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1 **Comparative study of concatemer efficiency as an isotope-labelled internal standard for allergen**
2 **quantification**

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19 **Abstract**

20 Mass spectrometry-based methods coupled with stable isotope dilution have become effective and
21 widely used methods for the detection and quantification of food allergens. Current methods target
22 signature peptides resulting from proteolytic digestion of proteins of the allergenic ingredient. The choice
23 of appropriate stable isotope-labelled internal standard is crucial, given the diversity of encountered food
24 matrices which can affect sample preparation and analysis. We propose the use of concatemer, an
25 artificial and stable isotope-labelled protein composed of several concatenated signature peptides as
26 internal standard. With a comparative analysis of three matrices contaminated with four allergens (egg,
27 milk, peanut, and hazelnut), the concatemer approach was found to offer advantages associated with
28 the use of labelled proteins, ideal but unaffordable, and circumvent certain limitations of traditionally
29 used synthetic peptides as internal standards. Although used in the proteomic field for more than a
30 decade, concatemer strategy has not yet been applied for food analysis.

31

32 **Keywords**

33 Food allergen analysis, mass spectrometry, isotope dilution, isotope-labelled internal standard, isotope-
34 labelled concatemer

35

36 1. Introduction

37 Food allergy is defined as an adverse health effect arising from a specific reproducible immune
38 response that occurs on exposure to a given food (Boyce et al., 2011). Several studies indicate an
39 increase in the prevalence of food allergy with nearly 5% of adults and 8% of children being affected
40 (Sicherer & Sampson, 2014). Given the absence of accepted treatment, the current solution for allergic
41 patients relies on allergen avoidance to circumvent allergic reactions. However, this essentially requires
42 correct food labelling and efficient risk management from food business operators to reduce the risk of
43 contamination by allergens to acceptable levels. European legislation (Regulation [EU] No 1169/2011)
44 requires the labelling of 14 allergenic ingredients when they are part of a foodstuff recipe. However, this
45 legislation does not cover the presence of hidden allergens that are due to cross-contamination during
46 food processing. Even if strongly requested by food producers and control laboratories, no harmonized
47 regulatory framework for managing hidden allergens or action thresholds have been enacted in Europe.
48 Some countries have set legal thresholds but with a high disparity among allergens and among countries
49 (Planque et al., 2019). A quantitative risk assessment was also developed by VITAL® (Voluntary
50 Incidental Trace Allergen Labelling) combining reference doses and exposure (Allen et al., 2014). The
51 thresholds for allergenic proteins in food are based on clinical data and are indicators of the action levels,
52 expressed as the total protein amount of the allergenic food (mg), below which only the most sensitive
53 allergic subjects might react (1% of allergic patients or 5% of them for the less common foods). These
54 values are often used by laboratories as a targeted limit of quantification (LOQ) in the absence of legal
55 thresholds.

56 The development of a quantitative allergen risk assessment requires quantitative allergen analysis.
57 During the last decade, mass spectrometry became the method of choice for allergen analysis (Ahsan,
58 Rao, Gruppuso, Ramratnam, & Salomon, 2016). Allergen analysis by mass spectrometry is
59 predominantly performed by specific analysis of peptides obtained by an enzymatic digestion of the
60 proteins of the sample, including the proteins of the allergenic ingredients. One of the advantages of
61 mass spectrometry-based methods is the possibility to simultaneously detect multiple peptides from
62 multiple allergens, thus enabling time- and money-saving multiplexed analysis. Such a targeted
63 approach, named multiple reaction monitoring (MRM), offers high sensitivity and specificity. Targeted
64 proteomics is often used for absolute peptide quantification in combination with isotope dilution, a
65 technique based on the use of an internal standard corresponding to the stable isotope-labelled version
66 of the analyte (Monaci, Losito, De Angelis, Pilolli, & Visconti, 2013; Nitride et al., 2019; Planque et al.,

67 2019). The introduction of this isotope-labelled internal standard corrects for variability and various
68 matrix effects during the actual analysis. Notably, ion suppression effects and, depending on the type
69 of internal standard, matrix effects and analyte loss during sample preparation may be corrected by the
70 use of isotope-labelled internal standards.

71 Peptides specific for allergen proteins are the analytes in mass spectrometry analysis of food
72 allergens; however, the initial analytes are proteins. Stable isotope-labelled internal standards can
73 therefore adopt different forms. In theory, a stable isotope-labelled protein is the ideal internal standard
74 as, when added to the food that needs to be analyzed, it can correct for sample losses during all the
75 steps of the sample preparation procedure (including protein extraction and digestion), as well as for
76 matrix effects during mass spectrometry analysis. Such an approach was proposed by Newsome and
77 Scholl (Newsome & Scholl, 2013) for the quantification of bovine milk α_{S1} -casein in baked goods. The
78 main limitation of this approach, besides technical issues for protein production, is its cost. When one
79 aims at multiplexed analysis, this necessitates the use of multiple isotope-labelled proteins, which is
80 unrealistic for laboratories performing routine analyses (Planque, Arnould, & Gillard, 2017). Therefore,
81 most laboratories rely on stable isotope-labelled synthetic peptides (Boo, Parker, & Jackson, 2018;
82 Henrottin et al., 2019; Planque et al., 2019). However, in food allergen analysis, the initial analytes are
83 proteins. Peptide internal standard and protein analytes can exhibit different behaviors during the
84 extraction, leading to different extraction yield. Moreover, the peptides do not undergo the enzymatic
85 digestion step which is known to be highly affected by the matrix effects (Korte, Oberleitner, &
86 Brockmeyer, 2019).

