

# Absolute Quantification of Allergen Glb33 in Rice by Liquid Chromatography–Mass Spectrometry using Two Isotope-Labeled Standard Peptides

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## Supporting Information

**ABSTRACT:** Allergen Glb33 is an important allergen in rice that can cause allergic reactions such as asthma and atopic dermatitis. However, knowledge of the content in rice is sparse. In the present work, an absolute protein quantification method was established for allergen Glb33 in rice samples using liquid chromatography–tandem mass spectrometry. After extraction of allergen Glb33 from rice grains using salt solution, the isotope-labeled peptide internal standard was added to the extract, followed by enzymatic digestion with trypsin. The signature peptide and its isotope-labeled analogue from the tryptic hydrolysates of allergen Glb33 and the internal standard were detected by liquid chromatography–tandem mass spectrometry. The quantitative bias caused by tryptic efficiency and matrix effect was corrected by using two isotope-labeled standard peptides. The method exhibited good linearity in the range of 1–200 nM, with coefficients of determination of  $R^2 > 0.998$ . A high sensitivity was observed, with a limit of quantification of 0.97 nM. Mean recoveries obtained from different rice matrices ranged from 82.7%–98.1% with precision <8.5% in intraday trials ( $n = 6$ ), while mean recoveries were from 75.1%–107.4% with precision <14.6% in interday trials ( $n = 14$ ). The developed method was successfully applied to the analysis of allergen Glb33 in 24 different rice cultivars.

**KEYWORDS:** allergen Glb33, absolute quantification, LC-MS/MS, isotope-labeled peptides, rice

## INTRODUCTION

Food allergy, usually used to describe an adverse immune response to food proteins,<sup>1</sup> is recognized as a significant public health concern with increased prevalence observed in an estimated 6% of young children and 3% to 4% of adults.<sup>2</sup> Most allergic patients are affected by consumption of milk, eggs, wheat, peanuts, and rice.<sup>3</sup> Rice is an important cereal consumed as both an energy and protein source by a large proportion of the population worldwide, especially in South and East Asian countries. Clinical studies have found that rice grains are responsible for severe asthma, eczema, and atopic dermatitis in some adult patients.<sup>4,5</sup> So far, several allergenic proteins, including  $\alpha$ -amylase/trypsin inhibitors and  $\alpha$ -globulin, have been identified and characterized biochemically and immunochemically from rice grains.<sup>3,6–13</sup> A novel type of rice glyoxalase I, named allergen Glb33, is one of the major allergens, due to strong immunoglobulin E reactivity in patients allergic to cereals.<sup>6</sup> As is well-known, the occurrence and severity of allergic reactions are mainly determined by the allergen content of the offending food, as well as the amount consumed.<sup>14</sup> Unfortunately, it is difficult for allergic consumers, especially those that depend on rice as a staple food, to avoid or minimize their intake of a particular allergen from their daily diet, since information pertaining to the content of this allergen in rice is lacking.

In recent decades, the United States, European Union, Japan, and South Korea successively introduced legislation

requiring information related to allergenic food ingredients to be included on labels to protect allergic consumers.<sup>14,15</sup> However, such legislation can be hard to implement due to the absence of thresholds for clinical reactivity, making it difficult to know whether some foods (especially mixed foods containing allergens) should be labeled or not.<sup>15,16</sup> Thus, it is important for food producers and regulators to have access to specific and sensitive methods that can detect allergen Glb33 at trace levels in rice.

Currently, analytical techniques for the determination of allergen Glb33 reported in the literature mainly use immuno-detection methods, such as enzyme-linked immunosorbent assay<sup>6</sup> or immunodot blot assay.<sup>8</sup> For instance, five rice allergens, including Glb33, were determined in the rice extracts by one-dimensional SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by multiplex immuno-detection as described by Lang et al.<sup>10</sup> Although most of immunological methods are commercially available and performed routinely in many laboratories due to their low cost and high sensitivity, these techniques also suffer from some intrinsic drawbacks; for example, although designed antibodies can recognize specific protein epitopes, homology between allergenic proteins can

