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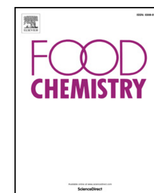

Development of a strategy for the quantification of food allergens in several food products by mass spectrometry in a routine laboratory

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ABSTRACT

Worldwide, mass spectrometry is widely used to detect and quantify food allergens, especially in complex and processed food products. Yet, the absence of a regulatory framework for the developed methods has led to a lack of harmonization between laboratories. In this study, ten allergens were analyzed in eight food products by UHPLC–MS/MS, in order to establish criteria for the retention time, variation tolerance, the ion ratio deviation, and the signal-to-noise ratio for allergen detection. The set of criteria should help laboratories to compare results and avoid false positives and negatives. Furthermore, a strategy combining standard addition and labeled peptide correction was used to quantify milk, soy, peanut, and egg allergens in eight food products. This strategy is particularly interesting for routine laboratories, which receive hundreds of samples and cannot use an external calibration curve for each sample.

1. Introduction

Food allergy is a pathological disorder of the immune system, affecting 5% of adults and at least 8% of children in western countries (Sicherer & Sampson, 2014). After an adverse reaction, the allergic population must strictly avoid consuming the offending food. Food labeling must thus be clear and reliable (Taylor & Hefle, 2006). EU legislation requires the declaration of 14 allergens (and products thereof) on food labels when they are incorporated as ingredients: milk, eggs, cereals containing gluten (wheat, rye, and barley), fish, crustaceans, mollusks, tree nuts (almonds, hazelnuts, walnuts, cashews, pecan nuts, Brazil nuts, pistachio, macadamia), soy, peanuts, sesame, lupin, mustard, celery, and sulfur dioxide (sulfites) (European Commission, 2011). Another important risk for food-allergic consumers, however, is the presence of hidden allergens due to cross-contaminations during food processing. The absence of a regulatory framework for managing hidden allergens and a lack of legal action thresholds have prompted the food industry to make excessive use of precautionary allergen labeling (PAL), leading to a loss of consumer trust (Allen, Remington, et al., 2014; Allen, Turner, et al., 2014; DunnGalvin et al., 2015; Pele, Brohée, Anklam, & Van Hengel, 2007).

Recently, various countries have set legal thresholds (e.g., Switzerland, Germany, Belgium, and the Netherlands), but considerable disparity is observed among these thresholds. In Australia and New Zealand, the Voluntary Incidental Trace Allergen Labeling (VITAL) system establishes eliciting doses (EDs) based on clinical studies for the protection of at least 95% of allergic people (ED_{05}) (Allen, Remington, et al., 2014; Allen, Turner, et al., 2014; Taylor et al., 2014). VITAL thresholds, which have no regulatory status, are set at 0.75 mg per kg for egg proteins, 2.5 mg per kg for milk or tree nut proteins, 5 mg per kg for peanut proteins, 25 mg per kg for soybean proteins, and 50 mg per kg for cashew proteins (portion size: 40 g). Laboratories often use them as target sensitivity thresholds to be reached by allergen detection methods.

Several methods have been developed for the sensitive detection of multiple allergens in processed or unprocessed matrices (Gomaa & Boye, 2015; Heick, Fischer, & Pöpping, 2011; Korte, Lepski, & Brockmeyer, 2016; Pilolli, De Angelis, & Monaci, 2017; Planque, Arnould, Dieu, et al., 2017). Per kilogram of incurred chocolate, for example, limits of quantification of 0.2–0.4 mg for milk, 1.0–4.0 mg for soy, 2.5–4 mg for peanut, and 1–3 mg for tree nuts have been obtained (Gu et al., 2018). In another study, a limit of detection (LOD) of 10 mg ingredient per kg was obtained for egg white, skimmed milk, peanut, soy, and tree nuts (almond, Brazil nut, cashew, hazelnut, pecan, pine nut, pistachio, and walnut) in incurred bread (40 min – 180 °C) or cookies (18 min – 180 °C) (New, Schreiber, Stahl-Zeng, & Liu, 2018). Boo et al. detected down to 5 mg egg, milk, or peanut ingredient per kg incurred sugar cookies (25 min – 190 °C) (Boo, Parker, & Jackson,

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2018). In processed cookies, tomato sauce (45 min at 95 °C), chocolate, and ice cream we have detected target allergens at 0.5 mg/kg for milk proteins, 2.5 mg/kg for peanut, hazelnut, pistachio, and cashew proteins, 3 mg/kg for egg proteins, and 5 mg/kg for soy, almond, walnut, and pecan proteins (18 min at 180 °C) (Planque, Arnould, Dieu, et al., 2017).

Although there is still room for improving allergen detection, one of the main current challenges is allergen quantification. An AOAC guideline (SMPR 2016.002) entitled "Standard Method Performance Requirements for Detection and Quantitation of Selected Food Allergens" has been published for the detection and quantification of allergens by mass spectrometry (MS). This guideline specifies a recovery of 60–120% and a relative standard deviation (RSD) of 20% (Paez et al., 2016). However, it is important to examine whether the strategies commonly used to quantify allergens in food matrices meet the AOAC specifications (Planque, Arnould, & Gillard, 2017).

Two main strategies are used in this area. The first does not use labeled internal standards. The concentration of the target allergen in a sample is determined with an external calibration curve (usually matrix-matched) or by standard addition, i.e. by adding known amounts of an allergen standard solution directly to the sample. After peptide analysis by UHPLC–MS/MS, a calibration curve is drawn and the allergen concentration in the sample is determined (Planque, Arnould, & Gillard, 2017). A study by Pilolli et al. used this approach in which the authors compared peak areas of milk, egg, soy, peanut, and hazelnut marker peptides in fortified cookies (raw ingredients added to the matrix after the process) and spiked cookie extracts (mixed allergen solution added to the matrix). Fortification/spiking was done at 300 and 600 mg ingredients per kg cookies and recoveries ranged from 51 to 95% (Pilolli, De Angelis, & Monaci, 2018). These recoveries do not totally meet the AOAC specifications, despite the lack of thermal processing, the use of the same food matrix, and the high concentration of allergens in the samples.

The second strategy is based on labeled peptide or protein quantification. It involves adding labeled peptides or proteins as internal standards at different stages of the protocol (prior to extraction, digestion, purification, or injection), in order to correct the peak area of the target peptide by means of the corresponding labeled peptide (Brun et al., 2007). This strategy allows correction of the matrix effect and some protocol steps, depending on the labeled internal standard used (Planque, Arnould, Dieu, et al., 2017). It is increasingly used to quantify allergens by mass spectrometry (Boo et al., 2018; Croote, Braslavsky, & Quake, 2017; Groves, Cryar, Walker, & Quaglia, 2018; Sayers et al., 2018). For example, Boo et al. used a matrix-matched calibration curve and labeled peptides to quantify milk, egg, and peanut allergens by LC–MS/MS in fortified cookies. They obtained a mean recovery of 77 ± 20% (Boo et al., 2018), but the AOAC recovery specifications (60–120%) were not always met, despite the use of labeled internal standards. New et al, who quantified milk, egg, peanut, and hazelnut allergens in several spiked matrices, obtained recoveries ranging from 5.9% to 119.3% after labeled peptide correction (New et al., 2018). The main disadvantage of the 'isotope-labeled peptides strategy' is the absence of correction for losses due to incomplete protein extraction or digestion (Croote & Quake, 2016).

To improve allergen quantification, two main types of labeled internal standard can be used, both of which require digestion: labeled proteins and long isotope-labeled peptides (several labeled peptides are linked together or a few amino acids are added at each end of the target labeled peptide). The prohibitive cost of labeled proteins limits their use, but they are often viewed as the "gold standard", as, in theory, they have the same structure/properties as the corresponding native proteins and thus allow correction of the entire protocol (Brun et al., 2007; Ma, McClatchy, Barkallah, Wood, & Yates, 2017).

Chen et al. compared the use of a short and a long isotope-labeled peptide (respectively VL[$^{13}C_6$,^{15}N]PV[$^{13}C_5$,^{15}N]PQK and QSVLSLSQSKVL[$^{13}C_6$,^{15}N]PV[$^{13}C_5$,^{15}N]PQKAVPYPQRQ) for peptide peak area correction. The latter allowed better recovery (98.8–106.7%) for the quantification of spiked allergens in cookies, probably thanks to correction of the digestion step (Chen et al., 2015). This strategy was tested on a single allergen in a single matrix, but as the recovery was promising, it might be a good alternative to the use of labeled proteins.