87 Here, we implemented an alternative method based on the synthesis of a concatemer used as a
88 stable isotope-labelled internal standard for allergen quantification. This strategy has been well adopted
89 by proteomics researchers, and the concatemers are known as QconCAT (Pratt et al., 2006), but, as far
90 as we know, these molecules have not yet been explored for food analysis. Concatemers are artificial
91 proteins composed of concatenated, proteotypic peptides originating from different proteins of interest.
92 The peptides themselves are typically first identified following mass spectrometry or are predicted from
93 theoretical peptide sequences. Concatemers are typically recombinantly produced in an environment
94 that allows labelling with stable isotopes (e.g., ^{13}C or ^{15}N). In contrast to synthetic peptides, concatemers
95 need to be proteolytically digested to release their peptides, and thus, this peptide release is also
96 affected by the interference caused by the matrix during the digestion step, in a manner similar to the
97 analyte of interest. Another advantage of concatemers is their potential for multiplexing. A single

98 concatemer can be composed of numerous proteotypic peptides and can therefore be used for
99 multiplexed allergen analysis. The limitation of this approach is fixed by the protein size reachable with
100 recombinant protein expression, which is more than 100 kDa (Chambers, Austen, Fulghum, & Kim,
101 2004). This approach can be cost-effective when compared with using synthetic peptides for multiplexed
102 analysis. For our study, we developed, produced, and purified a ¹⁵N isotopically labelled concatemer
103 composed of 19 proteotypic peptides, allowing for the analysis of 4 allergenic ingredients (egg, milk,
104 peanut, and hazelnut). We evaluated the performance of this concatemer by the analysis of three
105 uncontaminated food matrices spiked with increasing and defined concentrations (2.5 ppm to 50 ppm,
106 where ppm corresponded to mg of total allergen protein per kg of matrix) of the selected allergen
107 extracts. In addition, we compared the use of the concatemer with that of five synthetic peptides
108 corresponding to tryptic peptides from the four considered allergens and with β-lactoglobulin, a bovine
109 milk protein that was ¹⁵N isotopically labelled.
110

111 **2. Material and methods**

112 **2.1. Reagent and materials**

113 Gene synthesis and cloning were ordered from GeneCust (Boynes, France). Acetic acid, ammonium
114 bicarbonate, ampicillin sodium salt, chloramphenicol, dimethyl sulfoxide (DMSO), DL-dithiothreitol
115 (DTT), expression plasmid pET17b(+) Novagen, HiLoad® 26/600 Superdex® 200 pg, imidazole
116 hydrochloride, iodoacetamide (IAA), kanamycin monosulfate, Lennox broth (LB), Ni Sepharose® 6 fast
117 flow GE Healthcare, Origami™ B(DE3) pLysS competent cells Novagen, phenylmethanesulfonyl
118 fluoride (PMSF), Q Sepharose® Fast Flow, select agar, sodium chloride, sodium phosphate dibasic,
119 sodium phosphate monobasic, tetracycline hydrochloride, tetraethylammonium bicarbonate (TEAB),
120 trypsin from bovine pancreas, tris(hydroxymethyl)aminomethane (Tris) and urea were obtained from
121 Sigma-Aldrich (Bornem, Belgium). One Shot™ BL21(DE3) chemically competent *Escherichia coli*,
122 isopropyl β-D-thiogalactopyranoside (IPTG), SnakeSkin™ dialysis tubing, 3.5K MWCO, 22 mm were
123 purchased from Thermo Fisher Scientific (Waltham, MA, USA). Bioexpress cell growth media (U-15N,
124 98%) (10x concentrate) was obtained from Buchem B.V. (Apeldoorn, the Netherlands), Trypsin Gold,
125 Mass Spectrometry Grade from Promega (Madison, WI, USA), 4–20 Mini-PROTEAN® TGX™ precast
126 protein gels from Bio-Rad (Hercules, CA, USA), Sep-Pak C18 6 cc Vac solid-phase extraction (SPE)
127 cartridges from Waters (Milford, MA, USA), and 0.2 μm acrodisc syringe filters with supor membrane
128 from Pall Corporation (Port Washington, NY, USA). Water, acetonitrile (ACN), and formic acid (FA) were
129 obtained from Biosolve (Valkenswaard, the Netherlands). Labelled synthetic peptides
130 ADIYTEQV[¹³C₅¹⁵N]GR, FFVAPFPEVFGK[¹³C₆¹⁵N₂], GGLEPINF[Ring-D₅]QTAADQAR, LSF[Ring-
131 D₅]NPTQLEEQCHI, TANELNLLIL[¹³C₆¹⁵N]R were ordered from Eurogentec (Seraing, Belgium).

132 Food samples were analyzed by ultra-high performance liquid chromatography–tandem mass-
133 spectrometry (UHPLC MS/MS) using an Acquity liquid chromatograph equipped with a C18 Acquity
134 BEH130 column (2.1 x 150 mm; 1.7 μm) and coupled with a Xevo TQ-S micro triple quadrupole system
135 (Waters, Milford, MA, USA). Characterization of ¹⁵N isotopically labelled concatemer and β-lactoglobulin
136 was performed by ultra-high performance liquid chromatography–high resolution mass spectrometry
137 (UHPLC-HRMS) using an Acquity liquid chromatograph equipped with a C18 Acquity BEH130 column
138 (2.1 x 150 mm; 1.7 μm) and coupled to a Xevo G2-XS QToF quadrupole time-of-flight system (Waters,
139 Milford, MA, USA).