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**Table 1.** Parameters for Multiple Reaction Monitoring of the Allergen Glb33 Signature Peptide and Its Isotope-Labeled Signature Peptide

| protein        | peptide                           | sequence     | precursor ion ( $m/z$ ) | DP (V) | EP (V) | CXP (V) | product ion ( $m/z$ ) | CE (eV) |
|----------------|-----------------------------------|--------------|-------------------------|--------|--------|---------|-----------------------|---------|
| allergen Glb33 | signature peptide                 | VVLVDNADFLK  | 617.0                   | 106    | 10     | 9.5     | 822.5 <sup>a</sup>    | 27.83   |
|                |                                   |              |                         |        |        |         | 921.6 <sup>b</sup>    | 26.96   |
|                |                                   |              |                         |        |        |         | 1034.0 <sup>b</sup>   | 20.00   |
|                | isotope-labeled signature peptide | VVLV*DNADFLK | 620.0                   | 102    | 10     | 12.5    | 822.5 <sup>a</sup>    | 29.75   |
|                |                                   |              |                         |        |        |         | 927.7 <sup>b</sup>    | 27.08   |
|                |                                   |              |                         |        |        |         | 1040.5 <sup>b</sup>   | 25.90   |

<sup>a</sup>Quantitative ion. <sup>b</sup>Qualitative ion. DP, declustering potential; EP, entrance potential; CXP, collision cell exit potential; CE, collision energy.

result in false-positives via antibody cross-reactivity.<sup>16,17</sup> In addition, these techniques mainly provide qualitative assessment or can only be used for semiquantitative determination of the allergen contents.<sup>18</sup> Liquid chromatography (LC) coupled to mass spectrometry (MS) offers many advantages for allergen monitoring, including high specificity due to molecular or mass-to-charge ( $m/z$ )-dependent interactions, enabling the unequivocal identification of allergens and markers in food matrices with high sensitivity over a wide dynamic range.<sup>19,20</sup> Recently, several MS-based methods have been developed for screening peanut, milk, egg, and wheat allergens,<sup>21–25</sup> but quantitative determination of allergen Glb33 in rice has not yet been reported.

In this work, we developed a new LC–tandem MS (LC-MS/MS) method for absolute quantification of allergen Glb33 in rice samples using multiple reaction monitoring (MRM) of two isotope-labeled standard peptides. A simple enzymatic digestion of samples was performed after salt solution extraction of samples spiked with an internal standard peptide to enhance the digestion efficiency and minimize variability between experiments. A signature peptide from the tryptic digest was selected to represent the target protein, and an isotope-labeled signature peptide from the tryptic digest of the internal standard peptide was employed as the actual internal standard during MS analysis. Subsequently, the signature peptide and its isotope-labeled analogue were monitored by LC-MS/MS in MRM within positive ionization mode. The content of allergen Glb33 was determined based on the calibrated signature peptide. Finally, the validated method was applied to the measurement of allergen Glb33 content in grain samples from various rice varieties.

## MATERIALS AND METHODS

**Chemicals and Solvents.** Ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), dithiothreitol (DTT), and iodoacetamide (IAA) were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (FA), methanol, and acetonitrile (ACN) of HPLC-grade were from Merck (Darmstadt, Germany). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). Pure water (18.2 M $\Omega$ ) was produced by a Milli-Q water purification system (Millipore Co., Bedford, MA, USA) and used throughout all experiments.

**Synthetic Peptide Standards.** The signature peptide VVLVDNADFLK (corresponding to amino acid residues 278–288 of allergen Glb33), stable isotope-labeled signature peptides VVLV\*DNADFLK (V\*, Val–OH–<sup>13</sup>C<sub>5</sub>, <sup>15</sup>N), and internal standards DPDGWKVVLV\*DNADFLKELQ (V\*, Val–OH–<sup>13</sup>C<sub>5</sub>, <sup>15</sup>N) were synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). The purity was more than 98% for all peptide standards. Each standard solution was prepared at a concentration of 1.0 mg/mL by solubilizing them in 25% ACN aqueous solution (v/v) according to the manufacturer's instructions.

