1 *Article*

2 Feasibility of Utilizing Stable-Isotope Dimethyl 3 Labeling in Liquid Chromatography-Tandem Mass 4 Spectrometry-Based Determination for Food 5 Allergens—Case of Kiwifruit

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11 **Abstract:** Stable-isotope dimethyl labeling is a highly reactive and cost-effective derivatization procedure
12 that could be utilized in proteomics analysis. In this study, a liquid chromatography-tandem mass
13 spectrometry in multiple reaction monitoring mode (LC-MS-MRM) platform for the quantification of kiwi
14 allergens was first developed using this strategy. Three signature peptides for target allergens Act d 1, Act d
15 5, and Act d 11 were determined and were derivatized with normal and deuterated formaldehyde as external
16 calibrants and internal standards, respectively. The results showed that sample preparation with the phenol
17 method provided comprehensive protein populations. Recoveries at four different levels ranging from
18 72.5–109.3% were achieved for the H-labeled signature peptides of Act d 1 (SPA1-H) and Act d 5 (SPA5-H)
19 with precision ranging from 1.86–9.92%. The limit of quantification (LOQ) was set at 8 pg mL^{-1} for SPA1-H
20 and at 4 ng mL^{-1} for SPA5-H. The developed procedure was utilized to analyze seven kinds of hand-made
21 kiwi foods containing 0.0175–0.0515 mg g^{-1} of Act d 1 and 0.0252–0.0556 mg g^{-1} of Act d 5. This study
22 extended the applicability of stable-isotope dimethyl labeling to the economical and precise determination of
23 food allergens and peptides.

24 **Keywords:** stable-isotope dimethyl labeling; liquid chromatography-tandem mass spectrometry; food
25 allergen; kiwifruit
26

27 1. Introduction

28 Sensitive and reliable methods for the determination of food allergens are necessary to detect
29 the undeclared allergens resulting from contamination during food production and to enforce the
30 regulation of allergen labeling [1]. For these purposes, various techniques have been applied to
31 detect either the allergen protein itself or a marker indicating its presence [2]. Among those
32 methods, mass spectrometry (MS)-based proteomic methods, providing high sensitivity and
33 allowing the identification and quantification of allergenic protein, have been of great importance
34 for allergen detection [3].
35 In general, the methodology of MS-based proteomics absolute quantification is to analyze a
36 signature peptide using a synthetic analogue with a stable isotope label as the internal standard [4].
37 A signature peptide is a surrogate peptide that is unique to the target allergenic protein and fulfills
38 certain criteria, including the length of the peptide, accuracy of digestion, and absence of
39 posttranslational modification [5]. The stable isotope labels can be introduced into the signature
40 peptide metabolically, enzymatically, chemically, or provided by synthetic peptide standard [6].
41 However, many of these proposed methods suffer from several disadvantages, such as limited
42 biological applicability for labeling in cell culture, the requirement of a certain amino acid for some
43 chemical labeling methods, and high cost for isobaric tags, which restrict the applicability in routine
44 use for food samples [7].

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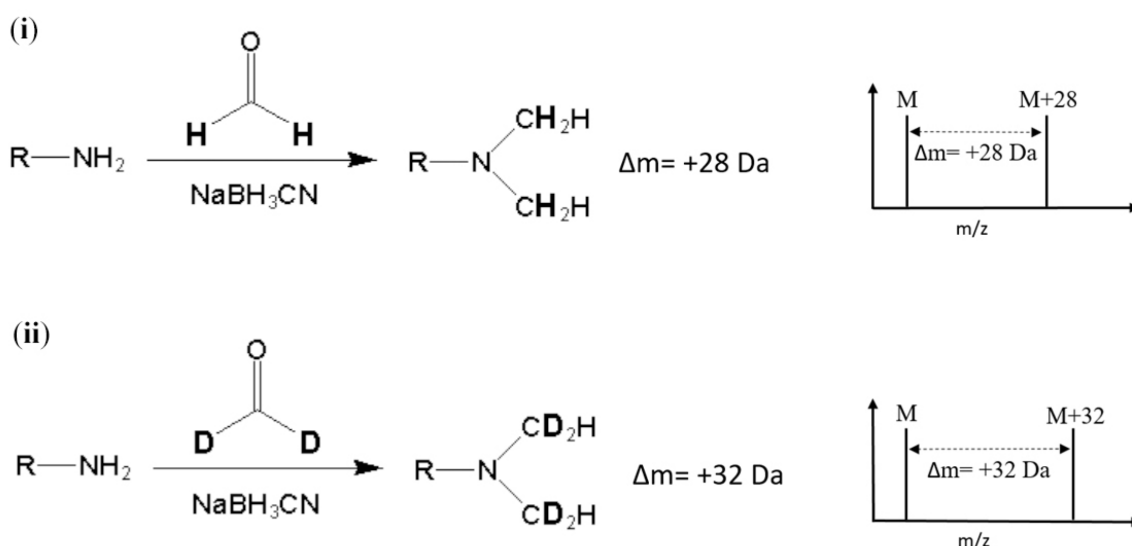
45 Stable-isotope dimethyl labeling, as a chemical labeling method for quantitative proteomics, is
46 achieved by the dimethylation of a primary amine (the N-terminus and side chain of lysine residue)
47 using different isotope forms of formaldehyde and sodium cyanoborohydride ($NaBH_3CN$) [8]. The
48 derivatization carried out with deuterated formaldehyde (CD_2O) produces a mass increase of 32 Da
49 for each reductive site (Fig. 1). On the other hand, dimethylation performed with normal
50 formaldehyde (CH_2O) generates a mass shift of +28 Da per primary amide. In addition, several
51 combinations of reagents with different isotope forms were proposed to produce various mass
52 differences [9]. The reaction of dimethyl labeling completes in minutes without any side product,
53 and this method is much more economical in cost than other developed methods [9, 10]. In
54 addition, dimethyl labeling can be practiced on protein from any species [7]. With these
55 advantages, stable-isotope dimethyl labeling has been applied in many quantitative proteomic
56 studies [11–14]. Nonetheless, to our knowledge, isotope dimethyl labeling has not been applied in
57 any analysis method for either allergenic proteins or food samples.



58

59 **Figure 1.** Reaction and *m/z* shift of (**i**) light (hydrogen) and (**ii**) heavy (deuterium) stable-isotope
60 dimethyl labeling. R represents the remainder of the peptide and M represents the *m/z* of the native
61 peptide with a single charge.

