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Determination of food allergens by LC-MS: Impacts of sample preparation, food matrix, and thermal processing on peptide detectability and quantification

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A B S T R A C T

Food allergies are a growing worldwide concern and the contamination of products with food allergens represents a significant health risk to allergic consumers. With the introduction of reference doses, quantitative methods are needed for the monitoring of allergen levels, and the potential of LC-MS/MS is of hugely growing interest. In this study, we demonstrate that relevant food matrices (bakery products and chocolates) and thermal food processing substantially influence the quantification of 18 marker peptides from various nut and peanut allergens via targeted proteomics. In addition, we characterize the individual release kinetics of marker peptides and provide examples for metastable marker peptide candidates. Matrix recovery rates overall ranged between 15 and 250% with the observed variation being linked to the individual peptide structure as well as to specific matrix interferences. In contrast, thermal processing considerably influences the detectability of allergens on the protein level as different marker peptides from the identical parent allergen are similarly affected, leading to a loss in signal of up to 83% in extreme cases after a 45-min simulated baking. Provided data are finally used for evaluation of different calibrators as well as the overall potential and challenges of LC-MS for the absolute quantification of food allergens.

Significance: With the scientific discussion moving towards a risk-based management of food allergens, including the establishment of threshold doses, robust methods for the absolute quantification of allergens in food samples are urgently needed. Because the currently used antibody- and DNA-based technologies show severe limitations in terms of specificity and reproducibility, LC-MS has emerged as a promising alternative. Its application to absolute quantification, however, first requires an understanding of the various impacts that affect quantification results, including different food matrices, sample preparation, and thermal processing of foodstuffs. Knowledge of these factors, which are assessed as part of a comprehensive survey in this study, is also an important prerequisite to evaluate means of calibration for an LC-MS-based quantification of food allergens.

1. Introduction

Food allergies pose a global risk to public health and may have fatal consequences to affected patients [1]. With prevalence rates recently estimated as high as 5% in adults and 8% in young children for westernized countries [2], European [3] and US [4] legislations have addressed this issue by the introduction of mandatory labeling for the most relevant allergenic foods when used as ingredients. Although unintended contamination with allergens, introduced, e.g., from potential cross-contact in the production facility, also represents a relevant health risk, this is not covered by hitherto regulations, and manufacturer address this issue by precautionary labeling of potential allergen traces [5]. This voluntary approach is prone to error and allergic patients therefore often do not follow these labels. to The introduction of harmonized, risk-based procedures aims to improve protection of allergic consumers [6,7]. Recently, an international expert board introduced a set of “reference doses” for the most relevant allergenic foods, following a NOAEL/LOAEL-based approach [8], thus forming a basis for possible threshold levels in the future.

To survey the compliance of food with such threshold levels, efficient and reliable methods are needed for the identification and quantification of allergenic contaminants. So far, ELISA and PCR are the

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most commonly used techniques for the determination of allergens. Both, however, show significant limitations, including cross-reactivity, low inter-assay reproducibility [9,10], and missing multiplexing ability (ELISA) [9–12], or the restrictions to specificity inherent to the DNA-based detection (PCR) [13,14], respectively. As an alternative, the development of LC-MS-based methods receives growing attention, representing a sequence-specific, protein-based approach [15]. Several multi-methods using multiple reaction monitoring (MRM) [16,17] or single-stage high-resolution mass spectrometry [18,19] have been published, which show promising sensitivity and a high linearity.

Absolute quantification of food allergens, however, requires proper calibration as well as suitable standards, to compensate effects on peptide detectability caused by the food matrix and production history, e.g., processing. The dependence of peptide LC-MS signal abundance on the food matrix has been described before [20,21], and there have also been reports on interferences with tryptic digestion and proteotypic peptide stability caused by matrix components [22–24]. The use of stable isotopically labeled (SIL) peptides, which are added to the tryptic digest prior to the measurement, has been repeatedly proposed for the exact quantification of allergenic contaminants [25,26]. While SIL peptides compensate matrix effects influencinge.g. analyte ionization, they are not appropriate for different effects arising during sample preparation, since they in general do not require enzymatic digestion for release [22,23,27,28].

Another challenge for the exact quantification of food allergens is introduced through food processing, which might affect protein structure as well as allergenicity, e.g., through unfolding, aggregation, chemical modification, or cross-linking to matrix components [29,30]. Most notably, thermal treatment is commonly applied to many allergenic ingredients, in particular to peanut and tree nuts, and heat induced structural changes and effects on allergenicity have, thus, been comprehensively investigated [30–33]. So far, only few studies are available on the effects of heat processing on the LC-MS analysis of allergens, which have all described a reduction in detectability, yet to a different extent, depending on the particular allergen and the mode of processing, respectively [10,21,34].

With the presented study, we aim to address some of the most important parameters that influence the quantitative determination of food allergens via LC-MS (food matrix, sample history, and sample preparation) using a method for the analysis of six allergenic nuts with high relevance for allergenicity. For simplification, all analyzed allergenic foods are described as nuts here, although peanut does belong to the legumes.

2. Materials and methods

2.1. Sample material

Almonds, cashews, hazelnuts, peanuts, pistachios, and walnuts as well as ingredients for the preparation of bread and cookies were purchased at local food stores. Chocolate products were obtained from the Schafschoki.de online shop for allergic people (Bremen, Germany) and were designated as “nut-free”. All foodstuffs were verified to be free of contaminations by nut allergens using LC-MS in MRM^3 mode and only one batch of each product was used throughout the entire study.

2.2. Preparation of reference materials

Nut-free bread was prepared from a dough made of 500 g of wheat flour, 7 g of conventional dry yeast from a retail store, a pinch of salt (approx. 0.3 g), and 300 mL of water, and baked for 45 min at 200 °C (top − /bottom heat, following preheating) in an ordinary domestic oven (Bosch, HEN200454). For the preparation of nut-incurred bread, nuts were ground to a fine flour and 550 mg of each nut was thoroughly mixed with the wheat flour before the dough was prepared. A final content of 903.1 mg/kg was calculated for each nut based on the weight after baking.

Cookie dough was prepared from 100 g wheat flour, 40 g of sugar, 25 mL of milk (1.5% fat), and one egg. The dough was split for the preparation of seven cookies and baked for 15 min at 190 °C. Nut-incurred cookies additionally contained 104 mg of each nut flour, resulting in a final content of 604.6 mg/kg after baking.