140 **2.2. ¹⁵N isotopically labelled concatemer production and purification**

141 Design and production of the concatemer were adapted from the method of Pratt (Pratt et al.,
142 2006). The first step focused on concatemer design and the selection of the concatenated peptides.
143 Here, we considered 19 peptides (Table 1) originating from seven proteins of four allergenic ingredients
144 (α _{s1}-casein and β -lactoglobulin from cow milk; ovalbumin, ovotransferrin, and vitellogenin-1 from hen's
145 egg; Cor a 9 allergen from hazelnut; and Ara h 1 allergen from peanut). These 19 peptides were selected
146 from a set of relevant peptide biomarkers identified by an empirical approach based on UHPLC-HRMS
147 analysis of incurred and processed samples. The applied food processing steps, sample preparation,
148 and selection criteria have already been detailed in our previous studies (Gavage et al., 2019, 2020;
149 Van Vlierberghe et al., 2020). The peptides were then *in silico* concatenated, and the resulting
150 polypeptide was flanked with an N-terminus initiator sequence including a methionine start and a
151 C-terminus hexahistidine purification tag (His-tag). Hydrophobicity of each of the 19 peptides was
152 evaluated based on their grand average of hydropathy (GRAVY) parameter. Hydrophobic and
153 hydrophilic peptides were alternated in the concatemer sequence to avoid the formation of high
154 hydrophobic clusters that can interfere with solvent accessibility of concatenated peptides and thus with
155 their subsequent proteolysis during the sample preparation. Translation-associated aspects such as
156 tRNA-mediated codon usage bias and mRNA secondary structure, known to impact the translation
157 process (Gorochoowski, Ignatova, Bovenberg, & Roubos, 2015), were also considered. Visual Gene
158 Developer (University of California-Davis, Davis, CA, USA) was used to predict and optimize the mRNA
159 secondary structure. The *in silico* designed DNA construct was finally chemically synthesized and cloned
160 into the pET17b(+) expression vector using *NdeI* and *XhoI* restriction sites to give the pET17b(+)-
161 concat1.

162 The *E. coli* BL21(DE3)/pET17b(+)-concat1 strain was inoculated in a 30 ml starter culture of ¹⁵N
163 labelled media (Bioexpress cell growth media [U-15N, 98%] with 100 μ g/mL ampicillin) and grown
164 overnight at 37 °C under 300 rpm orbital shaking. Cells were harvested by centrifugation (4000 x g,
165 5 min) and the pellet was resuspended in 1 mL of ¹⁵N labelled media. Next, a volume of 660 μ l of this
166 bacterial suspension was used to inoculate a 1L ¹⁵N labelled main culture. This culture was grown at 37
167 °C under 300 rpm orbital shaking until the optical density at 600 nm reached 0.6–0.8. Concatemer
168 expression was next induced with 1 mM IPTG and cells were cultured overnight at 25 °C under 300 rpm
169 orbital shaking. Cells were harvested by centrifugation (5000 x g, 15 min) and stored at –80 °C until
170 concatemer purification.

171 The cell pellet of the 1 L culture was resuspended in 40 mL of lysis buffer (50 mM Tris - 10 mM
172 imidazole - pH 8) with 1 mM PMSF. Cells were disrupted using a Vibra-Cell™ (Sonics, Newtown, CN,
173 USA) ultrasonic probe. The cell lysate was centrifuged twice (40000 x g, 20 min) and filtered through
174 0.2 µm syringe filters before to be submitted to metal affinity chromatography purification. The protein
175 solution was loaded on a 8 ml Ni Sepharose 6 Fast Flow column equilibrated with lysis buffer. An
176 intermediate washing step was performed in the presence of 20 mM imidazole and the His-tag labelled
177 concatemer was finally eluted by using a linear imidazole gradient from 20 mM to 250 mM. The elution
178 fractions were analyzed on SDS-PAGE (Supplementary data 1). The positive fractions were pooled and
179 dialyzed against the storage buffer (50 mM Tris - pH 8) to eliminate imidazole.

180 Total protein concentration was measured by absorbance at 280nm. A SDS-PAGE/densitometry
181 method based on ImageJ software was used to estimate concatemer purity. A total of 84.5 mg of ¹⁵N
182 isotopically labelled concatemer were produced and purified with an estimated purity higher than 90%.
183 Protein sequences, concentration calculations, and purity estimation are detailed in Supplementary
184 data 3.

185 **2.3. ¹⁵N isotopically labelled β-lactoglobulin production and purification**

186 The production of β-lactoglobulin, a cow milk protein, was adapted from the work of Loch and
187 collaborators (Loch et al., 2016) who implemented a method leading to the cytoplasmic accumulation of
188 correctly folded disulfide bond-dependent proteins. Briefly, two mutations (L2A/I3S) were introduced in
189 the β-lactoglobulin to facilitate *in vivo* cleavage of the N-terminal methionine allowing for correct protein
190 folding.) Further, the *E. coli* Origami B (DE3) pLysS strain, a glutathione reductase (*gor*) and thioredoxin
191 reductase (*trx*B) mutated strain, was used for conducting the cytoplasmic co-expression of the protein
192 of interest with DsbC, an *E. coli* cytoplasmic disulfide bond isomerase. The co-expression was achieved
193 with the same expression vector (pET17b(+)-DsbC-BLg) in which the two genes were transcribed from
194 individual T7 IPTG-inducible promoters.

195 To achieve the production of ¹⁵N labelled β-lactoglobulin, expression (starter culture, main culture,
196 and IPTG induction) conditions were similar as used for concatemer production. The antibiotics that
197 were used were tailored to 200 µg/mL ampicillin, 34 µg/mL chloramphenicol, 15 µg/mL kanamycin, and
198 12.5 µg/mL tetracycline, and the IPTG concentration was 0.5 mM. Harvested cells were resuspended
199 in 50 mM phosphate buffer, pH 6.5, with 1 mM PMSF and prepared for protein purification using the
200 same procedure as for the concatemer. The purification of ¹⁵N labelled β-lactoglobulin was performed

201 according to the procedure described by Loch and collaborator (Loch et al., 2016). Briefly, this protocol
202 combines anion-exchange chromatography (Q Sepharose® Fast Flow) with a NaCl linear elution
203 gradient (up to 2 M) followed by size-exclusion chromatography (HiLoad® 26/600 Superdex® 200 pg)
204 in initial conditions (50 mM phosphate buffer, pH 6.5). Eluates of these two purification steps were
205 collected in 1 mL fractions and analyzed on SDS-PAGE (Supplementary data 2).

206 Total protein concentration was measured by absorbance at 280nm. A SDS-PAGE/densitometry
207 method based on ImageJ software was used to estimate protein purity. Using this approach, a total of
208 2.4 mg of ¹⁵N isotopically labelled β-lactoglobulin were produced and purified with an estimated purity
209 higher than 70%. Protein sequences, concentration calculations, and purity estimation are detailed in
210 Supplementary data 3.