62 Kiwifruit (*Actinidia deliciosa*) is currently considered to be one of the common allergenic foods.
63 According to the World Health Organization and International Union of Immunological Societies
64 Allergen Nomenclature Sub-committee (WHO/IUIS-http://www.allergen.org/), 13 kiwifruit
65 allergenic proteins have been officially registered. Actinidin (Act d 1), a thiol-protease, is the major
66 allergen of kiwifruit [15]. Act d 2 belongs to the thaumatin-like protein family which includes
67 several fruit allergens [16]. Act d 3 is a glycoprotein, but its function is still unknown [17]. Act d 4
68 is characterized as phytocystatin, which is a cysteine protease [18]. Kiwellin (Act d 5), a major
69 protein component of kiwifruit, is a cell-wall-related protein [19]. Act d 6 and Act d 7 are a pectin
70 methylesterase inhibitor and pectin methylesterase, respectively [20]. Both Act d 8, a Bet v 1
71 homologue, and Act d 9, profilin, are pollen-related allergens [20, 21]. Act d 10 is a lipid transfer
72 protein, and Act d 11 belongs to ripening-related protein family [22, 23]. Act d 12, an 11S globulin,
73 and Act d 13, a 2S albumin, are two novel allergens that are located in the seeds [24]. Although
74 several kiwi allergens have been identified, few methods for their analysis have been proposed [25,
75 26]. Moreover, the low protein content within kiwifruit increases the difficulty of quantifying fruit
76 allergens, which means the development of a sensitive method is vital and necessary.
77 In this work, we report for the first time the application of stable-isotope dimethyl labeling to
78 the trace quantification of fruit allergens using liquid chromatography-tandem mass spectrometry in
79 multiple reaction monitoring mode (LC-MS-MRM). The identification of allergens was carried out

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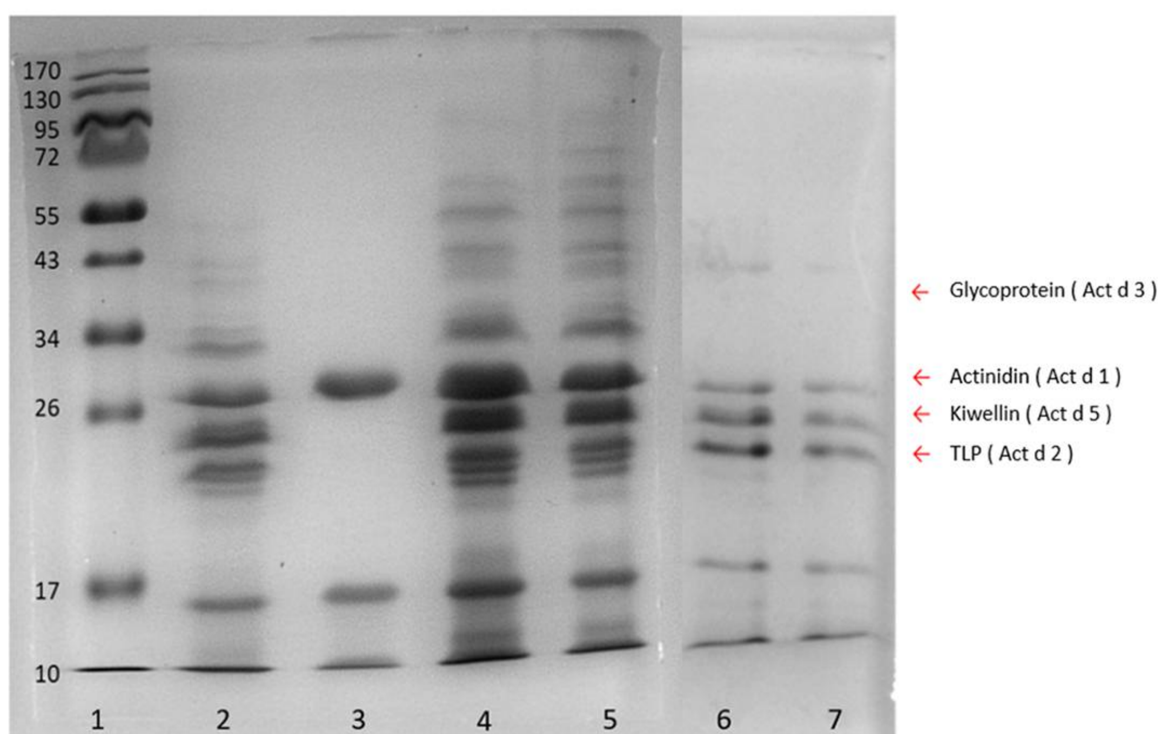
80 with liquid chromatography/electrospray ionization-quadrupole-time of flight mass spectrometry
81 (LC/ESI-Q-TOF). Eight of the thirteen proposed allergens were identified in this study, and Act d 1,
82 Act d 5, and Act d 11 were selected as target analytes. To develop the platform for the
83 quantification of kiwi allergens, the external and internal standards were prepared by isotopically
84 labeling the synthetic signature peptide standards with stable-isotope dimethyl labeling. In
85 addition, we optimized the protocol for protein extraction described in a previous study [27] and
86 proposed a more efficient procedure for sample preparation. Moreover, the established procedure
87 was validated with certain criteria and was applied to the self-made kiwi foods to examine the
88 applicability for foodstuff analysis.

89 **2. Results**

90 *2.1 Evaluation for protein extraction methods*

91 To determine the appropriate extraction method which could produce the most comprehensive
92 protein populations, kiwi proteins precipitated by phenol, trichloroacetic acid (TCA), ammonium
93 sulfate, and sodium chloride methods, were analyzed with SDS-PAGE (Fig. 2). Four allergenic
94 proteins, namely, actinidin (Act d 1), thaumatin-like protein (TLP) (Act d 2), glycoprotein (Act d 3),
95 and kiwellin (Act d 5) were chosen as the indicators to evaluate the extraction quality of each
96 method. Ammonium sulfate precipitation (Lane 3, Fig. 2) which gained only one (Act d 1) of the
97 four indicators showed poor extraction efficacy. On the other hand, the sodium chloride method
98 yielded three of them, while the phenol and TCA methods yielded all the indicators. In particular,
99 both the phenol and TCA methods spanned broadly in the region from 10 kDa to about 70 kDa
100 markers (Lanes 2, 4, and 5, Fig. 2), indicating that these two methods could provide the most
101 complete repertoire of proteins. To reduce the salt contaminants within the extracted proteins,
102 which might result in interference and ion suppression in further mass spectrometric assays [28], the
103 phenol method was eventually chosen for sample preparation.



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105 **Figure 2.** SDS-PAGE of the kiwifruit extracts from four different protein extraction methods. Lane 1,
106 protein ladder; lane 2, phenol extraction method; lane 3, ammonium sulfate precipitation method;
107 lanes 4 and 5, TCA extraction method; lanes 6 and 7, sodium chloride extraction method. Arrow

indicators on the right indicate four indicator proteins, glycoprotein (Act d 3) (40 kDa), actinidin (Act d 1) (30 kDa), kiwellin (Act d 5) (26 kDa), TLP (thaumatin-like protein) (Act d 2) (24 kDa).