All doughs were homogenized for at least 4 min using a hand mixer with dough hooks (bread) and beaters (cookies), respectively, followed by kneading by hand.

2.3. Sample preparation

All sample materials were ground and homogenized with a cutting mill prior to extraction. One gram of the nut flour was then added to 10 mL of extraction buffer (6 M urea, 1 M thiourea, 50 mM Tris, pH 8.0 adjusted with HCl) in a 50 mL plastic tube and dispersed for 2 min using an Ultra Turrax T-25 (ika, Staufen, Germany) with a 10 N dispersing element at 9500 rpm, yielding a finely granulated suspension. After centrifugation for 60 min at 4 °C and 12,000 × g, a 50 μL aliquot of the supernatant was reduced with 2.5 μL of dithiothreitol solution (200 mM) for 60 min in the dark, followed by alkylation with 10 μL of iodoacetamide solution (200 mM), which was stopped after 60 min by addition of another 10 μL of the dithiothreitol solution. After dilution with 388 μL of water, the sample was supplemented with 10 μg of sequencing-grade modified trypsin (Serva, Heidelberg, Germany) and incubated for 14 h at 37 °C under slow shaking.

The tryptic digest was subjected to SPE for desalting and purification, using Strata-X 33 μm RP 30 mg/1 mL columns (Phenomenex, Aschaffenburg, Germany). The cartridges were activated and equilibrated according to the manual. Samples were loaded onto the cartridge, washed with 1 mL of 1% formic acid (FA), and eluted with 1 mL of 90% MeOH 1% FA. Solvent was removed under a gentle nitrogen flow at 40 °C, and the samples were redissolved in 50 μL acetonitrile/water (3:97), corresponding to the initial HPLC conditions.

For experiments on the kinetics of tryptic digestion, the six nut flours were blended in equal parts before extraction (166.6 mg of each nut /10 mL) and subjected to incubation with trypsin for 0.5, 1, 2, 3, 4, 6, 10, or 20 h. Digestion was stopped by acidification and SPE, directly followed by LC-MS analysis run in MRM detection mode. The experiment was repeated twice on different days and was performed accordingly using an extract of the nut-incurred cookies.

2.4. Protein determination

Application of two complementary techniques, Kjeldahl method and Bradford assay, was necessary to determine protein concentrations in the untreated nuts as well as in their protein extracts. For the Kjeldahl method, a total of 0.6–2.0 g sample material was weighed in nitrogen-free paper bowls and transferred into a Kjeldahl flask. 30 mL of sulfuric acid and two catalyst tablets (Merck, Darmstadt) were added, and the mixture was heated until a clear green solution was obtained. 80 mL of water and 80 mL of 30% NaOH were added to release ammonia, which was then transferred into a flask containing 50 mL of boric acid (20 g/L) via steam distillation. After addition of 3 drops of Tashiro indicator (containing methyl red and methylene blue), the solution was titrated with 0.1 M HCl until the indicator changed from green to violet, and total protein was calculated using a conversion factor of 5.46 (peanut) and 5.30 (all other nuts), respectively.

For the Bradford assay, a total of 20 μL of protein solution was pipetted into the cavities of a 96-well microtiter plate, and 140 μL of water and 40 μL of Bradford reagent (Bio-Rad Protein Assay, Munich) was added. After a 5-min incubation in the dark, absorption at 595 nm was measured using an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). Quantification was performed by means of an external calibration with bovine serum albumin.

2.5. Liquid chromatography-mass spectrometry

Data were acquired on a QTRAP 6500 LC-MS/MS system (Sciex, Darmstadt, Germany) using an IonDriveTM Tubo V ESI source run in positive mode and coupled to an Agilent 1260 Infinity HPLC. LC separation was carried out on a Phenomenex Kinetex, 2.6 µm, C18, 100 Å (100 × 2.1 mm) column at a flow rate of 300 µL/min, employing a three-step acetonitrile/water gradient at a full duty cycle time of 34 min.

The mass spectrometer was run under control of the Analyst software (v1.6.2), operating in MRM and MRM^3 mode. Q1 and Q3 were both set to unit resolution (0.7 ± 0.1 amu) in MRM mode. In MRM^3 mode, Q1 was also set to unit resolution while Q3 was run in LIT mode. Ion fill time and excitation time were set to 75 and 25 ms, respectively, and the scan rate was 10,000 Da/s, resulting in a total scan time of 334–413 ms per MRM^3 experiment. To reduce cycle times, LC-MS runs were divided into multiple periods, with the acquisition of peptide-specific MRM^3 experiments limited to its period of elution. Data evaluation was performed using Analyst. For MRM^3 transitions, signal integration was based on the extracted ion chromatograms (XIC) from −0.5 up to +1.0 Da around the m/z of the second product ion. All peaks were smoothed by three points before integration.

For further details regarding the HPLC gradient, source parameters, MRM, and MRM^3 experiments, the reader is referred to Tables S1 and S2 in the supplementary material.

3. Results and discussion

3.1. Efficiency and completeness of the extraction procedure

With the aim of presenting a method suitable for the routine analysis of food allergens, our study needed a robust and efficient extraction procedure that also worked for complex food products, where allergenic proteins partially bind to to matrix components. We therefore utilized a chaotropic, high-molarity urea buffer, as it was proposed for the extraction of thermally processed food protein by various authors [35–37], in combination with a high-speed stirrer for a quick two-minute manual extraction. Following this procedure, extracted allergens are obtained in a denaturing buffer that can be directly applied to preparation of the tryptic digest without need for a buffer change or precipitation.

To investigate efficiency and completeness of the extraction, protein concentrations in the extracts were analyzed in triplicate by Bradford assay and correlated to the total protein content in the nuts without any bias from extraction, which was determined via Kjeldahl method in duplicate. The experimental results, presented in Fig. 1A, demonstrated an extraction efficiency of 80–113% for all analyzed nuts, with protein concentrations in the range of 24–47 mg/mL. Given the limits of variation around 100% and the different molecular principles between the Bradford and the Kjeldahl assays, we conclude an effective and largely exhaustive extraction. Furthermore, SDS-PAGE analysis (Fig. 1B) displayed all relevant seed storage protein bands in the extracts, including strong signals in the MW range of the 11S legumins, the parent protein family of most proteotypic peptides used as markers in this study.