**Standard Protein Expression and Purification.** Glb33 protein standards (>85% purity) were purchased from GenScript Biotech Corp. (Nanjing, China). The expression and purification procedure provided by the manufacturer were performed as follows. The DNA sequence of allergen Glb33 was optimized and synthesized and cloned into vector pET30a to include a His tag for protein expression, and the recombinant plasmid was transformed into *Escherichia coli* strain BL21 Star (DE3). A single colony was inoculated into Luria–Bertani medium containing antibiotics as required and cultured at 37 °C with shaking at 200 rpm. Protein expression was induced using isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). *E. coli* BL21 Star (DE3) cells from a glycerol stored preparation were inoculated into Terrific Broth medium containing appropriate antibiotics and were cultured at 37 °C. When the absorbance at 600 nm (OD<sub>600</sub>) reached 1.2, cells were induced with IPTG and culturing was continued for 16 h at 15 °C. Cells were harvested by centrifugation, and cell pellets were resuspended in lysis buffer, lysed by sonication, and centrifuged; the supernatant containing target proteins was kept for further purification. Target proteins were dialyzed and sterilized by passage through a 0.22  $\mu\text{m}$  filter before being stored in aliquots. Protein concentration was determined by the Bradford protein assay with bovine serum albumin (BSA) as a standard. Protein purity and molecular weight were determined by SDS-PAGE and confirmed by Western blotting (see Figure S1 in the Supporting Information).

**Rice Extracts.** Samples were extracted as previously described<sup>10</sup> with some slight alterations. Briefly, polished rice was pulverized three times for 1 min each time using a Tube Mill control (IKA, Staufen, Germany), and 100 mg of polished rice flour was placed in a 1.5 mL tube and mixed with 1.0 mL of salt solution (0.5 M NaCl, 30 mM Tris-HCl, pH 8.0). The tube was rotated on a rotary mixer at room temperature for 2 h. After the mixture was centrifuged at 10000g for 10 min at 4 °C, the supernatant was collected and the residue was re-extracted with salt solution for another 2 h. Extracts were combined, and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

**Preparation of Tryptic Hydrolysates.** Extracts were dissolved in 50 mM  $\text{NH}_4\text{HCO}_3$  and diluted to a final protein concentration of ~0.5 mg/mL. Next, samples (200  $\mu\text{L}$ ) were spiked with 10  $\mu\text{L}$  of 4  $\mu\text{M}$  internal standard DPDGWKVVLV\*DNADFLKELQ and mixed with 1.8 mL of 50 mM  $\text{NH}_4\text{HCO}_3$ . The mixtures were reduced with 10 mM DTT at 37 °C for 2.5 h, followed by alkylation with 50 mM IAA at room temperature in the dark for 40 min. Finally, digestion was performed by adding trypsin (trypsin/protein ratio of 1:20, w/w) and incubation for approximately 16 h at 37 °C. Digestion was terminated by the addition of FA to a final concentration of 1.0% (v/v).

Digested peptide mixtures were desalted by passing them through a 3 M Empore extraction disk cartridge (C18-SD, 7 mm/3 mL) pre-equilibrated with 1 mL of methanol. Subsequently, the cartridge was washed with 0.1% FA solution (v/v) to remove remaining salts and other polar molecules/ions. Desalted peptides were finally eluted twice with 1 mL of ACN aqueous solution (70%, v/v) containing 0.1% FA, and eluates were combined. The solution was passed through a 0.22  $\mu\text{m}$  nylon filter prior to LC-MS/MS analysis.