Newsome and Scholl, quantified allergenic bovine milk α_{S1}-casein in processed cookies (180 °C for 16 min), using a recombinant ^{15}N-αS1-casein protein (purity > 85%) as internal standard. They obtained recoveries between 60 and 80%, but they did not specify the recoveries obtained with spiked samples (Newsome & Scholl, 2013).

The starting point of the present work was a method previously developed in our laboratory: a sensitive method for detecting ten allergens (egg, milk, soy, peanut, almond, cashew, walnut, pecan nuts, hazelnut and pistachio) in complex and processed matrices (ice cream, sauce (95 °C for 45 min), cookie (180 °C for 18 min), and chocolate) (Planque, Arnould, Dieu, et al., 2017). Aiming to improve its use as a routine qualitative method, we have adapted it to analyze the 10 target allergens in a single run within a day. In order to limit the number of false negatives and positives, we have tested and set confirmation criteria, notably regarding the signal-to-noise ratio (S/N), the tolerance on the retention time, and the tolerance on the relative ion intensity for allergen detection. We have also tested and compared two quantification strategies: (1) using a single calibration curve to quantify allergens in several food products with the help of short or long labeled peptides (see the above-mentioned promising results of Chen et al., 2015) and (2) combining standard addition with the use of labeled peptides as internal standards. This second approach represents the main originality of this work as, to the best of our knowledge, it has never been used before to quantify allergens in several food products. As proof of concept, it was tested on eight spiked matrices and on incurred chocolate dessert matrices, to provide recovery results for "real samples". It allowed quantification of several allergens in several food products belonging to different food categories and is thus quite suitable for allergen quantification in routine laboratories.

2. Material and methods

2.1. Material and reagents

Tris(hydroxymethyl)aminomethane (Tris), urea, dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, and trypsin from bovine pancreas (T8802) were obtained from Sigma-Aldrich (Bornem, Belgium). Acetic acid was obtained from Acros Organics (Geel, Belgium) and hydrochloric acid was from Fisher Chemical (Loughborough, UK). Sep-Pak C18 solid-phase extraction (SPE) columns (6 mL, 500 mg – WAT043395) were used for peptide purification and enrichment and purchased from Waters (Milford, MA). Acetonitrile, 2-propanol, methanol (ULC–MS grade), waters, hexane, and formic acid were from Biosolve (Valkenswaard, the Netherlands). The labeled peptides TANELNLLI**L** [$^{13}C_6$$^{15}N$] R, FFVAPFPEVFG**K** [$^{13}C_6$$^{15}N_2$], GGLEPIN**F** [$D_5$] QTAADQAR, EAFG**V** [$D_8$] NMQIVR, GRFF**V**[$^{13}C_5$$^{15}N$]APFPEVFGKGG**L**[$^{13}C_6$$^{15}N$]EPINFQTAADQARGS, and GREAFG**V**[$^{13}C_5$ ^{15}N]NMQIVRTANE**L**[$^{13}C_6$ ^{15}N]NLLILRGS were from Eurogentec (Seraing, Belgium). Milk powder (NIST1549a 25.64% protein), soy flour (NIST 3234 53.37% protein), peanut butter (NIST 2387 22.2% protein) and whole egg (NIST 8445 48% protein) were obtained from the National Institute of Standards and Technology (NIST) (Gaithersburg, MD). Tree nuts (almonds, cashews, pecan nuts, hazelnuts, walnuts, and pistachios) were purchased from a local store before being finely ground under liquid nitrogen. An Acquity liquid chromatograph coupled with a Xevo TQS triple quadrupole system (Waters, Milford, MA) was used with a C18 Acquity BEH130 column (Waters; 2.1 × 150 mm; ref. 186003556).

2.2. Composition of target matrices

Eight target matrices were selected. Six were purchased from a local store (chocolate, compote, jam, chicken ham, Andalusian sauce and smoked paprika spices) and two were home-made (cookie and mayonnaise). Chocolate (containing 45% cacao, 35% sugar, and 20% rice powder), apple and pear compote (64.9% apple and 35% pear), jam (65% wood fruit and 33% sugar), Andalusian sauce (oil, 15% tomato, egg yolk, glucose syrup, vinegar, 3.5% mustard, sugar, lemon juice, salt, spices, and may contain milk), chicken ham, and spice (smoked paprika) were from the local store. Cookie dough was prepared by mixing 53.4% flour, 15.2% sugar, 16.2% oil, 14.8% water, 0.3% salt, 0.1% ammonium bisulfate, and 0.1% sodium bicarbonate with a blender. Cookies (40 g each) were finally cooked at 180 °C for 18 min. Mayonnaise was prepared by combining 33.3% oil, 33.3% cornstarch, 23.3% mustard, and 10% vinegar. To ensure homogeneity, the matrices were finely ground and then weighed for analysis. The eight target matrices were selected on the basis of the percentage of fat, carbohydrate, or proteins (based on the AOAC triangle (Phillips, Sharpless, & Wise, 2013)). Matrices with a high polyphenol content (ham and compote) or tannin content (spices and chocolate) were also selected to test the capacity of the method to detect and quantify allergens in a wide range of foodstuffs.

2.3. Preparation of labeled internal standard solutions

Stock solutions were prepared by dissolving labeled peptides in DMSO (10 mg/mL) before dilution to 1 mg/mL final concentration in 0.1% formic acid. Stock solutions were stored at −20 °C. Working solution was prepared in 0.1% formic acid with the stock solutions of FFVAPFPEVFGK [$^{13}C_6{}^{15}N_2$] (5.25 μg/mL), EAFGV [D_8] NMQIVR (9.25 μg/mL), TANELNLLI**L** [$^{13}C_6$ ^{15}N] R (9.25 μg/mL), GGLEPIN**F**[D_5] QTAADQAR (9.25 μg/mL), GRFF**V**[$^{13}C_5{}^{15}N$]APFPEVFGKGGL**L**[$^{13}C_6{}^{15}N$] EPINFQTAAD QARGS (10 μg/mL), and GREAFG**V**[$^{13}C_5{}^{15}N$]NMQIVRTANE**L**[$^{13}C_6{}^{15}N$]NLLILRGS (10 μg/mL).

2.4. Preparation of the standard protein working solution

On the basis of NIST or theoretical protein contents in ingredients, a solution (expressed in μg total proteins per mL) was prepared at 75 μg/mL for milk, 112.5 μg/mL for egg, 750 μg/mL for soy, and 375 μg/mL for peanut and tree nuts. The proteins were extracted with 200 mM Tris-HCl; pH 9.2, 2 M urea by shaking at 20 °C for 30 min (Agitelec, France) followed by ultrasound treatment at 4 °C for 15 min. After centrifugation at 4660*g* for 10 min at 4 °C, this solution was used to spike samples prior to applying the protocol.

2.5. Extraction, digestion and purification of samples

The protocol described in Planque et al. was used. Briefly, the proteins contained in 3 g sample, spiked beforehand with 100 μL labeled internal standard solution, were extracted with 30 mL extraction buffer (200 mM Tris-HCl; pH 9.2, 2 M urea), shaken at 20 °C for 30 min (Agitelec, France), and sonicated for 15 min at 4 °C (Planque, Arnould, Dieu, et al., 2017). After centrifugation at 4660*g* for 10 min at 4 °C, the proteins contained in 10 mL supernatant were diluted with 10 mL of 200 mM ammonium bicarbonate. Protein reduction and alkylation were performed with 1 mL of 200 mM DTT (45 min at 20 °C) and 1 mL of 400 mM IAA (45 min at 20 °C in the dark), respectively. Digestion was achieved by adding trypsin (1 mL of 1 mg trypsin/mL in 50 mM acetic acid) and incubated for 1 h at 37 °C. Digestion was stopped by addition of 300 μL of 20% formic acid. Peptides were concentrated and purified on C18 SPE cartridges. Cartridge conditioning was done with 18 mL acetonitrile followed by 18 mL of 0.1% formic acid. After centrifugation of the peptide extract at 4660*g* for 10 min at 20 °C, 20 mL supernatant were loaded on the column and impurities were flushed out with 18 mL of 0.1% formic acid. DMSO (30 μL) was added to avoid dryness in the collector tube before peptide elution with 6 mL acetonitrile/0.1% formic acid (80/20, *v/v*). After evaporation under a nitrogen flow in a water bath set at 40 °C, the peptides contained in the pellets were dissolved in 600 μL of 0.1% formic acid/acetonitrile (95/5, *v/v*). The extracts were centrifuged at 4660*g* for 5 min at 10 °C, transferred to a microtube, and centrifuged again at 11,754*g* for 5 min at 4 °C. The samples were then subjected to UHPLC–MS/MS in order to analyze 10 allergens simultaneously in a single injection run.