3.2. Release and stability of tryptic peptides

Exhaustive digestion of allergenic proteins to tryptic peptides is required for reproducible LC-MS analysis. Furthermore, the chosen marker peptides must not be prone to unspecific cleavage or degradation during digestion. Such decay would certainly impair the quantification of food allergens in matrix samples and has been described for tryptic peptides in several biological samples before [23,24,38,39].

To improve our understanding of the influence of tryptic digestion time on peptide recovery, we monitored the time-dependent release and degradation of all 18 proteotypic marker peptides included in the presented method (Table 1). An extract obtained from blended nut flours (multi nut extract) and an extract of cookies, incurred and baked with nuts as described in the experimental section, were each incubated

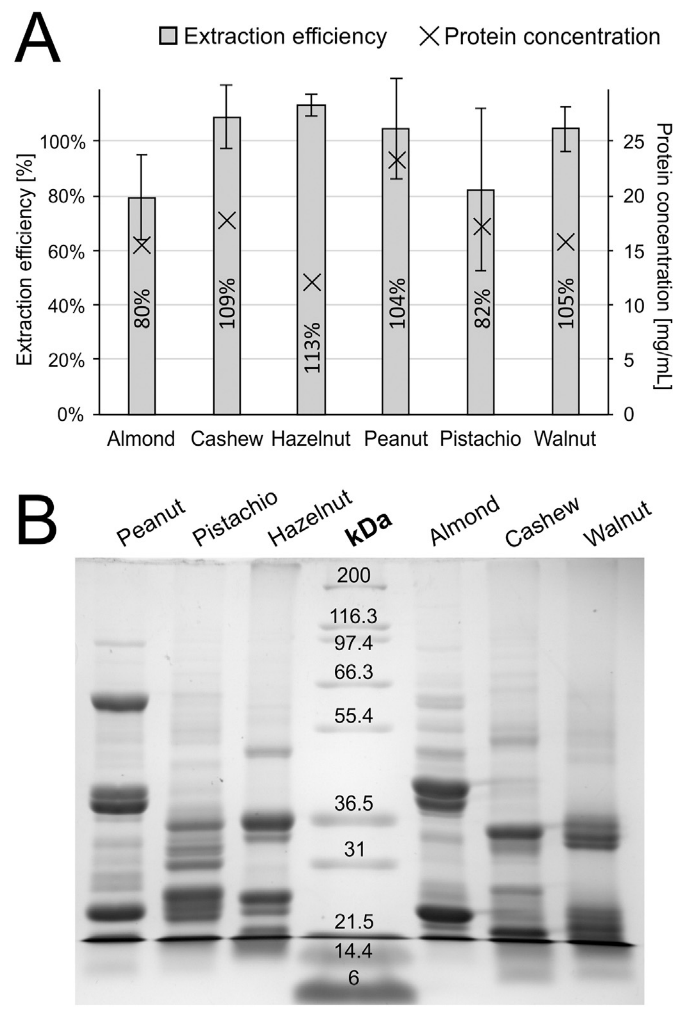
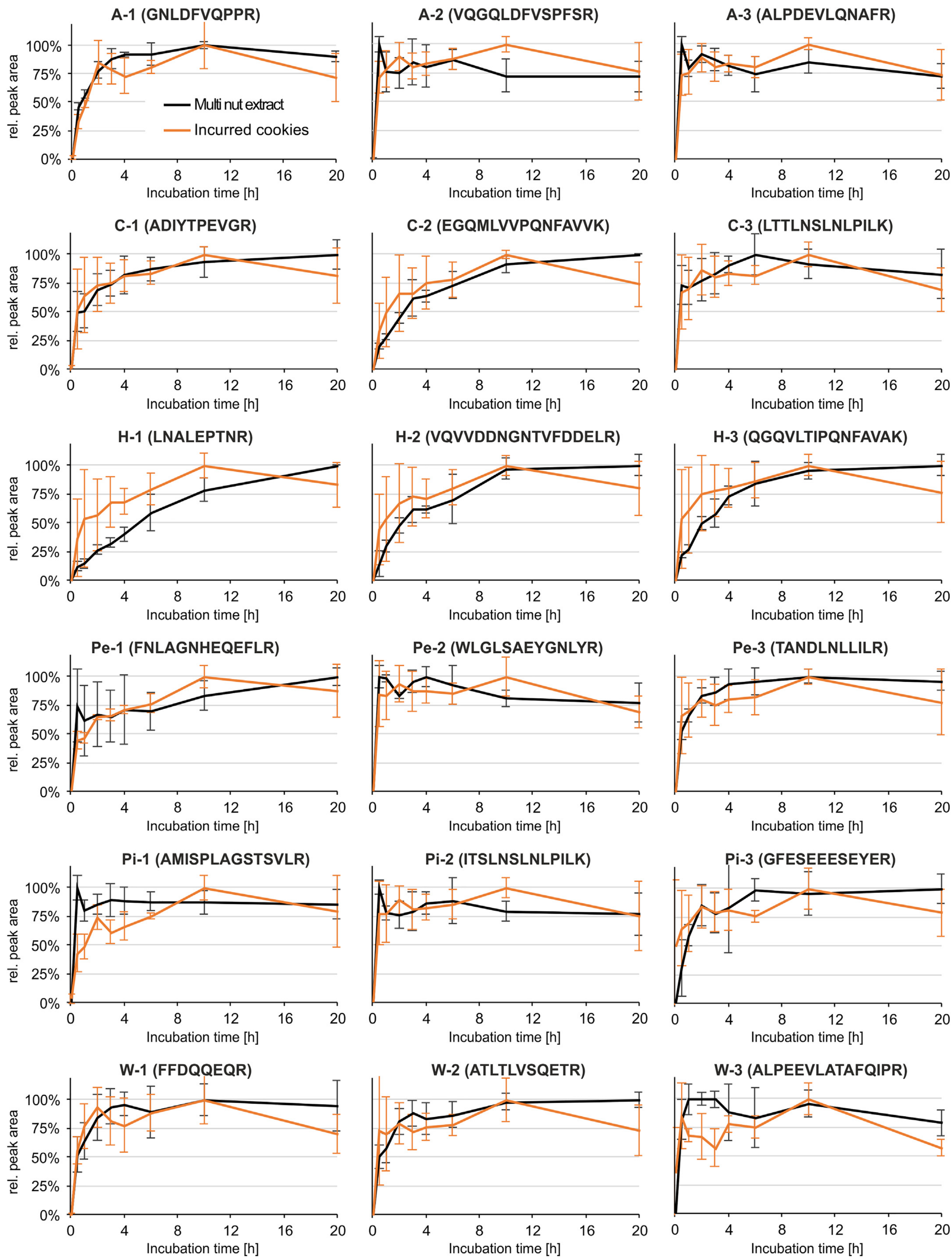