2.2 Determination of signature peptides for kiwi allergens

For the establishment of an LC-MS-MRM approach for protein quantification, selection of signature peptides from the tryptic peptides was needed. The protein extracts underwent tryptic digestion and were then analyzed using LC/ESI-Q-ToF. The mass spectral interpretation of proteins in the kiwifruit was performed with the Mascot Distiller, and eight of the kiwifruit allergenic proteins were identified (Table S1). Among the eight allergenic proteins, Act d 1, Act d 5, and Act d 11 were selected as target allergens for quantification due to high coverage and MASCOT score in protein analysis. Tryptic peptides verified from these three proteins served as candidate signature peptides (Fig. 3). Further screening was carried out in accordance with several criteria: 8 to 19 amino acids in length to fit the scan range limit of the mass spectrometer, the absence of cysteine and methionine, which could result in chemical modification, and the exclusion from internal tryptic cleavage sites and ragged end to stabilize the accuracy of tryptic digestion [27]. Peptides fulfilling the requirements described above were subjected to a BLAST search to confirm if they were specific to the respective allergens and species (Table S2). Peptide ^{135}SAGAVVDIK^{143}, which met all the criteria and was absent in other proteins and species turned out to be the signature peptide for Act d 1 (SPA1). Instead of peptide ^{106}IVALSTGWYNGGSR^{119}, which existed in other species, peptide ^{160}NNIVDGSNAVWSALGLDK^{177} was selected to represent Act d 5 (SPA5). For Act d 11, peptide ^{109}GEHNSVTWTFHYEK^{122} was determined as the surrogate peptide (SPA11). The three chosen peptides were artificially synthesized as standards for the establishment of the quantification methods.

(A) Act d 1

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  1 MGLPKSFVSMSLLFFSTLLILSLAFNAKNLTQRTNDEVKAMYESWLIKYG
 51 KSYNSLGEWERRFEIFKETLRFIDEHNADTNRSYKVGLNQFADLTDEEFR
101 STYLRFTSGSNKTKVSNRYEPRVGQVLPSYVDWRSAGAVVDIKSQGECGG
151 CWAFSAIATVEGINKIVTGVLISLSEQELIDCGRTQNTRGCNGGYITDGF
201 QFIINNGGINTEENYPYTAQDGECNVDLQNEKYVTIDTYENVPYNNEWAL
251 QTAVTYQPVSVALDAAGDAFKQYSSGIFTGPCGTAVDHAVTIVGYGTEGG
301 IDYWIVKNSWDTTWGEEGYMRILRNVGGAGTCGIATMPSYPVKYNNQNHP
351 KPYSSLINPPAFSMSKDGPVGVDDGQRYSA
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(B) Act d 5

```
  1 ISSCNGPCRDLNDCDGQLICIKGKCNDDPQVGTHICRGTTHSHQPGGCKP
 51 SGTLTCRGKSYPTYDCSPPVTSSTPAKLTNNDFSEGGDDGGPSECDESYH
101 NNNERIVALSTGWYNGGSRCGKMIRITASNGKSVSAKVVDECDSRHGCDK
151 EHAGQPPCRNNIVDGSNAVWSALGLDKNVGVVDITWSMA
```