2.6. Preparation of test samples

Blank matrices without target allergens were purchased from a local store (compote, jam, chicken ham, Andalusian sauce, and smoked paprika spice) or home-made (cookie and mayonnaise). They were analyzed in triplicate to check the specificity of the method. Afterwards, for each matrix, six samples called "C1 samples" were spiked at VITAL thresholds or lower (LOQs or LODs determined in a previous study (Planque, Arnould, Dieu, et al., 2017): 0.5 mg for milk proteins, 0.75 mg for egg proteins, 5 mg for soy proteins, and 2.5 mg for peanut and tree nut proteins per kg of food product. For each matrix, six samples called "C2 samples" were spiked at concentrations ten times as high as the corresponding C1 levels: 5 mg for milk proteins, 7.5 mg for egg proteins, 50 mg for soy proteins, and 25 mg for peanut and tree nut proteins per kg of food product. To determine the recovery, standard addition was performed on the six samples at 0, 0.5, 1, 2.5, 5, and 10 mg/kg for milk proteins, 0, 0.75, 1.5, 3.75, 7.5, and 15 mg/kg for egg proteins, 0, 5, 10, 25, 50, and 100 mg/kg for soy proteins, and 0, 2.5, 5, 12.5, 25, and 50 mg/kg for peanut and tree nut proteins. The eight food matrices were spiked (addition of extracted allergens after the thermal process), in order to avoid variation factors, such as the degradation of proteins by the thermal process. Standard addition was performed in triplicate for compote (C2 samples), in order to determine the experimental concentration of milk, egg, peanut, and soy proteins and to calculate the relative standard deviation (RSD) between replicates.

2.7. Analysis of peanut in chocolate dessert samples

Chocolate dessert matrices were produced by the University of Manchester in the framework of the "Integrated Approaches to Food Allergen and Allergy Management" (iFAAM) project. Chocolate matrices containing 0, 2, 4, 10, and 30 mg peanut proteins per kg were quantified.

2.8. UHPLC–MS/MS parameters for peptide analysis

Peptide separation was performed with an Acquity system (Waters, Milford, MA) on a C18 Acquity BEH130 Waters column (2.1 × 150 mm) at 50 °C at 0.2 mL/min. Peptide elution was carried out for 26 min as follows: 0–3 min: 92% **A**; 3–18 min: 92%–58% **A**, 18.0–18.1 min: 58% –15% **A**; 18.1–22.5 min: 15% **A**; 22.5–22.6 min: 15%–92% **A**, 22.6–26 min: 92% **A** (solvent **A**: 0.1% formic acid; solvent **B**: acetonitrile plus 0.1% formic acid). After sample analysis, the UPLC column was flushed with methanol/2-propanol/acetonitrile/water (25/25/25/25, v/v/v/v) for 15 min before returning to the initial conditions (92% solvent **A**) for 5 min, in order to avoid carry-over. MRM detection in positive electrospray mode was performed with a Waters Xevo TQS triple quadrupole system. The cone nitrogen flow was set at 150 L/h, the collision gas flow at 0.12 mL/min, the capillary voltage at 2.0 kV, and the source temperature at 150 °C. The desolvation temperature was set at 500 °C and the nitrogen flow at 1200 L/h.

3. Results and discussion

3.1. Selection of marker peptides

The sensitivity of the method was determined in a previous study for milk (casein and whey), egg (white and yolk), peanut, soy, and tree nuts (almond, hazelnut, walnut, pecan nuts, cashew and pistachio) in processed and complex food products (cookie (180 °C for 18 min), sauce (95 °C for 45 min), chocolate (tannin) and ice cream (fat)) (Planque, Arnould, Dieu, et al., 2017). The limits of quantification (LOQs), considering a signal-to-noise ratio above 10, were 0.5 mg/kg (casein) and 5 mg/kg (whey) for milk proteins, 2.5 mg/kg for peanut, hazelnut, pistachio, and cashew proteins, 3 mg/kg (egg white) and 60 mg/kg (egg yolk) for egg proteins, and 5 mg/kg for soy, almond, walnut, and pecan proteins (Planque, Arnould, Dieu, et al., 2017).

Most studies focus on detecting allergens in matrices containing a high percentage of carbohydrates (Planque, Arnould, & Gillard, 2017). Only a few have been tested on high-fat matrices such as poultry meat products (sausage or pâtés (25–30% fat)) (Montowska & Fornal, 2018). Routine methods must be able to detect allergens in all kinds of foodstuffs, including products with a high fat content (e.g., mayonnaise, sauce), a high protein content (e.g., meat, fish), or even both.

The main goals of the present study were (1) to set retention time, ion ratio and signal-to-noise ratio criteria for the detection of allergens in different categories of food matrices (cookies, chocolate, compote, jam, spice, chicken ham, mayonnaise and Andalusian sauce) and (2) to propose an allergen quantification strategy suitable for routine laboratories. To allow simultaneous analysis of 10 allergens in a single UHPLC–MS/MS run, we have retained only two to five of the most abundant peptides per allergen, with two MRM transitions per peptide, (Table 1). This low number of peptides/transitions contrasts with previous studies, where the main goal was to select abundant peptides for allergen detection in incurred and processed matrices.

While peptide selection and determination of method sensitivity were – and must be – performed with incurred and processed matrices, it is of utmost importance to work with spiked matrices, in order to validate the quantification strategy and to determine the percentages of recovery. Spiked matrices are not representative of "real food matrices", but 100% recovery is expected as there is no degradation of proteins (e.g., thermal processing) and they allow validation of the best quantification strategy.

3.2. Determination of the sensitivity

The ten allergens were analyzed in the eight target matrices by scanning two transitions per peptide for 35 selected peptides (Supplementary Material – Fig. 1). Analysis of the Andalusian sauce confirmed the presence of milk and egg allergens, as indicated on the label. Consequently, the sensitivity and specificity of milk and egg peptide detection could not be determined in this matrix. The LOQs obtained in spiked samples were compared with the LOQs previously determined in the four incurred and processed food products, as reported in Table 1 (Planque, Arnould, Dieu, et al., 2017). Although lower LOQs might have been expected in spiked matrices where the proteins are not affected by the thermal process, higher LOQs were observed in some matrices. This phenomenon was notably the case of the EAFGVNMQIVR soy peptide in sauce, mayonnaise, and paprika spice (Fig. 1). It was observed for 20.7% of the determined LOQs (56 out of 270) and was mostly associated with high-fat matrices (mayonnaise, 6.3%, and sauce, 4.1%) and spice (6.3%) (Supplementary Material – Fig. 2).

Nevertheless, the LOQs of egg yolk peptides were approximately 4 times lower in spiked than in processed cookies (Supplementary Material – Fig. 1). This result indicates that selection of marker peptides for allergen detection and determination of method sensitivity should always be done in processed matrices. Yet, as described above, standard addition (the matrix is spiked with target allergen after the process) should also be systematically performed during the first analysis of a food product, in order to guarantee detection of allergens at the LOQ. One should also note that differences have been observed between matrices that might be considered "comparable". In one study, for instance, the LOD for the β-casein peptide GPFPIIV was found to vary from 0.09 to 0.23 mg/L between five commercial white wines (Losito, Introna, Monaci, Minella, & Palmisano, 2013). Consequently, standard addition should also be done in the case of "similar" food products.

3.3. Acceptance criteria for positive samples

To declare a sample as positive or negative, several criteria must be taken in to account. The main question, largely debated among laboratories, is whether a sample should be considered positive when a single allergen peptide is detected or whether at least two peptides should be detected. To answer this question, different parameters must be considered, such as the signal-to-noise ratio (S/N), the tolerated retention time deviations, and the relative ion intensity.

We analyzed by UHPLC–MS/MS the eight complex and/or processed matrices spiked with ten different allergens at six concentrations (Supplementary Material – Fig. 1). To assess the rate of false positives, blank (allergen-free) matrices were also analyzed.

3.3.1. The signal-to-noise ratio

We first focused on the minimum S/N ratio for considering a sample positive. It is generally accepted in the literature that the S/N ratio must be higher than 3 for a limit of detection and above 10 for a limit of quantification (Peters, Drummer, & Musshoff, 2007). Here the samples were considered positive only if both MRM transitions gave a chromatographic signal at the same retention time, with an S/N ratio of 10 for the first transition and 3 for the second. In our data set, the LOQs (expressed in mg proteins per kg food) for hazelnut in mayonnaise (5 mg/kg), walnut in ham and smoked paprika spice (7.5 mg/kg), soy in smoked paprika spice (10 mg/kg), and egg in mayonnaise (4.5 mg/kg) were higher than those previously determined for processed and incurred food products.