Fig. 1. Extraction of allergenic nuts. (A) Extraction efficiency, calculated as the protein concentration of the extracts (via Bradford assay, given on secondary axis) divided by the protein content of the respective nut (via Kjeldahl method). (B) Laemmli SDS-PAGE (12%) analysis, 15 µg protein per lane.

Table 1
Peptide markers.[a]

Peptide[a]	Sequence	Allergen
A-1	GNLDFVQPPR	Pru du 6
A-2	VQGQLDFVSPFSR	Pru du 6
A-3	ALPDEVLQNAFR	Pru du 6
C-1	ADIYTPEVGR	Ana o 2
C-2	EGQMLVVPQNFAVVK	Ana o 2
C-3	LTTLNSLNLPILK	Ana o 2
H-1	LNALEPTNR	Cor a 9
H-2	VQVVDDNGNTVFDDELR	Cor a 9
H-3	QGQVLTIPQNFAVAK	Cor a 9
Pe-1	FNLAGNHEQEFLR	Ara h 3
Pe-2	WLGLSAEYGNLYR	Ara h 3
Pe-3	TANDLNLLILR	Ara h 3
Pi-1	AMISPLAGSTSVLR	Pis v 5
Pi-2	ITSLNSLNLPILK	Pis v 5
Pi-3	GFESEEESEYER	Pis v 5
W-1	FFDQQEQR	Jug r 2
W-2	ATLTLVSQETR	Jug r 2
W-3	ALPEEVLATAFQIPR	Jug r 4

[a] Species are abbreviated to initial letters (e.g., Pe = peanut).



(caption on next page)

Fig. 2. Time course of peptide release and degradation for 18 proteotypic peptides from six allergenic nuts, using a multi nut extract (black curves) and nut-incurred cookies (orange curves) for incubation with trypsin. Error bars indicate relative standard deviation of inter-day duplicate experiments, which were both measured in triplicate. For exact values, the reader is referred to Table S3 in the supplementary material.

with trypsin at intervals from 0.5 to 20 h. Progress of the tryptic digestion was determined via LC-MS/MS in MRM mode, using the most sensitive MRM-transition of each proteotypic peptide as quantifier and a further two qualifying transitions (Table S2, supplementary material). The whole experiment was repeated twice on different days. Each sample was measured in triplicate, peptide signals were averaged and normalized to the maximum value of the respective experiment.

The time courses given in Fig. 2 demonstrate a rapid increase in peptide concentrations, indicating an early digestion and peptide release in both sample types. In the multi nut extract, 12 out of 18 peptides reached a relative concentration above 85% of their maximum after an incubation time of 4 h or earlier, and four more peptides (C-1, C-2, H-2, H-3) reach this threshold after 6 or 10 h. This includes peptides that contain a high amount of proline (A-1) or acidic residues (H-2, Pi-3) in vicinity to the cleavage site, which has been discussed by various authors as a source of reduced digestion rates [40–42]. Only peptides H-1 and Pe-1 showed a significant signal increase after 10 h, demonstrating a reduced release kinetic from the parent allergen. Similar peptide release curves were in general obtained for the incurred cookie matrix, where allergen concentrations were considerably lower (604.6 mg/kg of each nut, respectively) compared to the multi nut mix (each nut diluted 1:5, ≙166,667 mg/kg) and which had been subjected to thermal processing before. A slightly faster formation was observed for several of the more slowly released peptides (e.g., H-1), which might result from the overall lower protein content in cookies compared to nuts. While no peptide showed a substantial trend to instability in the multi nut extract, some peptides showed a tendency to decreased signal intensity at later points. However, these results come at a rather high standard deviation between the two repetitions of the experiment, which will certainly also have an impact on absolute quantification.

To elucidate whether the observed digestion kinetics and peptide stabilities were representative for tryptic peptides in general, the same analysis was carried out for a selection of other tryptic peptides from the selected nuts that had been rejected from method development at an earlier stage for reasons such as insufficient sensitivity or matrix interference. Interestingly, we observed that some peptides were indeed markedly unstable in the digestion solution, which is exemplarily shown in Fig. 3 for the proteotypic peptides TSVLGGMPEEVLANAFQISR, IPSGFISYILNR and DLPNECGISSQR from cashew, peanut, and walnut, respectively. Peptides showing such digestion behavior are not appropriate to be used as markers for allergenic proteins, since the decrease in signal over time would certainly affect the sensitivity and reproducibility of allergen determination.

Altogether, our results demonstrate that the kinetics of tryptic peptide formation and degradation or potential interference with the food matrix in this regard may influence allergen quantification and have to be considered as a criterion for marker selection. For the marker collection used in this study, an optimum of 14 h incubation time was chosen for the following studies on matrix effects and thermal processing to consider delayed proteolytic release as for the marker peptides H-1 and Pe-1 as well as reduced signal intensities of some markers at later points.

To exclude side-specificities of the used trypsin, the experiment was also repeated for multi nut extract using trypsin from a different vendor (Sequencing Grade Modified Trypsin, Promega, Mannheim, Germany). Only marginal differences were observed compared to the digestion kinetics of Serva trypsin (data not shown), which is in agreement with the large-scale study on trypsin specificity as a source of variability in proteomics reported by Walmsley et al. [43].