(C) Act d 11

```
  1 MDLSGKMVKQVEILSDGIVFYEIFRYRLYLISEMSPVNIQGVDLLEGNWG
 51 TVGSVIFFKYTIDGKEKTAKDIVEAIDEETKSVTFKIVEGDLMELYKTFI
101 IIVQVDTKGEHNSVTWTFHYEKLKEDVEEPNTLMNFCIEITKDIETYHLK
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Figure 3. Protein sequences of (**A**) actinidin (Act d 1), (**B**) kiwellin (Act d 5), and (**C**) kirola (Act d 11). The tryptic peptides identified with LC/ESI-Q-ToF are underlined, and the selected signature peptides are presented in boldface type.

2.3 Sample derivatization and preparation of internal standards

An internal standard is another prerequisite for absolute quantification to calibrate the loss and error throughout the whole analysis procedure. For the preparation of internal standards, the signature peptides were reacted with CD_2O to form a dimethyl group on the N-terminus and lysine residue side chain. On the other hand, the external calibrants and the samples were derivatized with CH_2O. To investigate the exact mass shift and the efficiency of the labeling reaction, we analyzed the peptide markers with and without dimethyl label using a mass spectrometer. As shown in Fig. 4, +56 and +64 *m/z* shift for the H- and D-labeled SPA1 (SPA1-H, *m/z* 915 and SPA1-D, *m/z* 923) were observed when compared with SPA1 (*m/z* 859). In addition, the ion peak of the unlabeled SPA1 was not detected at the *m/z* of 859 in spectra of SPA1-H and SPA1-D, implying the complete labeling of peptide standards. On the other hand, the respective increase in *m/z* values for the H- and D-labeled SPA5 (SPA5-H, *m/z* 965 and SPA5-D, *m/z* 969) were 28 and 32 (Fig. S1). Relative to SPA11 (*m/z* 579), there was a +19 and a +21 *m/z* shift for the H- and D-labeled SPA11 (SPA11-H, *m/z* 598 and SPA11-D, *m/z* 600) (Fig. S1). The ion peaks of the unlabeled SPA5 were not detected at the *m/z* of SPA5 (*m/z* 937), nor detected at that of unlabeled SPA11 (*m/z* 579). Taken together, these MS results demonstrated that the synthetic peptides were mostly labeled with dimethyl, indicating that stable-isotope dimethyl labeling provided high labeling efficiency and that the H- and D-labeled peptides could be properly produced by stable-isotope dimethyl labeling as external calibrants and internal standards, respectively, for the development of the quantification methods.

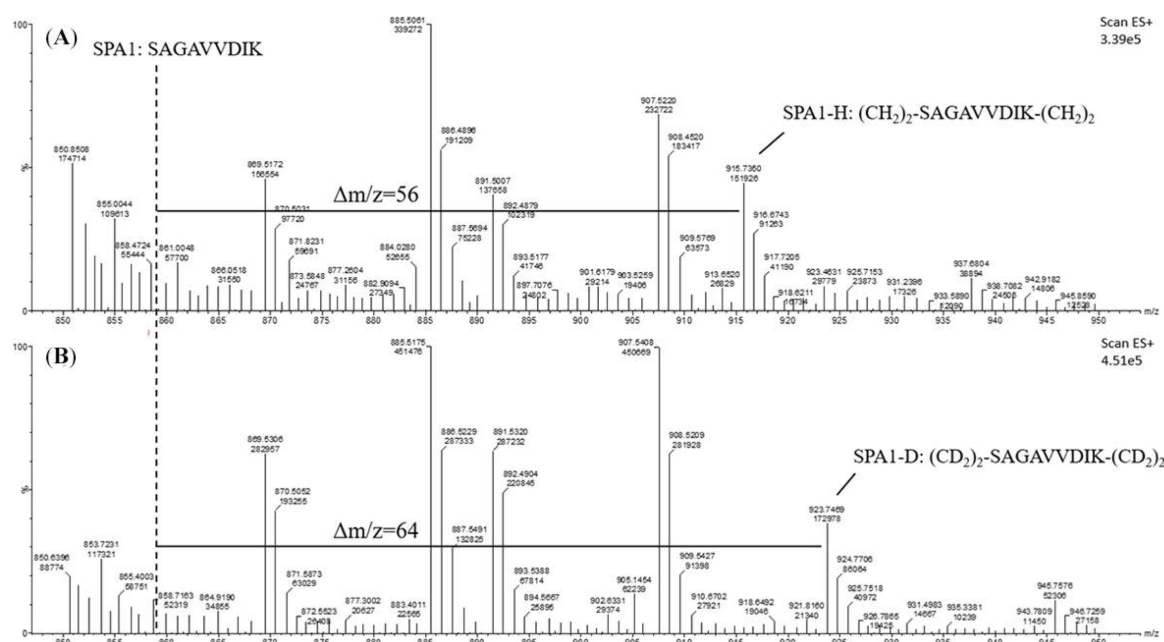

Figure 4. Mass spectra of (**A**) SPA1-H and (**B**) SPA1-D. The dotted line represents the *m/z* of SPA1 and Δ*m/z* shows the *m/z* shift of the signature peptide before and after stable-isotope dimethyl labeling.

2.4 Optimization for LC-MS-MRM parameters

In this study, LC-MS-MRM assay was applied for protein quantification. To develop the LC-MS-MRM methods, the conditions of both the mass spectrometer and the LC gradients were first

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optimized to ensure that the parameters could maximize the intensity of the peptides and that the peptides could be separated appropriately with the LC system.

The transitions and optimized parameters of MRM methods for the following data acquisition are summarized in Table 1. In addition to the optimization of MRM parameters, separation of peptides was accomplished with a linear gradient. The MRM chromatograms for SPA1-H and SPA1-D standards are shown in panels A and B of Fig. 5, respectively, and those for the labeled form SPA5 and SPA11 are shown in Fig. S2. The retention time of each peptide was obtained using the standards and is summarized in Table 1. It should be mentioned that SPA1-D, as the isotope-labeled internal standard, was coeluted with SPA1-H at almost the same retention time. When it comes to SPA5-D and SPA11-D, the same situation was also observed. These results indicated that the D-labeled peptides, coeluting with the target analytes, could correct not only loss during sample preparation but also instrumental errors in the LC and MS system.

Table 1. The parameters of LC-MS-MRM analysis for dimethyl-labeled peptides

Peptide code	Stable isotope dimethyl labeled peptide sequence	Retention time (min)	Precursor ion (*m*/*z*)	Cone voltage (V)	Product ion (*m*/*z*)	Collision energy (V)
SPA1-H	$(CH_2)_2$-SAGAVVDIK-$(CH_2)_2$	1.2	915	55	288	45
					628	40
SPA1-D	$(CD_2)_2$-SAGAVVDIK-$(CD_2)_2$	1.2	923	55	292	40
					632	40
SPA5-H	$(CH_2)_2$-NNIVDGSNAVWSALGLDK-$(CH_2)_2$	2.9	965	65	460	30
					584	25
SPA5-D	$(CD_2)_2$-NNIVDGSNAVWSALGLDK-$(CD_2)_2$	2.9	969	65	921	40
					1563	35
SPA11-H	$(CH_2)_2$-GEHNSVTWTFHYEK-$(CH_2)_2$	1.5	598	50	466	25
					302	30
SPA11-D	$(CD_2)_2$-GEHNSVTWTFHYEK-$(CD_2)_2$	1.5	600	45	527	20
					855	20

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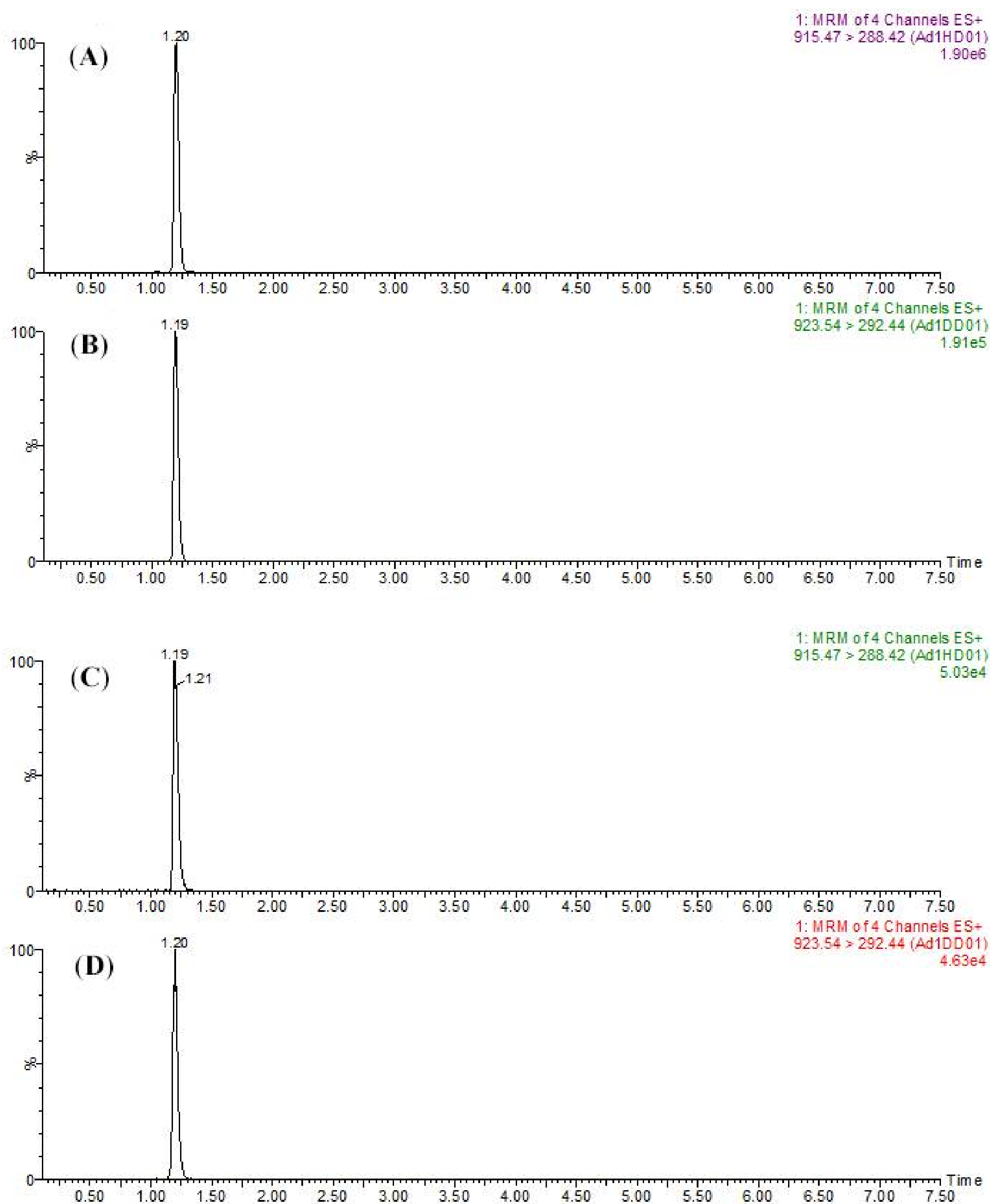

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175 **Figure 5.** MRM chromatograms of (**A**) SPA1-H (1 μg mL^{-1}) and (**B**) SPA1-D (500 ng mL^{-1}) for the
176 mixture of peptide standards and (**C**) SPA1-H (479.3 ng mL^{-1}) and (**D**) SPA1-D (500 ng mL^{-1}) for
177 kiwifruit raw extract sample.

178 *2.5 Method validation*

179 Prior to the quantification of the allergen commodities, this method was assessed for its
180 linearity, sensitivity, recovery, and repeatability. The calibration curves were generated by plotting
181 the ratio of the area of the external calibrants and internal standards to the concentration of the
182 spiked external calibrants. As shown in Table 2, good linearity ($R^2 \geq 0.99$) was observed in the
183 range of 0.008–4000 ng mL^{-1} for SPA1-H, in the range of 8–4000 ng mL^{-1} for SPA5-H, and in the range
184 of 20–4000 ng mL^{-1} for SPA11-H. Regarding the sensitivity, the LODs ranged between 8 pg mL^{-1}
185 and 8 ng mL^{-1}, and LOQs were between 8 pg mL^{-1} and 20 ng mL^{-1}, depending on the signature
186 peptides.

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187 **Table 2.** Coefficient of determination, LOD, LOQ, recovery, and precision of the target peptides.

Peptide code	R^2	LOD[a]	LOQ[a]	Spike[b]	Recovery (%)	Precision(% CV)
SPA1-H	0.9990	0.008	0.008	12500	109.9	1.86
				1562.5	83.9	5.11
				1250	78.9	9.92
				1000	75.6	8.51
				125	62.5	7.67
SPA5-H	0.9936	4	8	25000	75.8	3.44
				3215	86.3	9.55
				2500	84.9	9.37
				2000	72.5	3.87
				250	97.8	17.2
SAP11-H	0.9908	8	20	1250	39.8	11.7
				156.25	—[c]	—
				125	68.7	36.4
				100	341.9	72.3
				12.5	—	—