211 **2.4. Characterization of produced ¹⁵N isotopically labelled proteins**

212 Protein ¹⁵N stable isotope enrichment was evaluated by UHPLC-HRMS analysis of its constitutive
213 tryptic peptides. In separated containers, concatemer and β-lactoglobulin were diluted to 0.1 mg/mL with
214 50 mM TEAB, pH 9.2, to a final volume of 20 μl. Disulfide bridges of β-lactoglobulin were successively
215 reduced and alkylated with DTT (10 mM final concentration, 45 min incubation at 37 °C under 300 rpm
216 orbital agitation) and IAA (40 mM final concentration, 45 min incubation in the dark at 37 °C under 300
217 rpm orbital agitation). Concatemer and β-lactoglobulin were then proteolytically digested by adding 0.1
218 μg of trypsin gold (protein:trypsin ratio of 1:20). Digestion was conducted for 1 h at 37 °C under 300 rpm
219 orbital agitation and stopped by the addition of 1% (final concentration) of FA followed by centrifugation
220 (20000 x g, 5 min). Samples were ten-fold diluted with 5% ACN before UHPLC-HRMS analysis.
221 Peptides (5 μl of sample was injected) were first separated by reverse-phase liquid chromatography
222 using a 20 min water/ACN + 0.1% FA linear gradient from 5% to 40% of ACN. Data was acquired in
223 MS^E mode with 0.3 s scan time within the 50 to 2000 m/z mass range. The data were processed using
224 UNIFI software (Waters, Milford, MA, USA) and peptide mapping analysis type with traditional tryptic
225 cleavage rules and setting cysteine carbamidomethylation and ¹⁵N isotope labelling as a fixed
226 modifications.

227 For each identified tryptic peptide, the most intense charge state was considered to define the ¹⁵N
228 stable isotopic enrichment. The isotopic enrichment or isotope incorporation rate was evaluated for each
229 peptide by comparing the intensity (in counts) of the peak corresponding to the fully ¹⁵N labelled (U-¹⁵N)
230 peptide with other peaks corresponding to partially ¹⁵N labelled peptides. For practicality, we considered

231 a ^{13}C natural abundance of 1.1% and neglected hydrogen and oxygen isotopic distributions in our
232 calculations. Furthermore, only peaks corresponding to peptide with 1 ^{14}N isotope were considered in
233 our calculation. The proportion of U- ^{15}N peptide was then obtained after comparing the intensity of the
234 peak corresponding to the (U- ^{15}N & U- ^{12}C) peptide with the peak corresponding to the [(U-1)- ^{15}N & U-
235 ^{12}C] peptide. Protein isotopic enrichment was evaluated with the exponential trend given by the
236 proportion of the U- ^{15}N version of each peptide considering its nitrogen content.

237 **2.5. Food matrices preparation**

238 Three blank food matrices – thus, not contaminated with the considered allergenic ingredients –
239 were prepared to assess the variability due to the food sample used in our study. These blank matrices
240 were baked cookies, chocolate, and freeze-dried cookie dough.

241 Cookie dough was produced in batches of 3 kg by mixing (Kenwood Major Titanium, Stainless Steel
242 Dough Hook, 15 min, max speed) the following ingredients purchased from a local supermarket in the
243 respective weight proportions as follows: wheat flour (Carrefour type 55)/water (Milli-Q)/olive oil (Bertoli
244 Classico)/salt (sodium chloride ACS, $\geq 99\%$, Thermo Scientific™)/baking powder (Dr. Oetker
245 Baking)/Sugar (Grand Pont Crystal Sugar): 57%/18%/10%/0.2%/0.8%/14%. The dough was
246 subsequently rolled out to a thickness of 0.5 mm, and cookies with a diameter of 8 cm were pressed out
247 of the dough (weight = 25 ± 2 g). Cookies were baked for 25 min with the following program: 1–10 min:
248 180 °C heat from above and 180 °C heat from below; 11–25 min: 180 °C heat from above and 160 °C
249 heat from below. This was done to ensure that the warming of the baking plate would not result in uneven
250 cookie baking. Cookies were left at ambient temperatures to cool down, and subsequently milled and
251 sieved (Retsch® ZM 200 ultra-centrifugal mill [Retsch GmbH, Haan, Germany] with a 0.75 mm pore
252 size sieve, 14000 rpm). Cookie powder was stored at 4 °C in the dark until further use.

253 Cookie dough was produced as described above, rolled out to a thickness of 1 cm, stored at -20
254 °C, and subsequently freeze-dried. Freeze-dried cookie dough was then milled and sieved (Retch® ZM
255 200 ultra-centrifugal mill with a 0.75 mm pore size sieve, 14000 rpm). The freeze-dried cookie dough
256 powder was stored at 4 °C in the dark until further use.

257 Chocolate was made by warming chocolate walsenpowder (90%; Callebaut, Belgium) and cacao
258 butter (10%, Callebaut, Belgium) in a water bath at 40 °C (maximum temperature). The mixture was
259 stirred for 15 min, after which 2% ammonium phosphatide (kindly provided by Palsgaard, Julesminde,
260 Denmark) was added. This mixture was again stirred for 15 min and subsequently poured into chocolate

261 molds, resulting in chocolate chips of around 5 g each. The chocolate was left to cool down and solidify
262 at 4 °C for 2 h, and the chocolate chips were packed under vacuum and stored at 4 °C in the dark until
263 further use.