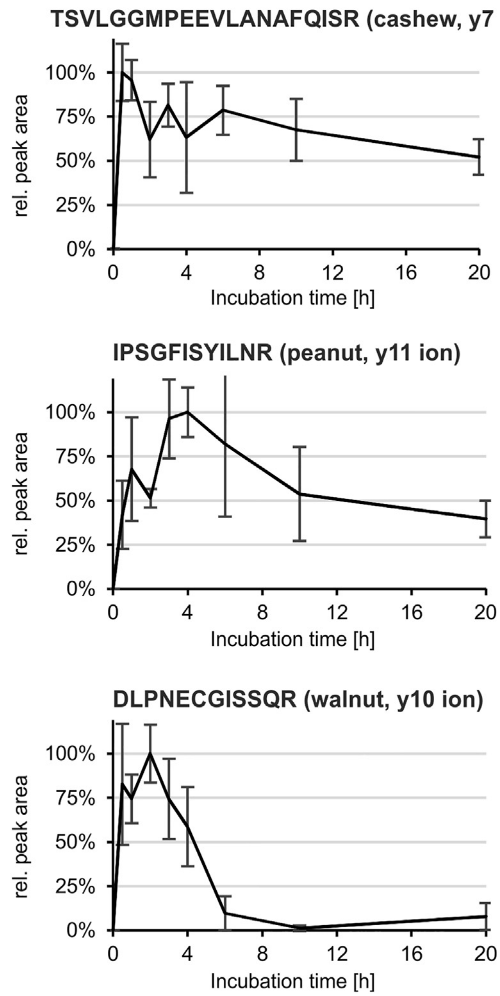


Fig. 3. Time course of peptide release and degradation for proteotypic peptides that show a delayed release or instability and were therefore rejected during method development. Data were obtained by tryptic digestion of a multi nut extract. Error bars indicate relative standard deviation of inter-day duplicate experiments, which were both measured in triplicate.

3.3. *Impact of the food matrix*

For the quantitative determination of food matrix effects, we followed the experimental scheme outlined in Fig. 4. Bakery (self-made bread and cookies) and chocolate products (allergen-free milk and dark chocolate) were used as two relevant groups of food matrices with a substantially different chemical composition and a high probability of contamination with nuts. Fortified matrix extracts were obtained by spiking 90% matrix with 10% of one single nut (*w*/w) prior to extraction. These fortified extracts were then mixed and diluted with blank matrix extract to a final concentration of 100 mg of each nut per kg of matrix (Fig. 4A). This way, a low-level contamination with nuts was simulated in a manageable way, covering a concentration that is well detectable by the applied method and clearly exceeds recently proposed reference doses [8]. Each such "fortified matrix sample" was prepared in triplicate and then subjected to tryptic digestion, so that matrix effects on peptide detectability during the full remaining sample preparation were covered. On the other hand, we also prepared blank matrix digests that were spiked with digested nut extracts directly

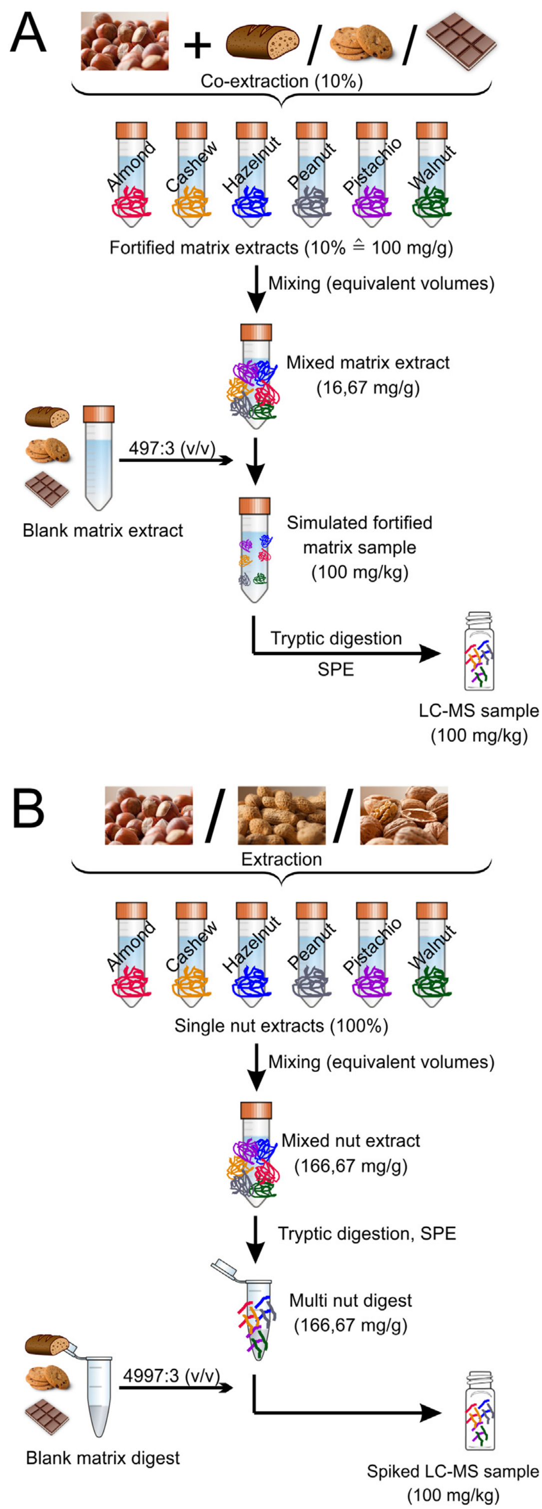


Fig. 4. Experimental workflow for the elucidation of food matrix effects through preparation of matrix samples (A) fortified prior to and (B) spiked after sample preparation.

before measurement, again yielding a final concentration of 100 mg/kg (Fig. 4B). These "spiked matrix samples" were prepared in duplicate and, due to their late spiking, only comprised matrix effects arising during the LC-MS measurement. To minimize bias of the LC-MS/MS data by unspecific signals, we used the multiple reaction monitoring cubed (MRM^3) mode for quantification, which offers dramatically increased specificity compared to MRM, particularly at such low allergen concentrations [17]. Two MRM^3 transitions were monitored for each peptide, one for quantification and one for additional qualification (Table S2, supplementary material). In addition, to each series of measurements, a reference sample of digested multi nut extract was analyzed, which was diluted according to Fig. 4B, using 3% acetonitrile instead of matrix digest, and every peptide signal was normalized to this reference to determine the recovery rate.