188 In addition, the assessment of accuracy and precision was performed with the kiwi matrices
189 spiked with H-labeled standards at five concentration levels and 500 ng mL^{-1} of internal standards.
190 Regarding the validation results of SPA1-H and SPA5-H, the lowest recovery was observed for
191 SPA1-H (65.5%), and the highest coefficient of variation was observed for SPA5-H (17.2%) (Table 2).
192 The range of recoveries was 75.6–109.3% for SPA1-H within the spiking range of 1000–12500 ng and
193 72.5–86.3% for SPA5-H within the spiking range of 2000–25000 ng, and the coefficients of variation
194 for SPA1-H and SPA5-H were 1.86–9.92% and 3.44–9.55%, respectively, which indicated that good
195 robustness could be achieved when analyzing SPA1-H and SPA5-H. On the other hand, the
196 percentage of SPA1-H recovery was from 39.8 to 341.9% with a coefficient of variation ranging from
197 11.7 to 72.3% (Table 2). The high variation for SPA11-H observed in the validation assessment
198 indicated that accurate and precise quantification of SPA11-H could not be carried out by means of
199 this analytical workflow, and SPA11-H was excluded in the further analysis of food samples. In
200 contrast, the quantification of SPA1-H and SPA5-H could be performed with this developed
201 procedure.

202 *2.6 Analysis of kiwi food samples*

203 The proposed workflow for sample preparation and LC-MS-MRM assay was applied to the
204 analysis of kiwifruit and kiwi foods. All the kiwi-based foods, including kiwi jam, hot-air-dried
205 kiwi, lyophilized kiwi, pasteurized puree, high-pressure-processed (HPP) puree, pasteurized juice,
206 and HPP juice, were self-made to control the amount of kiwifruit content in the foods. In addition,
207 to promote the operation and to reduce the reagents' use during sample preparation, some steps in
208 the literature [27] were modified for low-volume extraction. The amount of kiwi pulp used in the
209 step of sample preparation was reduced to 0.07 g, the total volume of extraction buffer used was
210 reduced to less than 500 μL, and the reaction time for the protein precipitation step was shortened to
211 4 h. Furthermore, to investigate the efficacy of the modified method, we performed one phenol
212 extraction plus three additional back extractions. The first three extractions gained approximately
213 73.7%, 20.2%, and 6.1% of the total extracted protein, respectively (Fig. S3). On the other hand, the
214 third back extraction produced little to no protein, which showed that almost all the extractable

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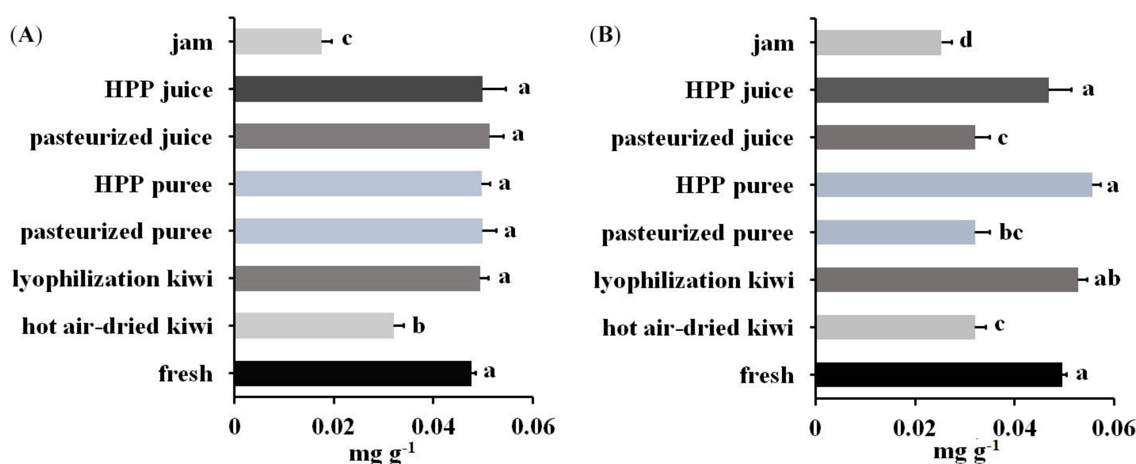
215 proteins could be obtained with two additional back extractions. After several modifications, the
216 optimized procedure and the procedure previously described [27] were carried out eventually to
217 investigate the extraction efficiency, and the former was found to achieve mostly the same extraction
218 efficiency (Fig. S4). Naturally, the modified procedure which was easy to operate and economical
219 in time was applied to the sample preparation in the following experiments.

220 Samples prepared with the procedure described above were then analyzed using LC-MS-MRM
221 (panels C and D of Fig. 5 and Fig. S2). The content of Act d 1 and Act d 5 was quantified with the
222 peak area ratio of the corresponding transition for the derivatized signature peptides. The content
223 of Act d 1 in kiwi products was in the range of 0.0175–0.0515 mg g^{-1} product weight (Panel A of Fig.
224 6). As can be seen, kiwi jam and hot-air-dried kiwi contained less Act d 1 with concentrations of
225 0.0175 and 0.0319 mg g^{-1}, respectively, than fresh fruit and other products with concentrations
226 between 0.0476 and 0.0511 mg g^{-1}. Panel B of Fig. 6 showed the content of Act d 5 ranged from
227 0.0252 to 0.0556 mg g^{-1}. The lower content of Act d 5 was observed in products with thermal
228 processing (kiwi jam, pasteurized juice, pasteurized puree, and hot-air-dried kiwi) when compared
229 with fresh fruit, which contained 0.0495 mg Act d 5 in one gram of kiwifruit. Moreover, all the
230 products with thermal processing contained less Act d 5 than those without thermal processing did,
231 indicating that Act d 5 was a heat intolerant allergen. However, regarding Act d 1, only some
232 products with thermal processing (hot-air-dried kiwi and kiwi jam) presented lower content of Act d
233 1 when compared with those without thermal processing, showing that Act d 1 was stable during
234 pasteurization and that a long period of hot-air drying and severe thermal process could result in the
235 decrease of the content of Act d 1.



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237 **Figure 6.** Content of (**A**) Act d 1 and (**B**) Act d 5 in one gram of kiwi foods produce by different
238 processing. There was no significant difference between treatments with the same letter (p-value <
239 0.05. n = 3).

240 **3. Discussion**

241 The sample preparation and protein extraction were key steps for trace peptide determination.
242 Compared with ammonium sulfate precipitation and sodium chloride precipitation methods, the
243 phenol extraction coupled with methanolic ammonium acetate precipitation carried out a more
244 comprehensive population of kiwi proteins in fruit juice samples (Fig. 2). This could result from the
245 two-step procedure in the phenol method, in which the first phenol extraction could exclude
246 interfering components such as water-soluble sugars and acids in food samples, and the following
247 ammonium acetate precipitation increased the purity of the proteins. On the other hand,
248 ammonium sulfate and sodium chloride methods were direct precipitations that could obtain more
249 hydrophilic compounds and result in poor protein quality. In addition, the phenol method could
250 further inhibit protein degradation and retain the protein repertoire as well [29]. Therefore, the

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phenol method was suggested to extract proteins from the complex matrix and food samples with low protein content.