Consequently, considering one positive peptide per allergen, this method generates 5.1% false negatives (4 peptides out of 78, i.e. 10 allergens in 8 matrices, excluding egg and milk in sauce). It is therefore of utmost importance to spike target food matrices at the LOQ to ensure reliable allergen detection. To consider a sample negative, there should be no signal at the relevant RT or the S/N ratios should be below 10 and 3. Yet defining such S/N ratio criteria immediately raises a second question: how do we decide that two samples have the same retention time?

3.3.2. Tolerated retention time variation.

The guideline SANTE/11813/2017 for pesticides states that an RT between a matrix-matched calibration standard and a sample should be lower than or equal to ± 0.1 min (European Commission, 2017). In Regulation 2002/657/EC for the analysis of veterinary drug residues, on the other hand, the tolerance is set at 2.5% for liquid chromatography analyses (European Commission, 2002).

In blank matrices, two MRM transitions at retention times similar to those of the target peptide were found for 11 peptides (pistachio: AMISPLAGSTSVLR in sauce, hazelnut: ALPDDVLANAFQISR in ham, almond: QETIALSSSQQR in mayonnaise, pecan nuts: LVGFGINGK in cookie, NFLAGQNNIINQLER in spice and ATLTFVSQER in mayonnaise, milk: LSFNPTQLEEQCHI in spice, peanut TANELNLLILR in ham and sauce, and RPFYSNAPQEIFIQQGR in ham and sauce) (Supplementary Material – Fig. 1).

We next analyzed the effect of applying one or the other recommended RT tolerance threshold (± 0.1 min or 2.5%) to the specificity of our detection method, considering detection of each allergen with a single peptide. We calculated the difference in RT between the target peptide at the LOQ and the impurities in the blank. Considering a

Table 1
Multiple reaction monitoring (MRM) parameters for the identification of milk, egg, soybean, peanut, and tree nut (walnut, pecan nuts, almond, cashew, hazelnut, and pistachio) proteins by UHPLC–MS/MS. The cone voltage was set at 35 V. The sensitivity reached for each peptide in processed and complex matrices (cookie (180 °C –18 min), tomato sauce (95 °C – 45 min), chocolate and banana ice cream) has also been specified (Planque, Arnould, & Gillard, 2017).

Allergen	Protein	Peptide	LOQ (mg of proteins per kg) (Planque, Arnould, & Gillard, 2017)	Precursor (charge state) (m/z)	Product ion (fragments)	Collision energy (eV)
Soy	Glycinin G2 PO4405 Gly m6	EAFGVNMQIVR	5	632.3 (+ +)	760.4 (y6)	17
					646.4 (y5)	22
	2S albumin P19594 Gly m 25 albumin	ELINLATMC[+57]R	5	610.8 (+ +)	865.4 (y7)	21
					638.3 (y5)	17
	Glycinin G1 PO4776 Glv m6	VFDGELQEGR	10	575.3 (+ +)	788.4 (y7)	20
					602.3 (y5)	20
Milk	Casein aS1 P02662 Bos d 8	FFVAPFPEVFGK	0.5	692.9 (+ +)	991.5 (y9)	18
					920.5 (y8)	18
		YLGYLEQLLR	0.5	634.4 (+ +)	934.5 (y7)	21
					771.5 (y6)	20
	Casein aS2 P02663	NAVPITPTLNR	2.5	598.3 (+ +)	911.5 (y8)	17
					285.2 (b3)	12
	P0β-lactoglobulin P02754 Bos d 5	VLVLDTDYK	10	533.3 (+ +)	853.4 (y7)	15
					754.4 (y6)	14
		LSFNPTQLEEQC[+57]Hl	5	858.4 (+ +)	1254.6 (y10)	26
					627.8 (y10)	27
Egg	Ovalbumin P01012 Gal d 2	GGLEPINFQTAADQAR	3	844.4 (+ +)	1121.5 (y10)	28
					666.3 (y12)	25
		LTEWTSSNVMEER	15	791.4 (+ +)	1052.5 (y9)	31
					951.4 (y8)	23
	Vitellogenin-2 P02845	EALQPIHDLADEAISR	60	593.3 (+ + +)	761.4 (y7)	19
					668.8 (y12)	15
		NIPFAEYPTYK	60	671.8 (+ +)	1115.5 (y9)	15
					558.3 (y9)	29
	Vitellogenin-1 P87498	YLLDLLPAAASHR	60	480.6 (+ + +)	709.4 (y7)	15
					582.3 (y11)	10
Peanut	Cupin Q8LKN1 Ara h 3/4	RPFYSNAPQEIFIQQGR	5	684.4 (+ + +)	748.4 (y6)	20
					836.4 (b7)	17
	Cupin Q647H4 Ahy-1	TANELNLLILR	2.5	635.4 (+ +)	854.6 (y7)	20
					741.5 (y6)	22
	Conglutin 7 Q6PSU2 Ara h2	NLPQQC[+57]GLR	5	543.3 (+ +)	858.4 (y7)	13
					429.7 (y7)	16
Walnut	Vici lin-like protein Q9SEW4	ATLTLVSQETR	12.5	609.8 (+ +)	620.3 (y5)	19
					832.5 (y7)	21
	Albumin seed storage protein P93198 Jug r 1	GEEMEEMVQSAR	5	698.3 (+ +)	949.4 (y8)	22
					820.4 (y7)	22
Pecan nuts	75 vicilin B3STU4 Car i 2	ATLTFVSQER	12.5	576.3 (+ +)	765.4 (y6)	16
					618.3 (y5)	18
		NFLAGQNNIINQLER	12.5	582.0 (+ + +)	659.4 (y5)	19
					772.4 (y6)	18
		LVGFGINGK	5	452.8 (+ +)	692.4 (y7)	12
					488.3 (y5)	13
Almond	Prunin Q43607 Pru du 6	GNLDFVQPPR	5	571.8 (+ +)	743.4 (y6)	19
					596.4 (y5)	14
		ALPDEVLANAYQISR	5	830.4 (+ +)	1035.6 (y9)	30
					922.5 (y8)	32
		QETIALSSSQQR	25	674.3 (+ +)	876.5 (y8)	26
					692.3 (y6)	27
	Pru2 protein Q43608 Pru du 6	TDENGFTNTLAGR	25	698.3 (+ +)	879.5 (y8)	25
					732.4 (y7)	23
Cashew	Allergen Ana o 2 Q8GZP6 Ana o 2	C[+57]AGVALVR	2.5	423.2 (+ +)	614.4 (y6)	13
					458.3 (y4)	14
		AMTSPLAGR	2.5	452.2 (+ +)	701.4 (y7)	13
					513.3 (y5)	15
	25 albumin Q8H2B8 Ana o 3	ELYETASELPR	2.5	654.3 (+ +)	773.4 (y7)	22
					672.4 (y6)	20
Hazenut	115 globulin- like protein Q8W1C2 Cor a 9	ADIYTEQVGR	5	576.3 (+ +)	689.4 (y6)	19
					588.3 (y5)	16
		QGQVLTIPQNFAVAK	5	807.5 (+ +)	1088.6 (y10)	27
					874.5 (y8)	23
		ALPDDVLANAFQISR	2.5	815.4 (+ +)	1019.6 (y9)	28
					906.5 (y8)	31

(continued on next page)

Table 1 (*continued*)

Allergen	Protein	Peptide	LOQ (mg of proteins per kg) (Planque, Arnould, & Gillard, 2017)	Precursor (charge state) (*m/z*)	Product ion (fragments)	Collision energy (eV)
Pistachio	115 globulin precusor B751_11 Pis v 5	ITSLNSLNLPILK	2.5	713.4 (+ +)	1011.6 (y9)	21
					470.3 (y4)	22
		AMISPLAGSTSVLR	2.5	701.9 (+ +)	1000.6 (y10)	23
					790.4 (y8)	28
	115 globulin B2KN55 Pis v 2	VTSINALNLPILR	2.5	712.4 (+ +)	838.6 (y7)	22
					1023.6 (b10)	23
	115 globulin precusor B7P073 Pis v 2	ALPLDVIK	2.5	434.8 (+ +)	684.4 (y6)	10
					342.7 (y6)	11

tolerated RT difference of 2.5% or less, the peptides LVGFGINGK (cookie) and TANELNLLILR (sauce) could be excluded from the false positive list. Consequently, considering one positive peptide per allergen, this method generates 10.3% false positives (8 peptides out of 78, i.e. 10 allergens in 8 matrices, excluding egg and milk in sauce). Assuming a (lower) tolerated RT difference of 0.1 min, 4 peptides should be considered present: NFLAGQNNIINQLER (spice), ATLTFVSQER (mayonnaise), LSFNPTQLEEQCHI (spice), and RPFYSNAPQEIFIQQGR (ham), amounting to a false positive rate of 5.1%. Despite the lower rate of false positives, we recommend setting an RT tolerance of 2.5% because of RT possible variations between similar matrices. The use of standard addition, however, solves the problem of retention time deviation tolerance, as it allows the distinguishing of impurities from target peptides, as shown in Fig. 1-Supplementary Material. In our data, there were two cases where impurities and a target peptide showed nearly identical retention times and thus allowed a doubt to subsist: pecan nut peptide ATLTFVSSQER in mayonnaise and peanut peptide RPYSNAPQEIFIQQGR in chicken. This amounted to a false positive rate of 2.6%.