The dashed columns given in Fig. 5 illustrate the effects on peptide detectability caused by interference of the food matrix with the LC-MS/MS measurement, e.g., by suppression or enhancement of analyte ionization (spiking of matrix **after** sample preparation). Recovery rates in the bakery products (Fig. 5A) were close to 100% for most peptides, with a few positive (A-2, Pe-2, Pe-3, Pi-1, W-2, W-3) and two negative outliers (H-1, H-2) of up to 50%. A more diverging distribution was observed for the chocolates (Fig. 5B), where some peptides showed a considerable signal increase of 50–150% (A-2, C-3, Pe-2, Pi-2, W-3) while multiple others were subject to matrix suppression by 30–50% of the reference signal (C-1, C-2, H-1, H-2, H-3, Pe-1, W-1). Substantially different results were obtained for those samples that were fortified **prior to** sample preparation (filled columns), and thus reflect the impact of matrix on all steps of sample preparation, including extraction, digestion, and solid phase extraction (SPE). For bread and cookie matrix, the signal of most peptides increased compared to the samples spiked after sample preparation, including several peptides that showed a considerable signal increase of > 50% (C-1, C-2, H-1, H-2, H-3, W-2), and in no case a significant reduction was observed. This outcome was completely different for the chocolates, where recovery rates dropped in case of every peptide, in the most extreme cases down to 15–30% related to the reference (H-1, H-2, H-3, W-1).

Taking into account the differences between fortification with matrix prior to and after sample preparation, the observed recovery rates cannot be explained solely by coelution of matrix components in LC-MS. Instead, the presence of food matrix apparently exerts an amplifying (bakery products) or a suppressing effect (chocolates) during sample preparation. Our data demonstrate, that this impact of matrix on peptide recovery is specific for a certain peptide, since peptides originating from the same protein did not exhibit a consistent behavior. It is, therefore, concluded that the observed effects do not predominantly originate from discrimination during protein extraction, as this would certainly affect all peptides of a given protein to the same extent. Other possible sources of discrimination include the rates of tryptic digestion, stability of tryptic peptides in the presence of matrix compounds, and purification by SPE. Notably, the exceptionally high recovery of peptide H-1 in bread and cookies could in parts be explained by the faster digestion depicted in Fig. 2. The mechanism underlying this huge increase in signal could, however, not be elucidated in our study.

3.4. Effects of thermal processing

Analyzing the effects of thermal processing on proteotypic peptide recovery, we pursued a similar strategy as to the determination of matrix recovery rates. Co-processed matrix extracts were prepared, as depicted in Fig. 6, by the extraction of bread and cookies that were incurred with nuts prior to thermal processing. The exact content of nuts was calculated based on the weight after baking, and extracts were further diluted with nut-free matrix extract to a concentration of 100 mg of nut per kg of matrix. Sample preparation and LC-MS measurements were carried out in triplicate and in MRM^3 mode again, and all peptide signals were normalized to a concomitantly run reference sample prepared as depicted in Fig. 4A, so that the only difference between sample and reference was thermal treatment.

The recovery rates, illustrated in Fig. 7, demonstrate a considerable impact of the baking process on peptide detectability. In the cookies, which were baked for 15 min, signals dropped to 40–80% relative to the unprocessed matrix samples. Loss of detectability was in all samples

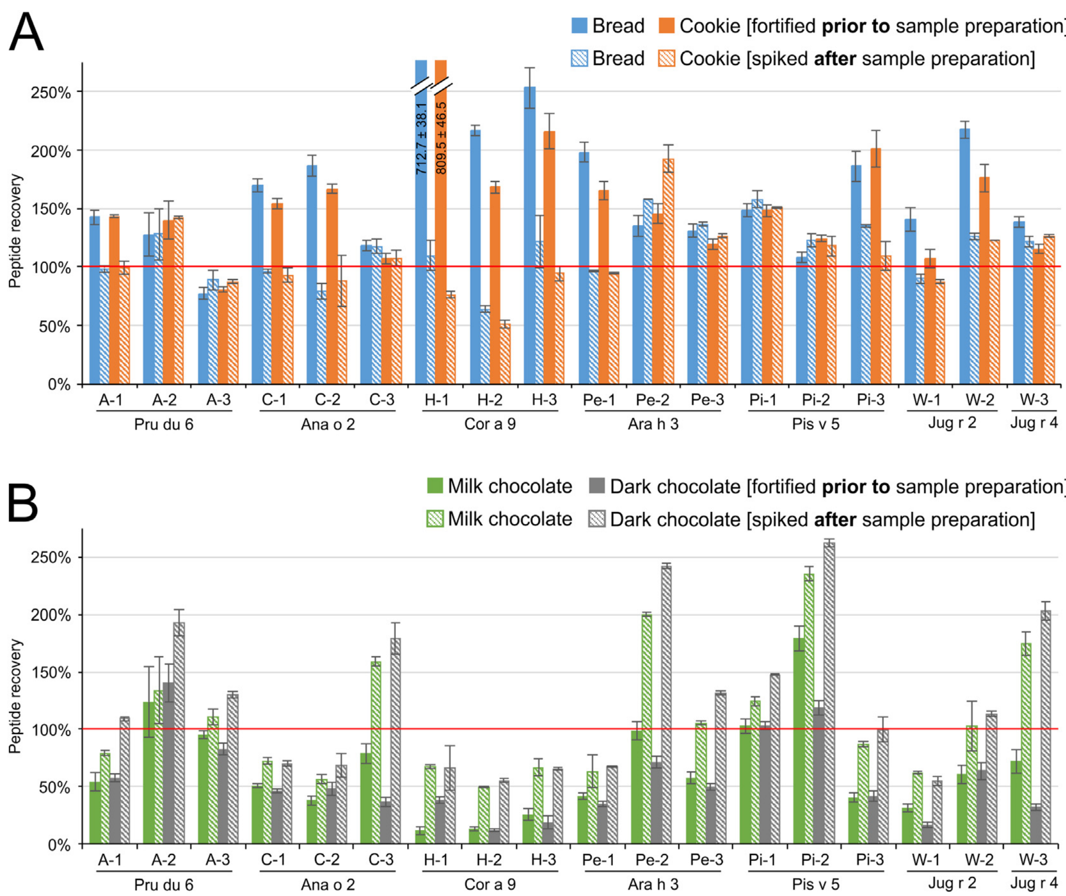


Fig. 5. Impact of different bakery products (A) and chocolates (B) on the detectability of proteotypic peptides. Recovery rates were calculated from peak areas of quantifier MRM^3 transitions normalized to a matrix-free reference sample of digested multi nut extract, which was diluted to the same concentration and run concomitantly. Filled columns represent samples prepared according to Fig. 4A (triplicates) and dashed columns according to Fig. 4B (duplicates), respectively. Parent proteins are indicated below the peptide acronyms. Error bars indicate relative standard deviation. For exact values, the reader is referred to Table S4 in the supplementary material.