**LC-MS/MS Analysis.** LC analyses were carried out using a LC-20ADXR HPLC System (Shimadzu Corp, Columbia, MD, USA).

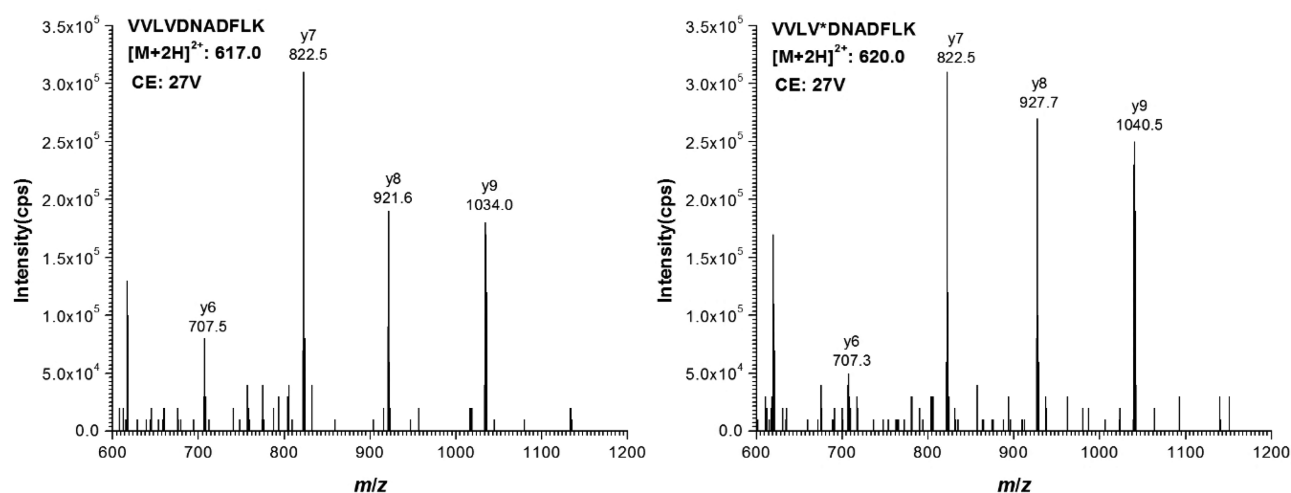


**Figure 1.** Typical product ion mass spectra of allergen Glb33 signature peptide VVLVDNADFLK and its corresponding isotope-labeled analogue VVLV\*DNADFLK.

Tryptic peptides were separated on an Agilent Poroshell 120EC-C18 column (2.1 mm × 150 mm, 2.7 μm; Agilent Technologies, Santa Clara, CA, USA) equipped with a guard column of the same material. The mobile phase consisted of solvent A (water containing 0.1% FA, v/v) and solvent B (ACN). The elution gradient was 10% B to 90% B over 18 min, 3 min at 90% B, followed by a return to 10% B and 5 min at 10% B for re-equilibration. The flow rate was 0.3 mL/min, and the column temperature was maintained at 40 °C; the injection volume was 2 μL.

Signature peptide and isotope-labeled signature peptide eluted from the LC column were introduced into a QTRAP 5500 mass spectrometer equipped with a Turbo V electrospray source (AB Sciex Instruments, Foster City, CA, USA). The mass spectrometer was operated in electrospray ionization positive ion mode with a source temperature of 450 °C, an ion spray voltage of 5.5 kV, and a curtain gas pressure of 20 psi, and GS1 and GS2 were both 40 psi. Data acquisition was performed in MRM mode. Three mass transitions (precursor/fragment ion pairs) were selected for each peptide for quantitation and confirmation. MS/MS parameters for both peptides are summarized in Table 1.

**Method Validation.** The method was validated based on specificity, matrix effects, sensitivity, linearity, accuracy, and precision. In these cases, the blank rice (including *indica*, *japonica*, and *glutinous* varieties) matrices were used. Specificity was evaluated by comparing the retention time of the synthetic signature peptide standard, natural peptides from tryptic-digested samples, and samples without enzymatic digestion.<sup>21</sup> Matrix effects were calculated from the ratio of the slope of matrix-matched standard curves and solvent-based standards using the following formula: matrix effects (%) =  $(B/A - 1) \times 100$ ,<sup>26</sup> where  $B$  is the slope of the matrix-matched calibration curve and  $A$  is the slope from spiked matrix-free solvent (70% ACN aqueous solution containing 0.1% FA). The influence of matrix effects was considered negligible when values were within ±10%, but where significant, there was signal suppression or enhancement caused by matrix interference.<sup>27</sup> Sensitivity was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ) values using the concentrations of target proteins after all sample preparation steps at which the signal-to-noise ratio was no less than 3:1 and 10:1, respectively. Calibration curves were constructed by linear regression of the signature peptide at five different concentrations (each containing 20 nM isotope-labeled signature peptide as the internal standard). The significance of the intercept at the 95% confidence level was evaluated by running a  $t$ -test. Accuracy (including intra- and interday accuracy) was expressed as the recovery assessed by measuring standard protein concentrations in rice samples spiked at four different levels. Recovery was determined by comparing the amount of standard protein added to the amount detected. Precision,

also including intra- and interday precision, was assessed by replicate analyses of the same spiked sample. Precision was calculated in terms of relative standard deviation (RSD) of the measured results. For both the accuracy and the precision trials, intraday data was determined by assaying six replicates on the same day, while interday data was obtained from 14 replicate runs on seven sequential days.

