Ara h 2) with a calculated LOQ of 9 ppm and TANELNLLILR (Ara h 3/4) with a LOQ of 5 ppm show the most promising sensitivity, in regards of the VITAL 3.0 Action level 1, even peptide WLGLSAEYGNLYR (Ara h 3/4) meets the needed LOQ for a serving size of 10 g. Ara h 1 peptide DLAFPGSGEQVEK and Ara h 3/4 peptide FFVPPSEQSLR seem to be only relevant for qualitative measurement, as LOQs are not sensitive enough.

For the three marker peptides for walnut deriving from Jug r 2 and Jug r 4 walnut peptides included in the validated method, the LODs is by far not relevant for the new action level set in the VITAL 3.0 reference dose. In Vital 2.0 the reference dose was set to 0.1 mg of protein instead of 0.03 mg of protein. For this reference dose the method would be sensitive enough if a food with a serving size smaller 10 g should be measured.

For the four marker peptides chosen for hazelnut, all four would be sensitive enough to measure even 10 g serving size as given in Table 37 for the Vital 3.0 action level 1. Cor a 9, with three peptides, and Cor a 11, with one peptide, are very promising candidates for MS-analysis for these quantification purposes.

For the detection and quantification of egg allergens in foods, only Gal d 2 and Gal d 3 can be detected. For Gal d 2 two peptides, namely LYAEER (LOQ of 6 ppm) and GGLEPINFQTAADQAR (LOQ of 7 ppm) give the best quantification. Both limits of quantification are low enough to quantify Gal d 2 in food in a relevant range for the application according to the VITAL 3.0 Action level 1. Allergen Gal d 3 can also be quantified in the relevant range with LOD of 3 ppm for AQSDFGVDTK and LOD of 7 ppm for YFGYTGALR.

Bos d 5 (IDALNENK) with a LOQ of 12 ppm, Bos d 9 (FFVAPFPEVFGK and NAVPITPTLNR) with a LOQ of 7 ppm and 9 ppm and Bos d 10 (NAVPITPTLNR) with a LOQ of 7 ppm can be detected in a very sensitive level. All 4 marker peptides can be detected below the necessary concentration in regards of VITAL 3.0. Detected marker peptide for Bos d 11 gives extremely high LODs and LOQs, which is not stringent with the preliminary LOD of around 6 ppm (factor 10 lower) evaluated without heavy peptide standards included in the measurement.

Table 37: Relevance of the measured LOQs for the marker peptides in regards of the VITAL 3.0 Action Level 1

				VITAL 3.0 Action level 1		
	Peptide	LOD ppm	LOQ ppm	Reference dose (mg of protein)	10 g serving size	5 g serving size
Ara h 1	DLAFPGSGEQVEK	11	43	0.2	< 20 ppm	< 40 ppm
Ara h 2	NLPQQCGLR	<1	9			
Ara h 3/4	TANELNLLILR	<1	5			
Ara h 3/4	WLGLSAEYGNLYR	4	19			
Ara h 3/4	FFVPPSEQSLR	9	51			
Jug r 2	ATLTLVSQETR	1	10	0.03	< 0.3 ppm	< 0.6 ppm
Jug r 2	EGVIIR	3	15			
Jug r 4	ALPEEVLATAFQIPR	3	13			
Cor a 9	INTVNSNTLPVLR	<1	5	0.1	< 10 ppm	< 20 ppm
Cor a 9	ALPDDVLANAFQISR	<1	6			
Cor a 9	TIEPNGLLLPQYSNAPELIYIER	<1	6			
Cor a 11	LLSGIENFR	<1	3			
Gal d 2	DILNQITKPNDVYSFSLASR	17	54	0.2	< 20 ppm	< 40 ppm
Gal d 2	LYAEER	1	6			
Gal d 2	GGLEPINFQTAADQAR	1	7			
Gal d 2	HIATNAVLFFGR	21	69			
Gal d 3	AQSDFGVDTK	<1	3			
Gal d 3	YFGYTGALR	<1	7			
Bos d 5	IPAVFK	15	44	0.2	< 20 ppm	< 40 ppm
Bos d 5	IDALNENK	5	12			
Bos d 9	FFVAPFPEVFGK	<1	7			
Bos d 9	YLGYLEQLLR	1	9			
Bos d 10	NAVPITPTLNR	<1	7			
Bos d 10	FALPQYLK	<1	-8			
Bos d 11	VLPVPQK	17	60			

Out of a pool of 151 marker peptides from 27 different allergens received from an in-silico digest, only 60 marker peptides for 20 different allergens were detected at a qualitative level via LC-MS/MS. This means only 40 % of theoretical available peptides covering 74 % of the different allergens deriving from 5 allergenic ingredients could be found in real samples.

Moving to a quantitative level, this number reduced dramatically. 25 marker peptides were implemented in the final quantification method, but only 14 marker peptides could be detected sensitive enough to be relevant for allergen detection at VITAL 3.0 action level 1. Only about 9 % of theoretical available peptides are detected in a relevant range.

In regards of a developed multi-allergen quantification method, where allergens from 3 plant sources and 2 animal sources should be detected, the aim was reached to develop a reference method sensitive enough for VITAL 3.0 and for current available ELISA quantification for the allergenic sources peanut, hazelnut, milk and egg. In case of walnut, reached LODs are comparable to commercial available ELISAs.

The big advantage of LC-MS/MS methods is, that the information level is allergen specific and can be even down to the level of epitope specificity.

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List of abbreviations

LOD	Limit of detection
LOQ	limit of quantification
MS	mass spectrometry
MS^2	tandem mass spectrometry
QQQ	tripple quadrupole
IT	ion trap
API	atmospheric pressure ionisation
QTOF	quadrupole time of flight
LC	liquid chromatographie
SMP	skimmed milk powder
EWP	egg white powder
WEP	whole egg powder
EP	egg powder
PN	peanut
HN	hazelnut
WN	walnut
nMP	native milk powder
Rpnf	roasted peanut flour
m/z	mass over charge

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