increased after baking for 45 min in bread matrix, where the relative signals ranged between 17 and 65%. Different to the effects caused by the food matrix during sample preparation (Fig. 5A, filled bars), thermal processing did not affect recovery rates of each peptide individually. Instead, peptides originating from the same parent protein showed a comparable signal suppression. This is particularly apparent for the peptides from walnut (W-1 and W-2 compared to W-3), which are, in contrast to the marker peptides of all other nuts, products of different parent proteins.

It is consequently assumed that the suppressive effect induced by thermal processing mainly results from a reduced protein extractability and not from the chemical modification of single proteotypic sequence elements. Reduced solubility has already been described for allergens from roasted peanuts [44] and walnuts [34], and utilization of a chaotropic buffer, as in this study, has been proposed as the most effective option for their extraction [35]. Notably, all marker peptides from our study are derived from cupin allergens (11S legumins and 7S vicilins), which have been discussed to show a higher tendency of aggregation and matrix binding, following thermal processing, than 2S albumins, the third important family of seed storage proteins in nuts [31,34,35,44,45]. 2S albumins are, however, less abundant in most nuts and, mostly, do not provide equally sensitive peptide markers [19].

3.5. Implications for the quantification of food allergens by LC-MS

SIL peptides as internal standards are progressively proposed as a means to compensate matrix effects for the absolute quantification of food allergens by LC-MS [25,26]. Our results concerning the impact of food matrices at different stages of the LC-MS method (Fig. 5) strongly indicate that such a standard (SIL peptide) would certainly be affected differently by the food matrix compared to the analyte (allergenic protein). A similar result was reported by Planque et al., who spiked SIL peptides to allergen samples prior to and after sample preparation, respectively, and found that the labeled peptides were only suitable to correct matrix effects directly affecting the LC-MS/MS analysis itself but are not useful standards compensating effects linked to the extraction and digestion steps [21]. We therefore conclude that SIL peptides used as sole internal standards would not sufficiently compensate the latter matrix effects, and that a large uncertainty would remain for the quantification of allergenic contaminants. Using SIL proteins as internal standards instead, which has been referred to as the "Gold Standard" for quantitative targeted proteomics [22], could be an option to deal with some of the observed matrix effects during sample preparation, though at considerably high costs. SIL extended peptides, representing a proteotypic peptide along with its chemical environment in the allergen, could be another, possibly cheaper alternative.

All mentioned options for calibration, however, cannot compensate the impact of thermal processing on peptide recovery, which presents an even more severe issue to food allergen quantification (Fig. 7). Here, a considerable difference between the baking of bread (45-min baking) and cookies (15-min baking) was observed, which in each case had a negative impact on peptide intensities in LC-MS, but to an overall stronger degree in bread. While the presented data show that the decrease in signal may vary between different bakery products, other

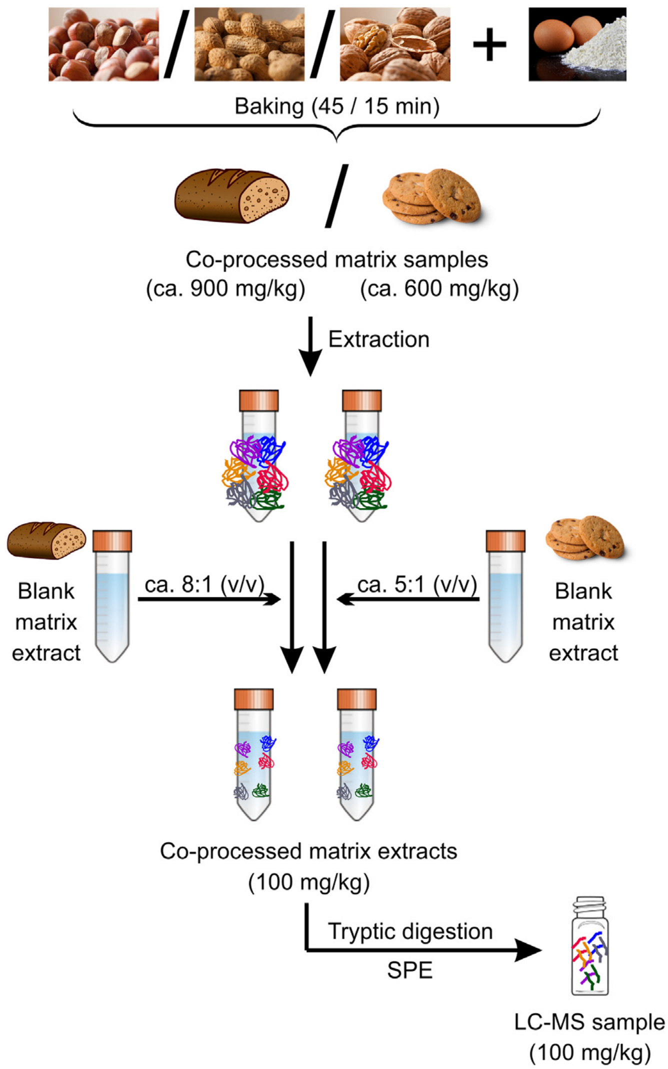


Fig. 6. Preparation of matrix samples that were co-processed with nuts, analyzing the impact of thermal processing on peptide recovery.

studies have reported similar results on the effects of boiling, roasting, and frying of food allergens, three modes of thermal processing, which result in different aggregate structures [31] and LC-MS recovery rates [35]. Beyond that, it has been repeatedly reported that thermal processing has a differential impact on the LC-MS detectability of different allergens in the same food [34,35], an observation that was also made for the walnut allergens Jug r 2 and Jug r 4 in this study. While some allergen families (e.g., cupins) appear to be more strongly affected than others (e.g., 2S albumins), the effect of a particular processing technique on the recovery rate of an individual protein can obviously not be predicted a priori. The multitude of case-specific variations (allergen; mode, temperature, and duration of heating) will certainly lead to some uncertainty and complicate an absolute quantification of allergenic contaminations in foodstuffs for all proposed analytical techniques.