264 **2.6. Sample preparation for UHPLC-MS/MS analysis**

265 Two series of samples were prepared and analyzed to be able to cover the three internal standards.
266 Concatemer and β -lactoglobulin were isotopically labelled with the same strategy (^{15}N uniform labelling)
267 and share common tryptic peptides, which cannot be distinguished after enzymatic digestion. Two series
268 of samples were prepared. Labelled peptides and β -lactoglobulin were spiked in the first series (only
269 one shared peptide LSFNPTQLEEQCHI) and labelled concatemer in the second one. For each series,
270 the three blank matrices (baked cookie, chocolate, and lyophilized unbaked cookie dough) were spiked,
271 before extraction, with the appropriate internal standard and with increasing amounts of a standard
272 extract of the four allergens (milk, egg, peanut, and hazelnut). These allergen amounts corresponded to
273 0, 2.5, 5, 10, 25 and 50 ppm level points expressed in total allergen protein per matrix kg. For each
274 series, each blank matrix and each point of the allergen curve, three biological sample replicates were
275 prepared and analyzed. Stock solutions containing the four allergen standards at 20 mg/mL were
276 prepared using a similar extraction protocol as that used for the samples (extraction, sonication, and
277 centrifugation; see below). These stock solutions were then combined and diluted in appropriate ratios
278 to spike samples at different contamination levels with a 100 μl volume. Combination and dilution were
279 calculated based on theoretical protein content of standards assuming 100% extraction yield. Each
280 internal standard was spiked at the similar molar level (0.25 nmol) with a 100 μl volume. Then, 1 mg/mL
281 stock solutions of the five considered labelled peptides (ADITYTEQV[$^{13}\text{C}_5^{15}\text{N}$]GR,
282 FFVAPFPEVFGK[$^{13}\text{C}_6^{15}\text{N}_2$], GGLEPINF[Ring-D₅]QTAADQAR, LSF[Ring-D₅]NPTQLEEQCHI, and
283 TANELNLLIL[$^{13}\text{C}_6^{15}\text{N}$]R) were combined and diluted at the appropriate concentration with 0.1% FA.
284 Concatemer and β -lactoglobulin solutions were also diluted to be spiked at 0.25 nmol level with a 100
285 μl volume. This level, converted in equivalent allergen ppm, ranged from 10 ppm for abundant proteins,
286 such as α_{S1} -casein, to more than 300 ppm for less abundant proteins, such as vitellogenin-1. This
287 estimate was based on the natural abundance of each considered protein in the corresponding
288 allergenic ingredient. Allergen standards and internal standard were added to blank matrices before
289 extraction.

290 Samples were prepared as previously described (Planque et al., 2016). Briefly, protein from 2 g
291 samples was extracted in 50 mL conical tubes with 20 mL of 200 mM Tris, pH 9.2, 2 M urea by shaking

292 at 20 °C for 30 min (Agitelec, J. Toulemonde, Paris, France) prior to ultrasonic treatment at 4 °C for 15
293 min. After centrifugation (4660 x g, 10 min), 10 mL of supernatant were diluted in digestion buffer (200
294 mM ammonium bicarbonate, pH 8.2). Protein disulfide bridges were successively reduced and alkylated
295 with 45 min incubation steps at room temperature with the addition of 1 mL of 200 mM DTT and 1 mL
296 of 400 mM IAA (in the dark). Protein was then enzymatically digested with the addition of 1 mL of trypsin
297 solution (trypsin from bovine pancreas, 1 mg/mL in 50 mM acetic acid, pH 2.8) and incubation for 1 h at
298 37 °C. The digestion reaction was stopped by adding 300 µl of 20% FA to the samples, which were then
299 centrifuged (4660 x g, 5 min). Obtained peptides were then purified and concentrated using C18 SPE
300 cartridges, which were first conditioned with 18 mL of ACN followed by 18 mL of 0.1% FA before loading
301 of 20 mL of the centrifuged sample. The cartridges were washed with 18 mL of 0.1% FA and eluted in
302 15 mL conical tubes with 6 mL of 80% ACN and 0.1% FA. A volume of 30 µl of DMSO was added to the
303 sample before evaporation (40 °C under nitrogen flow) to avoid dryness. The pellet was finally dissolved
304 in 600 µl of 5% ACN with 0.1% FA and centrifuged twice (4660 x g, 5 min in conical tube and 20 000 x
305 g, 5 min in 1.5 mL microtube, keeping the supernatant) before UHPLC-MS/MS analysis.

306 **2.7. UHPLC-MS/MS analysis and data analysis**

307 The peptides were separated by reverse-phase chromatography on-line connected to a triple
308 quadrupole mass spectrometer. The following 26 min solvent gradient (solvent A, 0.1% FA and solvent
309 B, ACN and 0.1% FA) was applied to the 20 µl injected sample volume: 0–3 min: 92% solvent A; 3–18
310 min: linear gradient from 92% to 58% solvent A; 18–22.5 min: 15% solvent A; and 22.5–26 min: 92%
311 solvent A, always at constant 0.2 mL/min flow rate. Eluted peptides were ionized using the positive
312 electrospray source and analyzed in MRM mode. The source gas flow was set at 50 L/h and the source
313 voltage at 2.5 kV for the capillary and 30 V for the cone. The source temperature was set at 150 °C and
314 the desolvation temperature at 400 °C with a gas flow at 1200 L/h. Targeted transitions are summarized
315 in Table 1. For each peptide, three transitions were analyzed, as well as the corresponding transitions
316 for the related isotopically labelled internal standard(s) (peptides, concatemer, and β -lactoglobulin). The
317 transitions were selected beforehand using criteria that included the MS signal intensity and the absence
318 of interference for the three considered matrices. The MS/MS acquisition method was generated using
319 the open source Skyline software (MacLean et al., 2010). The most intense transition was used for
320 internal standard comparison calculation and the two others as confirmatory transitions. Internal
321 standards were compared using the peak area ratio (for the most intense transition) between the peptide

322 from the allergenic ingredient and its corresponding isotopically labelled version from the internal
323 standard.

324 3. Results and discussion

325 3.1. Choice of the isotope labelling strategy

326 Stable isotope internal standard labelling and associated isotopic enrichment are key elements in
327 the design of quantitative mass spectrometry-based methods. The isotopic enrichment and mass shift
328 combination has to be sufficient to avoid any potential risk of false positive introduction. The resolution
329 of quadrupole analyzers is typically around 1 atomic mass unit (Georgiou & Danezis, 2015). Taking into
330 account that most of the peptide ion precursors carry multiple charges and that peptides contain tens of
331 carbons, which lead to widespread isotopic distribution (see Fig. 1), the mass shift introduced by the
332 stable isotopes has to be sufficient to be able to totally distinguish the natural analyte from its internal
333 standard. Considering these aspects, a mass shift of $m/z \geq 3$ is necessary. Furthermore, attention has
334 to be paid to the actual isotope enrichment. Depending on the labelling strategy, an insufficient isotope
335 enrichment may lead to the introduction of the unlabeled form of the internal standard, thus
336 corresponding to the natural analyte itself and contaminating the quantitative analysis.