Using the same criteria (S/N ratios of 10 and 3; a 2.5% RT deviation tolerance), we examined how considering two peptides for allergen detection instead of only one would affect the rates of false positives and false negatives. In our data set, TANELNLLILR and RPFYSNAPQEIFIQQGR were both detected in allergen-free ham. This means one false positive result out of 78, corresponding to a 1.3% false positive rate, much lower than the 10.3% rate obtained when the adopted criterion was one positive peptide per allergen. Yet, if two peptides per allergen must be detected with a signal-to-noise ratio of 10 for the first transition and 3 for the second selected transition, then 33.3% of the positive samples (26 cases out of 78) with a concentration higher than the LOQ previously determined in incurred matrices should be declared negative (Supplementary material – Fig. 1). Consequently, one should not require that two peptides per allergen be detected in order to declare a sample positive: although this criterion affords high specificity (1.3% false positives), it does not allow sufficient sensitivity (33.3% false negatives). Therefore, it appears preferable to consider a sample to be positive for an allergen if a single peptide is detected (especially if additional criteria allow reduction of the rate of false positives, as shown in the next section).

3.3.3. Tolerated relative ion intensity deviation

The ion ratio is the intensity ratio between the second and first transitions. After calculating each ion ratio, the ion ratio of the sample was compared to the ion ratio of the standard, and a "relative ion intensity" was determined, which is the difference between the two ion ratios, expressed as a percentage of the ion ratio of the standard

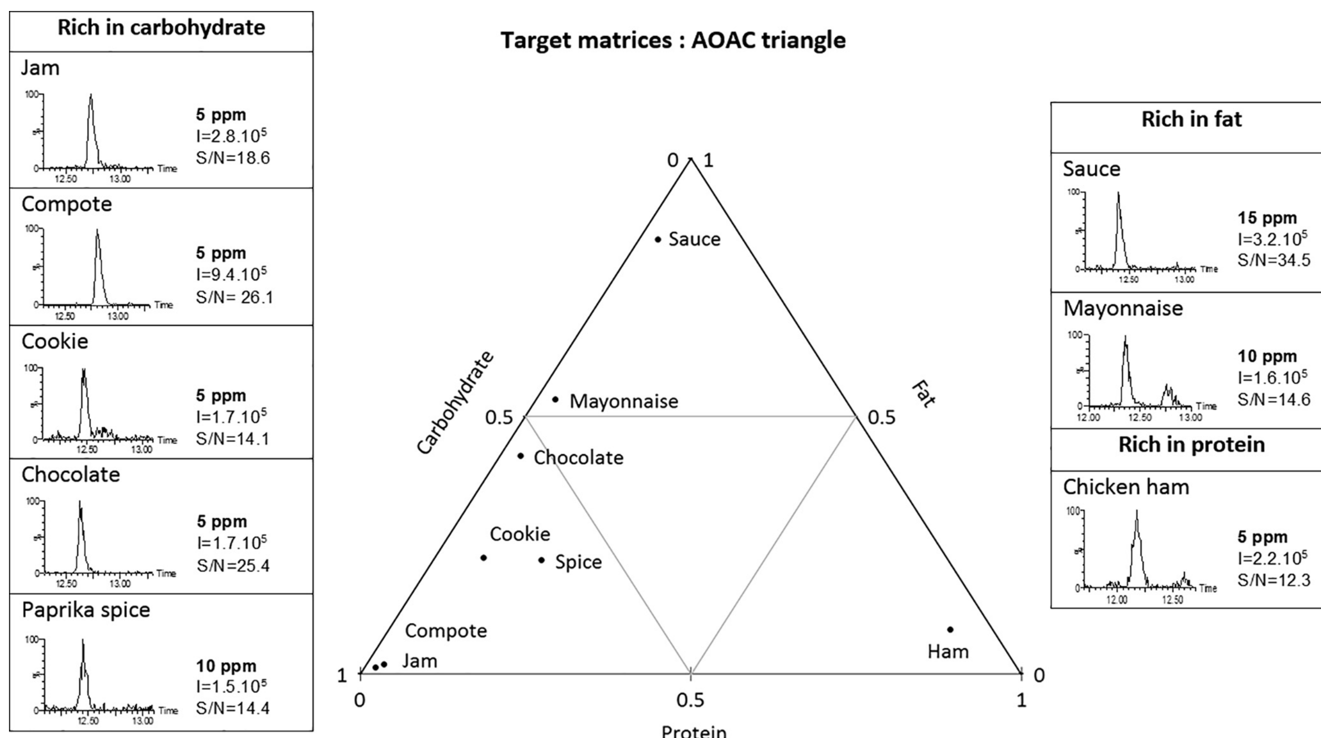

Fig. 1. Chromatograms of EAFGVNMQIVR soy peptide (632.3 > 646.4) spiked at the LOQ (S/N > 10) in eight matrices classified in function of the percentage of carbohydrate, protein and fat.