All allergenic nuts analyzed in this study are frequently consumed as ingredients of complex and highly processed foods, e.g., in cereal bars, spread, muesli, pastries, chocolate, or marzipan. Notably, foods that undergo industrial (thermal) processing are among those with the highest potential for technologically introduced contamination by nuts and, thus, hold a particular interest for the analysis of food allergens. Reduced detectability as a result of thermal processing does, however, not necessarily correlate to reduced allergenicity, since sample preparation conditions differ considerably from the processes that allergens undergo in the human gastrointestinal tract, and the susceptibility of allergen aggregates or complexes to extraction and tryptic digestion will, in consequence, not be equivalent. This point was underlined by a recent study from Downs et al., in which the authors investigated the reactivity of human IgE to roasted walnuts, and found that, although the overall solubility of the allergens dramatically decreased, the insoluble aggregates retained IgE reactivity [46].

With the scientific debate moving towards the establishment of threshold levels, sensitive and quantitative analytical methods are needed for the determination of allergenic contaminations. Given the numerous different impacts on food allergen detectability by LC-MS observed in this study, the absolute quantification of allergens in such food turns out to be challenging, and it appears that a more pragmatic approach is needed to guarantee that these products are safe for allergic consumers. Provided that allergen threshold levels are available, a safe approach to their monitoring could imply the consideration of specific recovery and processing factors for the analytical results, reflecting matrix interferences and the specific production history of food products, as they are already applied to the analysis of pesticides [48].

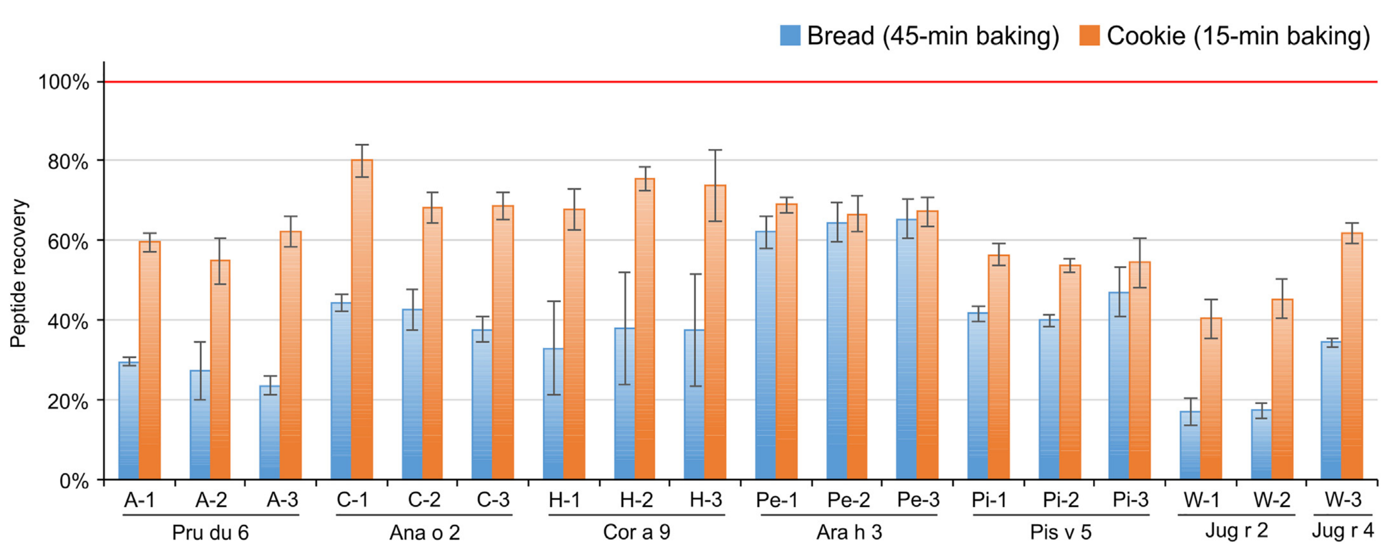


Fig. 7. Impact of thermal processing (baking) on peptide detectability. Recovery rates were calculated from peak areas of quantifier MRM^3 transition normalized to a concomitantly run reference sample fortified prior to sample preparation. Parent proteins are indicated below the peptide acronyms. Error bars indicate relative standard deviation. For exact values, the reader is referred to Table S5 in the supplementary material.

Such factors should, of course, be determined experimentally and differentiate between various treatments, food matrices, and allergenic ingredients.

4. Conclusions

We hereby present a comprehensive survey of the central parameters that influence LC-MS-based allergen analysis and draw conclusions towards the source of variation in quantitative results.

The impact of the food matrices on the detectability of food allergen marker peptides are shown to significantly differ between chemically heterogeneous foods, with recovery rates of the 18 studied marker peptides from nut allergens ranging mostly between 15 and 250%. While a, by trend, increased peptide recovery is observed in bakery products, chocolate matrices rather reduce signal intensities of marker peptides. Moreover, thermal processing is shown to drastically influence the detectability of allergens, leading to a loss in signal of 20–83% after baking of bread and cookies, depending on the studied allergen and the baking time/product type, respectively. As the observed variation of the LC-MS signal cannot be explained solely by matrix effects on peptide ionization, it is concluded to arise from the sample preparation procedure, e.g., from different rates of tryptic digestion in the presence of matrix compounds, and from a reduced protein extractability after food processing, respectively.

Given the results from our study, it is concluded that SIL peptides, which have repeatedly been proposed as internal standards for the exact quantification of allergenic contaminants, by themselves cannot sufficiently compensate the observed matrix and processing effects. Considering the challenges demonstrated for the absolute quantification of food allergens by LC-MS, method development has to bear in mind the inclusion of alternative and novel approaches beyond stable isotope dilution. It will possibly focus on developing a more pragmatic approach to monitor the compliance with future allergen threshold levels, which could involve specific recovery and processing factors to compensate for an a priori unpredictable variation of analytical results.

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Appendix A. Supplementary data

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