337 Several strategies have been developed to produce isotopically labelled proteins, including selective
338 labelling using auxotrophic *E. coli* strains and growth medium supplemented with isotopically labelled
339 amino acids (Mondal, Shet, Prasanna, & Atreya, 2013) or post-translational protein deuteration (Galan
340 et al., 2018). In this study, we decided to use a rich bacterial cell growth medium specifically designed
341 for ^{15}N labeling protein using *E. coli* as a host cell for recombinant protein expression. This original
342 medium is an algal hydrolysate that contains the same level of amino acids as LB medium. This strategy
343 allowed for stable and protein sequence independent labelling (as each amino acid contains at least
344 one nitrogen) with a high isotopic enrichment. As one of the peptide biomarkers selection criteria
345 concerned the actual peptide length (peptides should have at least 8 amino acids), $m/z \geq 3$ mass shift
346 precaution is respected for triply charged precursor. Indeed, selected peptide biomarkers are tryptic
347 peptides, with a lysine or an arginine in C-terminal position, holding two and four nitrogen atoms,
348 respectively.

349 3.2. Characterization of ^{15}N isotopically labelled proteins

350 The isotopic enrichment in the concatemer and β -lactoglobulin was evaluated following analysis of
351 their constitutive tryptic peptides by UHPLC-HRMS. The proportion of the fully ^{15}N labelled version of
352 each tryptic peptide was estimated by comparing the intensities of the monoisotopic peak ($\text{U-}^{15}\text{N}$ & U-
353 ^{12}C) and those of its isotope containing one ^{14}N isotope ($[\text{U-1}]^{15}\text{N}$ & $\text{U-}^{12}\text{C}$). As shown in Fig. 1, the

354 intensities of the peaks from peptides with more than one ^{14}N isotope were found to be negligible
355 (relative peak intensity <1% compared to the $[\text{U-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}]$ peak). Given the resolution of the MS
356 system (40000), carbon and nitrogen isotopes could not be distinguished. As a result, the monoisotopic
357 peak ($\text{U-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$) was combined with the peak corresponding to the peptide with one ^{14}N and one
358 ^{13}C isotope ($[\text{U-1}]\text{-}^{15}\text{N} \ \& \ ^{13}\text{C}_1$). The proportion of fully ^{15}N labelled peptide was evaluated by comparing
359 ($\text{U-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$) and ($[\text{U-1}]\text{-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$) peak intensities. The part of the peak intensity corresponding
360 to the ($\text{U-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$) isotope therefore had to first be discriminated from the combined ($\text{U-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$)
361 and ($[\text{U-1}]\text{-}^{15}\text{N} \ \& \ ^{13}\text{C}_1$) isotopes' peak intensity. Since isotopes with more than one ^{14}N were found to be
362 negligible, we assumed that the ($[\text{U-1}]\text{-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$) isotope peak would only correspond to this
363 combination of isotopes. The peak intensity of the ($[\text{U-1}]\text{-}^{15}\text{N} \ \& \ ^{13}\text{C}_1$) isotope could therefore be predicted
364 from the ($[\text{U-1}]\text{-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$) isotope peak intensity assuming a 1.1% natural abundance of ^{13}C isotopes
365 and knowing the number of carbon atoms in the peptide. With this prediction, the ($\text{U-}^{15}\text{N} \ \& \ \text{U-}^{12}\text{C}$) isotope
366 peak intensity could be deduced from the combined isotopes' peak intensity.

367 The proportion of fully ^{15}N labelled peptide was evaluated for all the 19 concatenated tryptic peptides
368 of the concatemer and for all identified tryptic peptides from β -lactoglobulin. As shown in Fig. 2, the
369 relation between the labelling proportion and the number of nitrogen atoms in the peptides follows an
370 exponential decay. The associated exponential decay constant corresponds to the natural logarithm of
371 the isotopic enrichment. Indeed, for a given isotopic enrichment (φ), the proportion of fully ^{15}N labelled
372 peptide with n nitrogens is given by φ^n , which can be transformed into $e^{\ln(\varphi)*n}$. Isotopic enrichment is
373 deduced from this mathematical transformation by equating $\ln(\varphi)$ to experimentally obtained
374 exponential arguments (-0.00446 for the concatemer and -0.00411 for β -lactoglobulin). These results
375 give an isotopic enrichment of 99.5% for the concatemer and 99.6% for β -lactoglobulin, and are in
376 agreement with the >98% isotopic enrichment of the growth medium.

377 By using a method for efficient isotopic labelling of recombinant protein, we demonstrated that the
378 purified ^{15}N isotopically labelled concatemer and β -lactoglobulin internal standards fulfilled the required
379 criteria regarding isotopic enrichment and the introduced mass shift. With this ^{15}N uniform labelling
380 strategy, the introduced mass shift was sufficient to distinguish the internal standard from the natural
381 analyte using the quadrupole analyzer. The lowest mass shift corresponded to the double charged
382 FYTVISSLK peptide (from egg white ovotransferrin), one of the 19 concatenated peptides, which
383 contained 10 nitrogen atoms and an associated mass shift of a m/z of 5. Such a mass shift and obtained
384 isotopic enrichment combination prevented the risk of false positive introduction.

385 **3.3. Comparison of isotopically labelled internal standards**

386 Performance of the three types of isotopically labelled internal standards (peptides, concatemer,
387 and protein) were evaluated following analysis of three food matrices (baked cookie [cookie], chocolate,
388 and lyophilized unbaked cookie dough [dough]). In theory, a perfect internal standard would have the
389 same exact behavior as its corresponding analyte during sample preparation and analysis. Hence, any
390 analyte loss or matrix effect (during sample preparation or UHPLC-MS/MS analysis) which affects the
391 analyte should equally affect the internal standard. Consequently, for a given natural analyte
392 concentration and internal standard spike level, the signal ratio between a natural analyte and the
393 internal standard would remain constant, independent of analyte losses and matrix effects. The three
394 internal standards considered in this study were compared based on this correlation.