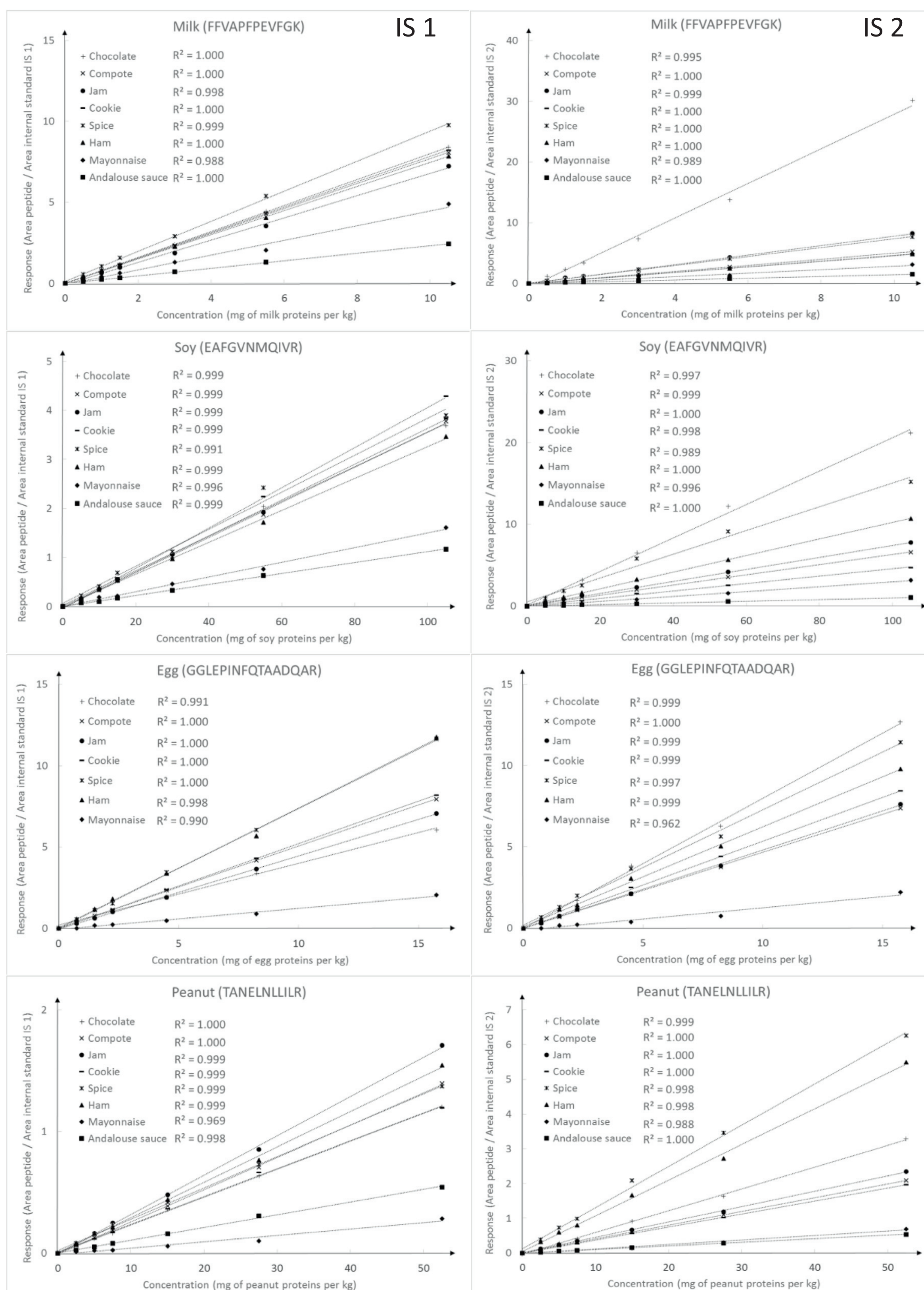

Fig. 2. Linear regression of peptide peak areas corresponding to the highest MRM transition corrected with (A) the labeled IS 1 peptide ((FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] (milk), GGLEPINF[D_5]QTAADQAR (egg), TANELNLLIL[$^{13}C_6$ ^{15}N]R (peanut), EAFGV[D_8]NMQIVR (soy)) or (B) the long labeled IS 2 peptide (GRFFV [$^{13}C_5{}^{15}N$]APFPEVFGKGGL[$^{13}C_6{}^{15}N$]EPINFQTAADQARGS (milk and egg) and GREAFGV[$^{13}C_5{}^{15}N$]NM QIVRTANEL[$^{13}C_6{}^{15}N$]NLLILRGS (soy and peanut)) as a function of the concentration of food allergen proteins in spiked chocolate, compote, jam, cookie, spice, ham, and mayonnaise. The linearity was checked for milk casein FFVAPFPEVFGK (692.9 > 920.5), egg white GGLEPINFQTAADQAR (844.4 > 666.3), peanut TANELNLLILR (635.4 > 741.5), and soybean EAFGVNMQ-IVR.

(Supplementary Material – Fig. 3). According to guideline SANTE/11813/2017 for pesticide analysis, a peptide shall be confidently detected and considered positive if the ion ratio deviation between different allergen concentrations does not exceed 30%. Regulation 2002/657/EC for the analysis of veterinary drug residues adopts the same criterion, with an added nuance: when the ion ratio is low (i.e. the intensity of the second transition is no more than 10% lower than the intensity of the first), a greater ion ratio deviation between allergen concentrations can be tolerated.

The ion ratio deviation between two different concentrations (LOQ and 20 × LOQ) was calculated and compared with the tolerated deviation set by Regulation 2002/657/EC. The choice of the concentration 20 × LOQ was justified by a high matrix effect in some matrices (Supplementary Material – Fig. 3). For peanut peptide RPYSNAPQEIFIQQGR, the relative ion intensity deviation observed between the LOQ (from 2.5 to 27.5 mg) and 52.5 mg peanut proteins per kg was 5.9–19.6%, except in the case of chicken ham, characterized by a 965.7% relative ion intensity deviation (Supplementary material – Table 1). This led us to exclude the RPYSNAPQEIFIQQGR peptide for the determination of peanut-positive chicken ham samples.

A similar problem was observed for the ATLTFVSSQER pecan nut peptide. The relative ion intensity deviation between the LOQ (from 2.5 to 27.5 mg) and 52.5 mg pecan nut proteins per kg ranged from 1.3 to 14.7%, except in the case of mayonnaise, for which a 36.8% relative ion intensity deviation was observed. As the ion ratio for ATLTFVSSQER peptide in mayonnaise was higher than 50%, the tolerated ion ratio deviation should not exceed 20% (2002/657/EC) or 30% (SANTE/11813/2017). This made it necessary to exclude this peptide for the detection of pecan nut in mayonnaise. The two false positive samples observed in blank matrices (peanut peptide RPYSNAPQEIFIQQGR in ham and ATLTFVSSQER pecan nut peptide in mayonnaise) were excluded on the basis of the relative ion intensity criterion. Hence, to avoid false positives, this criterion should be applied systematically.

In conclusion, it is possible to avoid false positives by combining the standard addition strategy, signal-to-noise ratios of 10 and 3 for the first and second transitions, respectively, a 2.5% tolerance for the retention time, and by setting criteria for the tolerated relative ion intensity deviation.

3.4. Quantification strategy

The SMPR 2016.002 "Standard Method Performance Requirements for Detection and Quantitation of Selected Food Allergens" sets a recovery of [60–120%] and a maximum relative standard deviation (RSD) of 20% for the validation of allergen methods based on mass spectrometry.

3.4.1. External calibration curve with labeled internal standard correction

The ideal quantification strategy for routine laboratories would be to use a single calibration curve performed in a solvent or even a matrix, no matter the kind of food to be analyzed. This strategy implies careful selection of the internal standard, in order to correct the matrix effect and the different steps of the protocol. The first tested internal standard (IS 1) in this study contained the following short labeled peptides: FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] for milk, GGLEPINF[D_5]QTAADQAR for egg, TANELNLLIL[$^{13}C_6{}^{15}N$]R for peanut, and EAFGV[D_8]NMQIVR for soy. This standard proved adequate for correction of the matrix effect, the purification step, and UHPLC–MS/MS analysis variability. It did not adequately correct the extraction and tryptic digestion steps, whose correction is crucial to using a single calibration curve (Planque, Arnould, Renard, et al., 2017).

In the present study, a second internal standard (IS 2) containing the following long labeled peptides was tested: GRFF**V**[$^{13}C_5{}^{15}N$]APFPEVFGKGG**L**[$^{13}C_6{}^{15}N$]EPINFQTAADQARGS for milk and egg and GREAFG**V**[$^{13}C_5{}^{15}N$]N MQIVRTANE**L**[$^{13}C_6$ ^{15}N]NLLILRGS for soy and peanut. As long labeled peptides must be digested prior to MS analysis, this should allow correction of the digestion step.

The IS 1 or IS 2 labeled peptides were introduced before the extraction step and the eight target matrices were spiked at 6 concentrations. The peak areas of milk, egg, soy, and peanut peptides in the eight matrices were corrected by means of the corresponding short or digested long labeled peptide (IS 1 or IS 2) (Linear curves obtained after correction with the IS 1 or IS 2 labeled peptides were drawn for milk, egg, soy, and peanut peptides; Fig. 2).

To test the possibility of using a single calibration curve for the quantification of allergens in foodstuffs, the strategy was evaluated in terms of the linearity of the calibration curve (R^2) and the RSD between samples (n = 8, 1 replicate per matrix) spiked at the highest concentration (20 × LOQ). Determinations were done without and with IS 1 or IS 2 internal standard correction. The regression coefficient (R^2) was higher than 0.99 for 27 linear regressions out of 31 when no internal standard was used, but internal standard correction with IS 1 or IS 2 raised this number to 29 or 28, respectively.

As shown in Fig. 2, the calibration curves obtained after correction with either IS 1 or IS 2 did not coincide for the eight matrices tested. This shows the importance of matrix interference in allergen detection and made it impossible to use a single calibration curve for all the targeted food matrices in the case of milk, egg, soy, and peanut peptides. Considering the eight matrices, the RSDs calculated at 20 × LOQ ranged from 27% to 43% and from 112% to 74% for the 4 target allergens after IS 1 and IS 2 correction, respectively. The RSD was lower after IS 1 correction than after IS 2 correction (Supplementary Material – Table 2).

As shown in Fig. 2, after correction of peptide peak areas with IS 1, the calibration curves for mayonnaise and sauce appeared separate from the other calibration curves. Two groups of matrices were created: those with a carbohydrate content higher than 50% (jam, compote, cookie, chocolate, and spice) and those with a fat content higher than 50% (mayonnaise and sauce). When only the group of matrices containing more than 50% carbohydrate was considered, the RSD for milk, egg, soy and peanut dropped to the 6% to 26% range (calculated on the basis of the highest level of concentration after IS 1 correction) instead of the initial 27%–43% range for the eight matrices (Supplementary Material – Table 2). As the RSD exceeded 20% (SMPR 2016.002), a single calibration curve per group of matrices cannot be used for the quantification of allergens in food (Paez et al., 2016).