## RESULTS AND DISCUSSION

### Selection of Signatures Peptide for the Glb33 Allergen.

Because a bottom-up proteomics strategy was

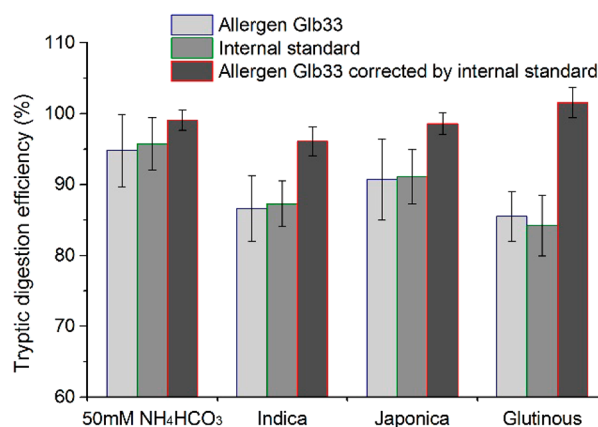


**Figure 2.** Tryptic digestion efficiency of allergen Glb33, the internal standard, and allergen Glb33 corrected using the internal standard in different matrices. Error bars represent means ± standard deviation (SD;  $n = 5$ ).

adopted in the developed method, the selection of suitable tryptic signature peptides specifically representative of the target protein was the most essential and crucial step. Herein, specific peptides for allergen Glb33 were chosen and identified by comparing endogenous and theoretical peptides from trypsin-digested allergen Glb33. Endogenous peptides generated from the enzymatic cleavage were identified by the nanoLC-LTQ-Orbitrap and sequence database (Proteome Discoverer Protein Database, Thermo Scientific, Scotts Valley, CA, USA) searches, while theoretical peptides of allergen Glb33 were obtained by computational prediction using online PeptideMass tools ([http://web.expasy.org/peptide\\_mass](http://web.expasy.org/peptide_mass)).

**Table 2. Spiked Recovery and Precision Testing of the Developed LC-MS/MS Method for Determination of Allergen Glb33**

| sample type      |     | intraday experiment<br>(%, <i>n</i> = 6) |           | interday experiment<br>(%, <i>n</i> = 14) |           |
|------------------|-----|------------------------------------------|-----------|-------------------------------------------|-----------|
|                  |     | recovery                                 | precision | recovery                                  | precision |
| <i>Indica</i>    | 1   | 84.4                                     | 6.4       | 80.8                                      | 9.6       |
|                  | 5   | 87.2                                     | 8.0       | 75.1                                      | 8.3       |
|                  | 25  | 93.3                                     | 3.6       | 90.7                                      | 6.7       |
|                  | 100 | 88.0                                     | 2.3       | 94.2                                      | 3.2       |
| <i>Japonica</i>  | 1   | 82.7                                     | 7.2       | 79.5                                      | 14.6      |
|                  | 5   | 97.9                                     | 8.5       | 107.4                                     | 10.5      |
|                  | 25  | 92.3                                     | 5.3       | 85.3                                      | 5.7       |
|                  | 100 | 95.6                                     | 2.9       | 92.0                                      | 6.4       |
| <i>Glutinous</i> | 1   | 89.8                                     | 6.9       | 84.6                                      | 12.3      |
|                  | 5   | 93.5                                     | 5.7       | 98.1                                      | 8.8       |
|                  | 25  | 91.4                                     | 4.6       | 90.7                                      | 7.7       |
|                  | 100 | 98.1                                     | 3.8       | 83.8                                      | 2.9       |

This resulted in the identification of 15 peptides (Table S1) from allergen Glb33 digests, possessing the same charge state distribution and corresponding molecular weight as theoretical tryptic cleavage products. Subsequently, each of these peptides were further verified by performing online BLAST searches against UniProt ([www.uniprot.org](http://www.uniprot.org)) and NCBI ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) databases. The results showed that only one peptide, VVLVDNADFLK (amino acid residues 278–288), was unique for allergen Glb33 and was accordingly synthesized as a signature peptide. During LC-MS/MS analysis, its mass transitions were optimized as  $m/z$  617.0 > 822.5,  $m/z$  617.0 > 921.6, and  $m/z$  617.0 > 1034.0 from product ion mass spectra, corresponding to  $y_7$ ,  $y_8$ , and  $y_9$  fragment ions, respectively (Figure 1).