395 Similar matrix-matched calibration curves were prepared for the three matrices. These curves
396 included a blank and five allergen concentrations ranging from 2.5 to 50 ppm (expressed in mg total
397 allergen protein per kg of matrix), with each sample prepared in triplicate. For each combination of matrix
398 and allergen contamination level, the appropriate internal standard(s) (isotopically labelled peptides and
399 U-¹⁵N β-lactoglobulin for the first sample series, and U-¹⁵N concatemer for the second one) was spiked
400 at the same concentration. Results are presented separately for each targeted peptide and its
401 corresponding internal standard (five synthetic peptides, 19 allergenic tryptic peptides from U-¹⁵N
402 concatemer digestion, and four tryptic peptides from U-¹⁵N β-lactoglobulin digestion). Representative
403 peptides of each internal standard are shown in Fig. 3, and complete results are shown in
404 Supplementary data 4. Performance of the different internal standards were evaluated by comparing
405 the peak area ratio for the most intense transition (highlighted in Table 1) between the analyte and its
406 corresponding internal standard for the three considered matrices. As shown, for a given analyte and
407 internal standard concentration, the signal ratio remained constant when the internal standard was
408 effective. The overlay of the generated linear regression lines was therefore used to evaluate internal
409 standards performance. Overlapping regression lines indicated, for each allergen contamination level,
410 a constant peak area ratio among matrices and thus, an effective internal standard, compensating for
411 matrix effects. In addition to visual evaluation, overlapping regression lines were evaluated using the
412 coefficient of variation (CV) between the slopes of the linear regression lines.

413 Overall, the best results were obtained for the isotopically labelled protein, U-¹⁵N β-lactoglobulin.
414 Assuming that recombinant protein folding was similar to the native protein and that the introduced N-
415 terminal mutations had no significant impact, as previously demonstrated (Loch et al., 2016), this

416 approach seemed to be the one best suited one for quantifying allergen proteins. Aside from their mass
417 (given the mass shift introduced by isotope labelling), both the analyte protein and the internal standard
418 protein must have had the same properties. This was confirmed by the analysis of four constitutive
419 tryptic peptides from β -lactoglobulin. Regression lines overlapped with all CV values below 15%. This
420 confirmed that the internal standard had efficiently balanced matrix effects during sample preparation
421 and UHPLC-MS/MS analysis, further supported by the fact that the analyte absolute peak area varied
422 by a factor of up to 10 among the three considered matrices, depending on the peptide (data not shown)
423 while the analyte/internal standard peak area ratio remained constant. However, the labelled protein
424 was spiked into the different samples after food processing, which is known to impact peptide
425 detectability and quantification (Korte et al., 2019; Parker et al., 2015). Peptide biomarker selection is
426 therefore a crucial preliminary step in the development of a quantitative method, and selected peptides
427 have to be robust to the food process.

428 The results obtained with isotopically labelled peptides and the concatemer were less
429 straightforward to interpret. For some targeted peptides, such as LSFNPTQLEEQCHI with labelled
430 peptides, or TNDNAQISPLAGR with the U-¹⁵N concatemer, the internal standard efficiently
431 compensated for matrix effects with observed CV values below 15%. However, for some other targeted
432 peptides, such as GGLEPINFQTAADQAR with both U-¹⁵N concatemer and labelled peptides, the
433 analyte and internal standard signal ratio was highly matrix-dependent. In these cases, internal
434 standards did not correctly balance matrix effects, potentially leading to biased allergen quantification.
435 These results are consistent with those reported by Planque and co-workers (Planque et al., 2019). No
436 significant difference was observed for the three peptides which were common to synthetic peptides and
437 concatemer used as internal standards.

438 Isotopically labelled peptides are not subject to one of the crucial steps during sample preparation,
439 this being the proteolytic digestion with trypsin. The composition of the food matrix directly impairs the
440 efficiency of enzymatic digestion at least in two different ways. First, different matrices have different
441 protein concentrations, directly affecting the protein/enzyme ratio. Labelled peptides do not balance for
442 this aspect. Second, some other sample components, such as polyphenols and tannins, may also affect
443 the efficiency of trypsin digestion (Gonçalves, Mateus, Pianet, Laguerre, & De Freitas, 2011), which
444 might help to explain why the chocolate matrix gave lower signals for most of targeted peptides. Contrary
445 to the labelled peptides, the U-¹⁵N concatemer needed to be digested by trypsin to yield peptides that
446 could be detected upon UHPLC-MS/MS analysis. Therefore, factors such as the sample protein content

447 or the presence of tannins should be balanced when using such an internal standard. However, our
448 results indicated that the performance of the concatemer was peptide-dependent. For some peptides,
449 such as TNDNAQISPLAGR from hazelnut Cor a 9 allergen and FFVAPFPEVFGK from milk α _{S1}-casein,
450 matrix effects were efficiently balanced with linear regression lines CV below 15% between the matrices.
451 However, for other peptides, such as NVNFDGEILK from egg vitellogenin-1 and TPEVDDEALEK from
452 milk β -lactoglobulin, the associated CVs were much higher (>30%).

453 Matrix effects can also affect analytes by other means. Robustness to food processing was one of
454 the criteria for peptide biomarkers selection (Gavage et al., 2019, 2020; Van Vlierberghe et al., 2020)
455 and can therefore be excluded. Variation in protein extraction can also be excluded as, for all selected
456 proteins, multiple peptides were included in the U-¹⁵N concatemer, and no general trend of the matrix
457 effect was observed for all the peptides of a given protein. Indeed, if protein extraction of the analyte
458 and/or the internal standard was affected by the matrix, all peptides from a given protein should be
459 equally impacted, which was not observed.