3.4.2. Standard addition with labeled internal standard correction

Standard addition at 6 different concentrations was performed on the eight matrices already spiked with milk, egg, soy, and peanut allergens at the LOQ and at 10 × LOQ (2.6 Preparation of test samples). Peptides were quantified in the matrices spiked at these concentrations, using two strategies: standard addition without and with correction of peptide peak areas by means of internal standards (IS 1 or IS 2). The RSD of the recovery (without or with labeled peptide correction) was calculated on three independent replicates of compote spiked at 10 × LOQ (C2).

3.4.2.1. Standard addition: 6 concentration. The slope and intercept of the regression line obtained without internal standard correction and after correction with IS 1 and IS 2 were used to determine the initial concentrations of milk, egg, soy, and peanut allergens. As shown in Fig. 3, the concentration was calculated by dividing the intercept by the slope (Supplementary Material – Table 3A and B). Recoveries were calculated by dividing the estimated concentration by the theoretical concentration and multiplying by 100. Without correction with an internal standard, 35.0% of the calculated recoveries (21.7% at C1 and 13.3% at *C*2) were outside the range [60–120%] specified by AOAC guideline SMPR 2016.002 (Fig. 4). Correction with IS 1 or IS 2 allowed a significant reduction of this percentage, to 18.3% (15.0% C1 and 3.3% C2) with IS1 correction and 16.7% (10.0% C1 and 6.7% C2) with IS2 correction.

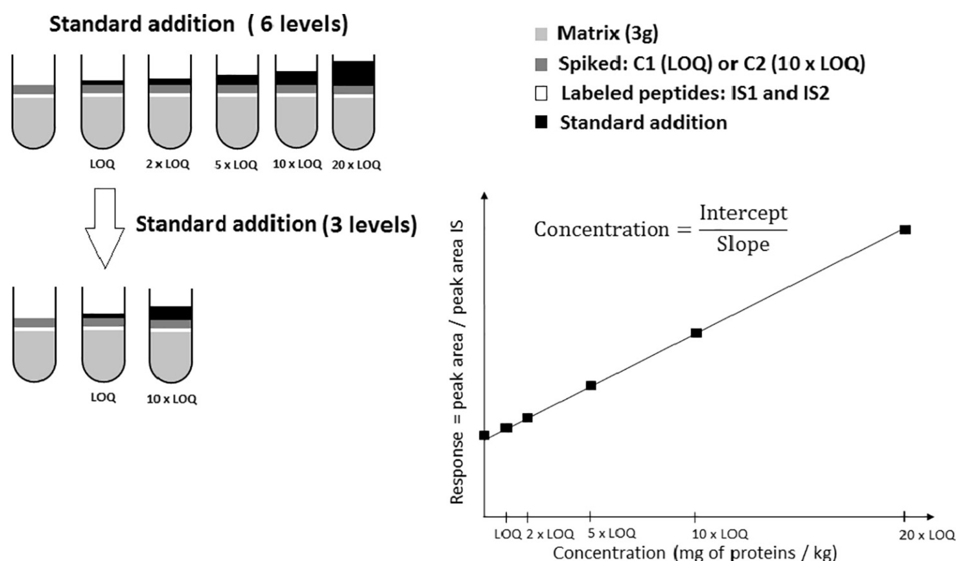

Fig. 3. Strategy for quantifying allergens in foodstuffs: standard addition + labeled internal standard IS 1 or IS 2. Quantification at six levels of concentration was compared to quantification at 3 levels (sample, sample spiked at the LOQ, and sample spiked at 10 × LOQ), the goal being to reduce the number of samples for the development of a routine method.

3.4.2.2. Calculation of the RSD between recoveries. The recoveries obtained for the three technical replicates of compote matrix spiked at C2 with IS 1 or IS 2 correction and without correction were compared (Supplementary Material – Table 4A and B). Without internal standard correction, recoveries ($n = 3$) were sometimes outside the range specified by the AOAC: milk (116 ± 14%), egg (126 ± 24%), soy (129 ± 25%), and peanut (115 ± 18%). Correction of peptide peak areas with IS 1 or IS 2 labeled peptides made it possible to respect the AOAC specifications, decreasing the relative standard deviation between replicates and giving rise to recoveries of 103 ± 5% for milk, 103 ± 4% for egg, 107 ± 4% soy, and 100 ± 1% for peanut, with IS 1 correction, and 103 ± 4% for milk, 105 ± 8% for egg, 111 ± 1% for soy, and 107 ± 1% for peanut, with IS 2 correction.

The use of either type of labeled peptide thus decreased significantly the percentage of out-of-range recoveries. At the LOQ, however, this percentage remained high. Some analytical regulations such as SANTE/11813/2017, concerning pesticide residue analysis in food and feed, recommend tolerating a 50–120% recovery range at the LOQ.

3.4.2.3. Standard addition: 3 concentrations. For a routine laboratory, standard addition should ideally be done with a limited number of spiking concentrations. Therefore, recoveries were also calculated for only three calibration points (0, 0.5 and 5 mg/kg for milk proteins, 0, 0.75 and 7.5 mg/kg for egg proteins, 0, 5 and 50 mg/kg for soy

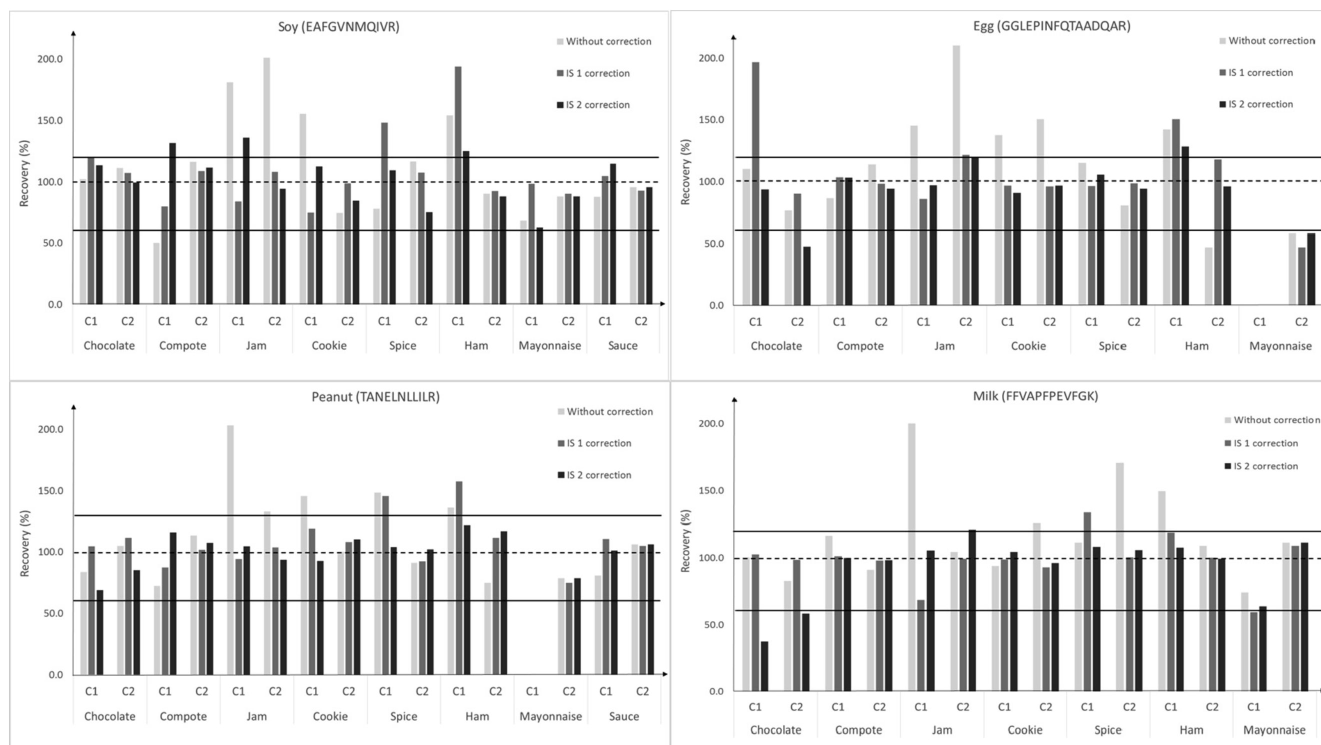

Fig. 4. Recoveries obtained by standard addition (6 levels of concentration) for soy, peanut, egg, and milk peptides added at the LOQ (C1) and at 10 × LOQ (C2) without internal standard correction, with labeled IS 1 peptide correction (FFVAPFPEVFGK[$^{13}C_6{}^{15}N_2$] (milk), GGLEPINF[D_5]QTAADQAR (egg), TANELNLLIL[$^{13}C_6{}^{15}N$]R (peanut), EAFGV[D_8]NMQIVR (soy)); and with long peptide IS 2 correction (GRFFV[$^{13}C_5{}^{15}N$]APFPEVFGKGGL[$^{13}C_6{}^{15}N$] EPINFQTAADQARGS (milk and egg) and GREAFGV[$^{13}C_5{}^{15}N$]NMQIVRTANEL[$^{13}C_6{}^{15}N$]NLLILRGS (soy and peanut). The recovery range specified by AOAC [60–120%] is delimited by two black lines.