A signature peptide, the critical element for the protein identification and quantification, should fulfill several criteria to guarantee its stability and specificity. It was reported that peptide containing arginine and asparagine was susceptible to deamidation, which could lead to errors in MS-based quantitative methods [30]. However, in this study, the quantification of SPA5-H, which contains three asparagines was not influenced by deamidation, where high recovery and low coefficient of variation were observed in the validation assessment for SPA5-H (Table 3). This is probably because SPA5-D, as the chemically analogous internal standard, would show almost the same degree of deamidation as the SPA5-H and thus adjusted the variation resulting from deamidation. In other studies using an isotope internal standard strategy, good robustness could be achieved as well when analyzing signature peptides containing arginine and asparagine [31, 32], which supported our contention that the isotope internal standard could correct the errors caused by deamidation and indicated that a peptide being prone to deamidation could be selected as a signature peptide for protein quantification with the employment of an isotope internal standard.

For accurate and precise quantification, a chemically analogous isotope internal standard was essential to compensate for recovery loss and systematic bias within the analysis procedure. In this study, the artificial synthetic signature peptides were derivatized with CD_2O as the isotope internal standard. The peptides methylated with CH_2O served as the standards for calibration curves. It was observed that the dimethylation of signature peptide standards had been fully reacted without any unlabeled peptide detected (Fig. 4 and Fig. S1), and highly linear calibration curves were established. In addition, all the signature peptides in the kiwi food samples were labeled with CH_2O as well to minimize possible variation and errors resulting from the incomplete reaction and to correct and maintain the recovery in sample preparation and the derivatization process. Fortunately, there were no unreacted allergen peptides detected within food samples after dimethylation, which indicated that the reaction of food samples was highly effective and efficient. Moreover, the isobaric tag for relative and absolute quantification (iTRAQ) which labels isotopic *N*-methylpiperazine onto peptides is commercially available, and it has been widely utilized for various types of sample matrices such as blood, plant tissues, and food samples [33, 34, 35]. Unlike the quantification by dimethylation which labeled the precursor ions of H- and D-labeled peptides and their sequence fragment ions, the iTRAQ method was carried out by means of detecting the *N*-methylpiperazine tag as the reporter ion of the peptides. Hence, the efficiency of the labeling reaction would highly influence the number of reporter ions and the recovery of target proteins. The results in which no unlabeled peptides were detected in the MS spectra (Fig. 4 and Fig. S1) revealed that formaldehyde methylation was an effective and efficient derivatization and thus suggested that dimethyl labeling could be an alternative method to iTRAQ.

To assure the reliability of this study, certain parameters such as precision and accuracy should be evaluated. In the validation experiments, the H-labeled peptide standards and the D-labeled internal standards were spiked before the trypsin digestion to examine the recovery and reproducibility for sample preparation. After the experiments, good robustness could be observed when analyzing SPA1-H and SPA5-H (Table 3). These results represented that, with respective labeling of CH_2O and CD_2O on the analytes and the internal standards, the systematic errors such as recovery loss within the desalting process and inconsistent ionization efficiency for analytes could be adjusted, and accurate and precise quantification could be achieved. Furthermore, compared with other commonly used labeling approaches such as metabolic labeling and the iTRAQ method, dimethyl labeling was more convenient and accessible due to its low cost and ease of operation [9]. Taken together, the dimethyl labeling method could be a recommended strategy for routine analysis.

The analytical procedure was validated for SPA1-H and SPA5-H, however, a lower S/N ratio and a high coefficient of variation was observed when it came to SPA11-H. This result could be attributed to the poor performance of SPA11-H on charge competition during the ionization within ESI and led to poor ionization. As reported previously, the nonpolar residues contributed to the

fraction of peptides from ESI droplets, and hydrophobic residues could reduce the ability for peptides in charge competition [36]. Thus, SPA11-H containing only two nonpolar amino acids could be less successful in competing for charges with the matrix and led to unstable ionization efficiency. To prevent this disadvantage, an amino acid with hydrophilicity was suggested to be taken into consideration in the selection of the signature peptide for quantitative protein analysis using dimethyl labeling coupled with LC/ESI-MS/MS.

The workflow in this study was finally applied to kiwi foods, which were low in protein content. This extended the applicability of stable-isotope dimethyl labeling to the MS-based quantification of kiwi allergens at trace level and revealed the potential of this labeling method in protein analysis of food samples. Unlike blood or urine samples, in which the matrix is normally composed of water, proteins, and electrolytes, the matrix of food samples varies based on the food materials. In fruits and vegetables, there would be more cellulose and low proteins, but carbohydrates, lipids, and proteins would be the major components when it comes to soybean seeds. For meats and seafood, the samples could have high protein and lipid content. Therefore, the sample extraction and preparation steps would be critical for different food samples to remove compounds that could lead to interference in the following sample preparation and instrumental analysis. With an appropriate extraction procedure such as the phenol method in this study that removed the contaminants, complete derivatization reaction could be achieved and resulted in accurate analysis with the stable-isotope dimethyl labeling method.