460 Proteolytic digestion of extracted proteins is a key step in sample preparation and could be a source
461 of the observed variability. Even if the concatemer internal standard needs to be digested to release its
462 constitutive peptides, multiple factors could influence the digestion kinetics. For instance, amino acids
463 surrounding trypsin recognition sites are known to influence the efficiency of peptide bond hydrolysis
464 (Siepen, Keevil, Knight, & Hubbard, 2007). Cleavage sites are described using the nomenclature
465 formulated by Schechter and Berger (Schechter & Berger, 1967), as P4-P3-P2-P1-P1'-P2'-P3', in which
466 cleavage of the peptide bond occurs between P1 and P1'. Arginine, lysine, and proline in position P1'
467 have, for instance, a negative effect on the digestion efficiency. The acidic amino acids aspartate and
468 glutamate also negatively influence digestion when they are present near the cleavage site. These
469 aspects were taken into account during peptide biomarkers selection, and sequences known to
470 negatively affect trypsin digestion were rejected. However, peptide biomarkers were synthetically
471 stitched together in the concatemer. Considering a given peptide in the concatemer, its cleavage site is
472 surrounded at the N-terminal side (P4 to P1) by amino acids from this peptide but also by amino acids
473 from its neighboring peptide at the C-terminal side (P1' to P3'). Consequently, at a local scale, enzymatic
474 digestion of the concatemer only partially reflects digestion of the natural proteins. This difference
475 between natural analytes and concatemers might lead to differences in enzymatic digestion kinetics and
476 could have been a source of the observed variations. A relatively simple solution to overcome this would
477 be the introduction of amino acids between each targeted peptide of the concatemer. Such introduced

478 amino acids could be the flanking amino acids in the corresponding natural protein sequence, a solution
479 known as a peptide-concatenated standard (PCS) (Kito, Ota, Fujita, & Ito, 2007). However, amino acids
480 surrounding the cleavage site in the three-dimensional structure of the protein might also affect trypsin
481 digestion. Hence, cleavage sites surrounded by acidic amino acids, characterized by a greater average
482 exposed area, are more subject to missed-cleavages.

483 Besides flanking amino acids, structural parameters also interfere with enzymatic digestion of a
484 protein. According to the work of Hamady and co-workers (Hamady, Cheung, Tufo, & Knight, 2005),
485 secondary protein structures affect trypsin digestion efficiency. Cleavage sites within unstructured
486 domains are more prone to be cleaved incorrectly, whereas cleavage sites in alpha-helices are more
487 favorable. The structures of proteins targeted by the UHPLC-MS/MS method, when available, were
488 analyzed to define whether observed variability among peptides could be linked to findings of Hamady
489 and co-workers or not (Hamady et al., 2005). No general trend emerged from our data, limited to the 19
490 concatenated peptides. However, three-dimensional and structural aspects could be included in a future
491 peptide biomarker selection, in addition to all other criteria already considered in this study.

492 **4. Conclusions**

493 Mass spectrometry-based detection and quantification of food allergens in processed food products
494 remains challenging. Currently, no threshold values for allergen trace-level contamination have been
495 established in European legislation, but these are highly expected by all stakeholders involved in the
496 food chain, from producers to control laboratories, and will require quantitative analysis methods.
497 Quantitative methods based on stable dilution techniques need isotopically labelled internal standards.

498 Here, we presented and compared the performances of three different types of isotopically labelled
499 internal standards for allergen analysis in processed food products: synthetic peptides, concatemer, and
500 protein. These internal standards were compared through the analysis of three matrix-matched
501 calibration curves (cookie, chocolate, and unbaked lyophilized cookie dough) for four targeted allergens
502 (egg, milk, peanut, and hazelnut). An effective internal standard needs to behave similar to the natural
503 analyte and is therefore identically impacted by matrix effects during sample preparation and UHPLC-
504 MS/MS analysis. As expected from a theoretical point of view, the isotopically labelled protein that was
505 used as an internal standard gave the best results. A constant signal ratio between the analyte and the
506 internal standard peak areas was observed in all matrices tested for the four tryptic peptides generated
507 from the studied protein. However, we need to emphasize that these results only come from one
508 investigated protein, β -lactoglobulin from milk.

509 Results from our studies using peptides and the concatemer were more equivocal and seemed to
510 be peptide-dependent. For some synthetic peptides or some tryptic peptides from the concatemer,
511 matrix effects during sample preparation and UHPLC-MS/MS analysis could be efficiently countered by
512 the applied internal standards, whereas for other peptides, significant matrix effects were observed.
513 However, the non-inferiority of the results obtained for the tryptic peptides from the concatemer was
514 established, when compared to synthetic peptides. Moreover, the addition of any synthetic peptide in a
515 method represent an additional cost, limiting therefore the number of targeted peptides for routine
516 laboratories. The concatemer production costs are relatively independent of the number of concatenated
517 tryptic peptides. From a rough estimate of ten peptides, the use of a concatemer as internal standard is
518 financially advantageous and supersedes synthetic peptides.

519 Even though isotopically labelled synthetic peptides are currently the most commonly used internal
520 standard for allergen analysis, they do not exactly reflect the natural situation as they do not need to be
521 subjected to proteolytic digestion, while part of the variability observed in our study could have come
522 from proteolytic digestion. Concatemers clearly need to be digested to release their constituting

523 peptides. However, our data seem to indicate that the digestion of the concatemer could be improved
524 to more efficiently represent analyte protein digestion. In this respect, introducing flanking amino acids
525 between each individual peptide (i.e. the PCS strategy) could be a future asset. Moreover, for our
526 concatemer construct, peptide biomarker selection was mainly focused on robustness to food
527 processing and local sequences, but additional criteria, such as protein structure and the local digestion
528 site environment, could be included in the peptide selection process. Such possible future improvements
529 strongly suggest that isotopically labelled concatemers could represent relevant internal standards, as
530 they overcome limitations of the use of synthetic peptides, while combining advantages of the use of
531 labelled proteins and, further, allowing for multiple allergen quantification by mass spectrometry.

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