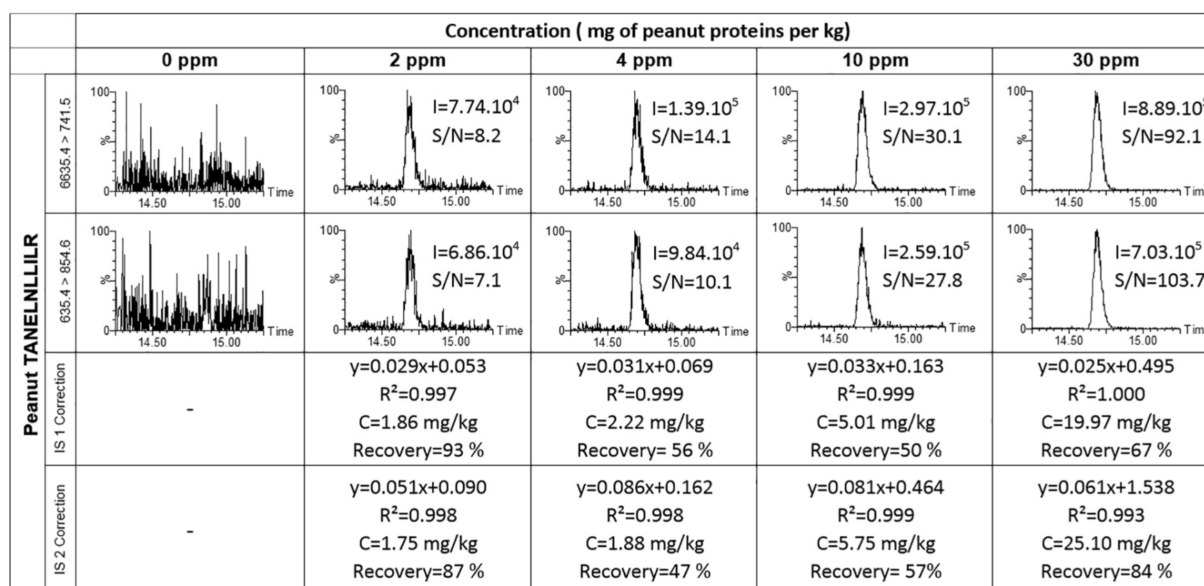

Fig. 5. Detection and quantification of peanut with TANELNLLILR peptide in iFAAM chocolate dessert matrices containing 0, 2, 4, 10, and 30 mg peanut proteins per kg. Recovery was calculated with IS 1 and IS 2 labeled peptide correction.

proteins, and 0, 2.5 and 25 mg/kg for peanut proteins). Without internal standard correction, 50.0% of the recoveries (27.4% at C1 and 22.6% at C2) were outside the range (60–120%) specified in AOAC guideline SMPR 2016.002 (Fig. 4). Correcting with IS 1 or IS 2 made it possible to reduce the percentage of out-of-range to 13.3% (11.7% at C1 and 1.7% at C2) or 18.3% (13.3% at C1 and 5.0% at C2), respectively (Supplementary Material – Table 5A and B). After correction of peak areas with the help of labeled peptides, the percentages of out-of-AOAC-specification recoveries observed with 3 calibration points were similar to those observed with 6 calibration points. In order to reduce the time of analysis (3 samples instead of 6), standard addition at 1 × and 10 × LOQ was retained for the quantification of real samples.

3.5. *Quantification of peanut in chocolate dessert samples*

The standard addition strategy combined with the introduction of IS 1 or IS 2 labeled peptides was used to quantify allergens in incurred chocolate desserts. Chocolate desserts containing 0, 2, 4, 10, and 30 mg peanut proteins per kg were spiked with peanut proteins at 0, 2.5, and 25 mg/kg. The corresponding MRM chromatograms are presented in Fig. 5.

The developed method can detect peanut in chocolate dessert at 2 mg peanut proteins per kg with a S/N ratio higher than 3. The slope and the intercept of the calibration curves obtained for peanut peptide (TANELNLLILR) in chocolate desserts were used to determine the recovery. The recovery from incurred chocolate desserts ranged from 50 to 93% with IS 1 correction and from 47 to 87% with IS 2 correction. With IS 1 and IS 2 correction, respectively, the recoveries previously determined for peanuts spiked in chocolate were 104.5% and 69% at the LOQ and 111.6% and 85.1% at 10 × LOQ. As shown previously, the recovery range (60–120%) set by a panel of experts in the AOAC guideline can be hard to reach for incurred food products (Newsome & Scholl, 2013; Sayers et al., 2018), but a calibration curve prepared under conditions similar to those used for samples in the study of Gu et al., gives better recoveries (60.1–92.4% for milk, soy, peanut and tree nut proteins). This is totally unrealistic for routine laboratories, because of the number of samples to be analyzed (Gu et al., 2018). In routine laboratories, furthermore, the process conditions and, in some instances, the recipe is not specified for the samples received, so the product cannot be reproduced.

4. Conclusion

The developed method in this study can detect 10 allergens (egg, milk, soy, peanut, almond, hazelnut, walnut, pecan nuts, cashew, and pistachio) in eight matrices belonging to different food product categories (high in fat, carbohydrate, protein, tannins, or polyphenols). Developed for a routine laboratory, the method uses a single protocol to detect 10 allergens within a day. We have previously reported sensitive methods for processed samples (sauce heated at 95 °C for 45 min and cookie baked at 180 °C for 18 min) and incurred samples (chocolate and banana ice cream) (Planque et al., 2016; Planque, Arnould, Dieu, et al., 2017), but in high-fat matrices or spices, some allergens were not detected at the determined LOQs after spiking matrices with the target allergens at the LOQ. The complexity of some food products leads to interferences influencing the sensitivity of MRM signals. We have evaluated several factors liable to influence the rate of false positive or negative results, such as the retention time, the S/N ratio, and the relative ion intensity. On the basis of the data obtained, we recommend using the following criteria for allergen detection: signal-to-noise ratios above 10 and 3 for the first and second transitions, respectively, a 2.5% retention time deviation between matrix-matched samples and a relative ion intensity deviation according to guideline SANTE/11813/2017. Furthermore, the selection of marker peptides and the determination of method sensitivity (LOD, LOQ) should always be done with processed and incurred food products, because of the impact of the thermal process on allergen detection. Standard addition by spiking the matrices with target allergens at the LOQ is imperative to ensure detection of allergens at the LOQ and to decrease the rates of false positives and negatives.

In the second part of this work, we have compared two strategies for the quantification of milk, soy, peanut, and egg allergens in the eight food products. For a routine laboratory, the ideal quantification strategy involves the use of a single calibration curve. Yet, neither the use of short labeled peptides (IS 1) nor the use of long isotope-labeled peptides (IS 2) allowed using a single calibration curve for the quantification of all target allergens. The strategy combining a labeled internal standard with standard addition appears promising, since 81.7% (IS 1 correction) and 83.3% (IS 2 correction) of recoveries determined at two concentrations (LOQ and 10 × LOQ) in eight matrices for milk, egg, soy, and peanut allergens met the AOAC recovery specification of 60–120%. On the basis of the results obtained, we recommend

tolerating a wider recovery range at the LOQ. We have also used the same strategy to quantify peanut proteins in an incurred chocolate matrix containing 0, 2, 4, 10, and 30 mg peanut proteins per kg. The recoveries obtained with IS 1 correction were between 50% and 93% and those obtained with IS 2 correction were between 47 and 87%. Unexpectedly, we observed no improvement of recovery with long isotope-labeled peptides combining milk with egg or peanut with soy peptides. To reach the goal of using a single calibration curve, the use of labeled proteins should next be tested, but our strategy combining standard addition with labeled peptides is already a very efficient alternative, allowing allergen quantification in all kinds of foodstuffs with good recovery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.08.095.

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