In conclusion, we first introduced the stable-isotope dimethyl labeling method to develop a reliable and economical LC-MS/MS quantitative procedure for kiwi allergens. The high efficiency of the dimethyl labeling was proved, and the developed procedure was validated and was used on kiwi food samples for trace allergen analysis. This dimethyl labeling strategy could be further utilized and applied to the quantification of allergens in different food matrices and could have potential for the analysis of bioactive peptides and of peptide markers for food authenticity.

4. Materials and Methods

4.1 Materials and reagents

Dithiothreitol (DTT), iodoacetamide (IAA), ethanol, ammonia, formic acid, formaldehyde (37% solution in H_2O), formaldehyde-D_2 (20% solution in H_2O), phenol, sodium deoxycholate (SDC), trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, and *triethylammonium bicarbonate buffer (TEAB)* were purchased from Sigma-Aldrich (St. Louis, MO). Acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), *N*,*N*′-methylene-bis-acrylamide, glycine, and *N*,*N*,*N*′,*N*′-tetraethylmethylenediamine were obtained from Bio-Rad (Hercules, CA). MS grade trypsin was provided by Fisher Scientific (Houston, TX). Acetonitrile (MS grade), acetone, Coomassie brilliant blue, methanol, Tris-Base, and ammonium acetate were from J.T.Baker (Phillipsburg, NJ). Sodium cyanoborohydride was purchased from Alfa Aesar (purity 95%, Haverhill, MA). Urea was from VWR International (Radnor, PA). Ammonium sulfate was obtained from Taiwan Fertilizer Co. (Hsinchu, Taiwan). The peptide standards, IVALSTGWYNGGSR (SPA1), NNIVDGSNAVWSALGLDK (SPA5), and GEHNSVTWTFHYEK (SPA11) were synthesized at the National Institute of Infectious Diseases and Vaccinology (Taipei, Taiwan). Kiwifruits were purchased from a local market in Taipei city.

4.2 Phenol extraction

The phenol extraction method was modified according to a previously described method [27]. Seventy μL of the extraction buffer (1.8 M sucrose, 240 mM Tris-HCl, pH 8.0, 24 mM EDTA, and 0.96% (v/v) β-mercaptoethanol) was added to 0.07 g homogenized kiwi powder, and 100 μL of saturated sucrose in phenol was added subsequently. Being mixed for a few seconds, the mixtures were placed in an ultrasonicator bath for 20 min at 40 °C and centrifuged at 3900*g* for 15 min at 25 °C. Afterward, the supernatants (the phenol phase) were transferred to a new tube, and the lower aqueous phase was extracted twice with the same steps described above. Proteins were

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precipitated from the collected phenol phase with five volumes of 0.1 M ammonium acetate in methanol at –20 °C for 4 h and then pelleted by centrifugation at 21100*g* for 10 min at –9 °C. The protein pellets were further washed with 0.1 M ammonium acetate in methanol, 80% ice-cold (v/v) acetone, and cold 70% (v/v) ethanol. Being dried with a vacuum dryer, the protein pellets were finally stored at –20 °C.

4.3 TCA precipitation

Kiwi puree (4 g) was added to 25 mL TCA buffer (10% (w/v) TCA and 2% (v/v) *β*-mercaptoethanol in acetone). Extracts were mixed for a few seconds and precipitated for 1 h at –20 °C. The precipitated proteins were pelleted by centrifugation at 5000*g* for 30 min at 4 °C and washed twice with 10 mL ice-cold acetone. The pellets were dried with a vacuum dryer and resuspended in 15 mL tank buffer (25 mM Tris-base, 0.2 M glycine, and 0.1% (w/v) SDS). The extracts were centrifuged at 5000*g* for 10 min at 4 °C, and the resulting supernatants were transferred to a new tube and stored at –20 °C.

4.4 Ammonium sulfate precipitation

Kiwi pulp (250 g) was blended with 250 mL of extraction buffer (100 mM Tris-HCl, 2 mM NaCl, and 10 mM EDTA). After the centrifugation (4500 rpm, 15 min, 25 °C), the supernatants were added with ammonium sulfate to 95% saturation and precipitated overnight at 4 °C. The precipitated protein was collected by centrifugation (9300*g*, 15 min, 4 °C). Afterward, the protein pellets were suspended in dialysis buffer (200 mM Tris-HCl, 20 mM EDTA, 2 mM NaCl, 0.8% (v/v) *β*-mercaptoethanol) and dialyzed against water for at least 24 hours. The treated solution was finally stored at –80 °C.

4.5 Sodium chloride extraction

This method was carried out as described previously [28]. One mL extraction solution (0.5 M NaCl, pH 8.3, 10 mM DTT) was added to 0.7 g of homogenized kiwi pulp. Extracts were placed on ice for 1 h and centrifuged at 16000*g* for 10 min at 4 °C. The supernatants were transferred to a new tube and stored at –20 °C.

4.6 SDS-PAGE analysis

Extracted proteins were separated via polyacrylamide gel electrophoresis (PAGE) using 12% SDS-PAGE gels at a voltage of 60–130 V. After the separation, the proteins were visualized with Coomassie blue G-250, and the gels were finally digitized with a scanner.

4.7 Tryptic digestion

For samples subjected to LC/ESI-Q-TOF analysis, the protein pellets collected from the phenol extraction were resuspended with 0.5 mL of resuspending buffer (9 M urea in 1 M TEAB buffer). The protein solution was then diluted to the concentration range of 1.5–2.4 $\mu g\ \mu L^{-1}$. Reduction of the disulfide bonds was performed with 0.6 μL of 100 mM DTT, and the alkylation of the cysteine residues was carried out in the dark for 30 min with 0.9 μL of 500 mM IAA. Afterward, 4 μL of 100 mM DTT was added, and the treated solution was incubated for 10 min to quench the alkylation reaction. Prior to tryptic digestion, the solution was first diluted with 182 μL of water. Finally, 2.5 μL of trypsin (0.1 mg mL^{-1}) was added to the samples for 16-hour digestion at 37 °C.

The digestion protocol for samples for LC-MRM analysis was as follows. The protein pellets from the phenol method were added to 100 μL of resuspension buffer (5% SDC in 50 mM TEAB buffer) and 1 μL of 500 mM DTT in 50 mM TEAB. The protein pellets were then resuspended by pipetting. For the protein solution, disulfide bonds were reacted with DTT for 30 min at room temperature, and the cysteine residues were alkylated with 8 μL of IAA (50 mM in TEAB buffer) for 30 min in the dark at room temperature. The alkylation reaction was quenched with 6 μL of DTT (500 mM in TEAB buffer) for 10 min at room temperature. The treated protein solution was then

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diluted with 885 μL of TEAB buffer. Afterward, 35 μL of the diluted protein solution was transferred to a new tube and added with 15 μL of internal standard solution, formed by mixing the D-labeled peptide standards, SPA1-D, SPA5-D, and SPA11-D, with the concentration of 25 μg mL^{-1} of each in the ratio 1:1:1 (v/v/v). The mixed solution was finally added with 2.5 μL of trypsin (0.1 mg mL^{-1}) and incubated for 7 h at 37 °C.

4.8 Stable isotope dimethyl labeling

This procedure was modified from the study previously proposed [8]. For the preparation of standards, five μL of 4% (v/v) formaldehyde solution (light, CH_2O; heavy, CD_2O) was added to 25 μL of the peptide standards (1 mg mL^{-1}) diluted with 100 μL of TEAB buffer. Immediately after the addition of formaldehyde, five μL of freshly prepared 0.6 M $NaBH_3CN$ was added. After incubation for 1 h at 20 °C, the derivatization reaction was quenched by adding 20 μL of 1% (v/v) ammonia. Then, ten μL of 5% (v/v) formic acid was added to acidify the solution. It should be noted that the acidification needs to be performed on ice. After that, the solution of peptide standards was finally diluted to a concentration of 25 μg mL^{-1} with 835 μL of water.

For the preparation of samples for LC-MS-MRM analysis, the dimethylation was carried out after the tryptic digestion. Samples undergoing tryptic digestion were added with 1.5 μL of 4% (v/v) CH_2O, and 1.5 μL of freshly prepared 0.85 M sodium cyanoborohydride was then added right after the addition of formaldehyde. The reaction was quenched with the addition of 6 μL 1% (v/v) ammonia after 1 h of incubation at 20 °C and was then acidified with 3.5 μL 5% (v/v) formic acid. The treated samples were finally centrifuged at 15700*g* for 2 min to pellet SDC, and 52 μL of the supernatants were transferred to a new tube.

4.9 Zip-Tip desalting

Prior to LC-MS/MS analysis, samples were desalted using the Zip-Tip C18 pipette tips. The tips were prewetted with acetonitrile and then equilibrated with 0.1% (v/v) formic acid before use. The peptides were bound to the tips by approximately 30 times of up-down pipette draws. The tips were then washed with 0.1% (v/v) formic acid for desalting. After that, the peptides were eluted with 20 μL of 60:40 acetonitrile/0.1% formic acid (v/v) by aspirating and dispensing the elute solution for about 20 times. The eluted solution was finally diluted with 180 μL of water and stored at –20 °C for further analysis.

4.10 LC/ESI-Q-TOF analysis

Samples treated with digestion and desalting were subjected to LC/ESI-Q-TOF analysis. The peptide mixtures were separated with a Waters Acquity solvent delivery system. Mobile phase A was 0.1% (v/v) formic acid in water whereas mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The separation was carried out on a *ACQUITY* UPLC® *Peptide BEH* C18 nanoACQUITY column (1.7 μm, 75 μm × 250 mm) at a stationary flow rate at 0.25 mL min^{-1} with 120 min total run time, and the injection volume was 5 μL. The mobile phase gradient started from 2% to 10% mobile phase B over 0.1 min; mobile phase B was then linearly ramped 40% over 90 min; from 90.1 min to 95 min the amount of mobile phase B increased linearly from 40% to 85% and this proportion was maintained for another 5 min. Following this, mobile phase B was changed to 2% in 5 min, and the column was re-equilibrated under the initial condition for 15 min. The eluted peptides were ionized with positive-ion electrospray ionization and analyzed using a Waters nanoACQUITY-SYNAPT G2 HDMS hybrid quadrupole-time of flight mass spectrometer. The capillary voltage was set at 2.75 kV, sample cone at 40 V, desolvation temperature at 300 °C, and source temperature at 100 °C. Data of full scan and MS/MS scan were obtained in the range of *m*/*z* 350–1700 and of *m*/*z* 50–2000, respectively.

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4.11 LC-MS-MRM analysis

The samples for quantification were analyzed using a Waters ACQUITY UPLC-Quattro Primer XE mass spectrometer. The *ACQUITY* UPLC® *Peptide BEH* C18 nanoACQUITY column (1.7 μm, 2.1 mm × 50 mm) was run at a flow rate of 0.25 mL min^{-1}. Mobile phase A was 5 mM ammonium acetate in water with 0.1% (v/v) formic acid and mobile phase B was 0.1% (v/v) formic acid in acetonitrile. The column was equilibrated using 15% mobile phase B and the samples were injected with 10 μL injection volume. A linear gradient was employed from 15% to 40% mobile phase B over 3 min; mobile phase B was then ramped to 90% from 3 min to 3.25 min and was maintained for 1 min with this proportion. Afterward, mobile phase B was changed to 15% in 0.5 min and was re-equilibrated for 5 min. The mass measurement system was operated in positive mode. Solutions were sprayed through a capillary held at 2.8 kV. The source temperature was set at 120 °C, desolvation temperature at 450 °C. The quadrupoles were scanned in MRM mode with a dwell time of 0.05 s. The cone voltage and collision energy for each transition were derived from repetitive tuning to obtain the highest signal intensity and are summarized in Table 2. Chromatograms and mass spectra were recorded and processed using MassLynx (Waters; v. 4.1).

4.12 Validation experiments

This analytical method was validated for the three allergens in accordance with International Conference on Harmonization of Technical Requirements of Registration of Pharmaceuticals for Human Use guidelines Q2 (R1).

The calibration curves were established with diluted peptide standards over the concentration range of 0.008–4000 ng mL^{-1} and internal standards at the concentration of 500 ng mL^{-1}. The LOD and LOQ were determined on the basis of sign-to-noise ratio (S/N), where the LOD > 3 and the LOQ > 10. For the assessment of the accuracy and precision, five different concentration levels of the H-labeled peptide standards were spiked before tryptic digestion was analyzed. The spiked amounts of the quality control standards are 125–12500 ng for SPA1-H, 250–25000 ng for SPA5-H, and 12.5–1250 ng for SPA11-H.

4.13 Statistical analysis

All the error bars of the bar charts were ±SD of at least three independent experiments by performing duplicates. The difference between experimental groups was determined with one-way analysis of variance. A *p*-value under 0.05 was considered a statistically significant difference.

Supplementary Materials: The following are available online, Table S1 and S2, Figure S1-S4.

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