**Optimisation and Synthesis of Isotope-Labeled Peptides.** Although LC-MS/MS technology based on quantitative MRM has been extensively used for protein quantification in complex biological matrices, the performance (e.g., accuracy, precision, and sensitivity) of MRM assays is prone to being affected by matrix effects caused by matrix coextracts, exogenous substances, and especially by digestion efficiency and variability of peptides from target proteins due to differences in proteolytic cleavage between experiments.

In our case, two isotope-labeled standard peptides were employed for allergen Glb33 quantification. One of the isotope-labeled peptides was synthesized as an isotope-labeled signature peptide with the sequence VVLV\*DNADFLK and was used to generate calibration curves with unlabeled signature peptide. Its mass transitions were optimized as  $m/z$  620.0 > 822.5,  $m/z$  620.0 > 927.7, and  $m/z$  620.0 > 1040.5 from product ion mass spectra, also corresponding to  $y_7$ ,  $y_8$ , and  $y_9$  fragment ions, respectively (Figure 1). Since the mass-to-charge ratios of precursor ions of the isotope-labeled signature peptide were +3 compared with those of unlabeled signature peptide (Table 1), both could be easily distinguished by MS, even at low (unit) resolution. Furthermore, the results revealed no cross-talk effects between them in MRM analysis (data not shown), confirming peptide VVLV\*DNADFLK as an ideal isotope-labeled analogue of the signature peptide.

In addition to the isotope-labeled signature peptide, another isotope-labeled peptide, DPDGWKVVLV\*DNADFLKELQ, was synthesized and served as an internal standard. This peptide was composed of the isotope-labeled peptide with an

additional three or six native amino acids flanking the cleavage sites. When the internal standard DPDGWKVVLV\*DNADFLKELQ was digested using the same enzymatic cleavage protocol employed for allergen Glb33 during sample preparation, the isotope-labeled signature peptide was released. Variability/loss during the tryptic process as well as matrix effects observed in LC-MS/MS analysis are discussed below.

**Determination of Digestion Efficiency.** Trypsin is the most commonly used protease for production of MS-amenable peptides, due to its high cleavage efficiency and high specificity targeting the C-terminal side of basic amino acid residues (lysine and arginine).<sup>28</sup> However, its digestion efficiency can be substandard due to the presence of endogenous interference, including trypsin inhibitors,<sup>29</sup> thereby resulting in underestimation of the target protein. To investigate the tryptic digestion efficiency, standard proteins (10, 50, and 200 pmol) and internal standards (40 pmol) were spiked into 2 mL of lysis buffer and digested using the same digestion protocol described herein. Each spiking experiment was performed with five replicates. The product tryptic fragments of VVLVDNADFLK and VVLV\*DNADFLK were both monitored using LC-MS/MS after tryptic digestion. The tryptic digestion efficiency of standard proteins and the internal standard were evaluated using an amount of tryptic cleavage products equivalent to the known amount of standard proteins or internal standard. As shown in Figure 2, digestion efficiencies were  $94.8\% \pm 5.1\%$  and  $95.7\% \pm 3.7\%$  for the standard protein at all spiking concentrations and its synthetic internal standard, respectively. However, both digestion efficiencies were correspondingly reduced to 85.5%–90.7% and 84.2%–91.1% (Figure 2), respectively, when the standard protein and internal standard were spiked into different rice (*indica*, *japonica*, and *glutinous*) matrices. Interestingly, the tryptic digestion efficiencies in the same matrix spiked with standard protein or synthetic internal standard were evaluated by running a *t*-test, but no significant differences were apparent within the 95% confidence intervals ( $p > 0.05$ ). This suggests that the tryptic digestion efficiency of the internal standard is representative of that of the standard protein. Therefore, to obtain an accurate quantitation for allergen Glb33 in the tested samples, the tryptic digestion efficiency was multiplied by a correction factor for each test sample matrix. The correction factor was obtained by calculating the molar mass ratios of the added DPDGWKVVLV\*DNADFLKELQ peptide and its fragment VVLV\*DNADFLK measured by LC-MS/MS. It can be seen from Figure 2 that the corrected digestion efficiency of the standard protein reached 96.1%–101.6%. Meanwhile, the variability between experiments was also much reduced compared with that of uncorrected data and even with that of matrix-free data.

Alternatively, the tryptic digestion efficiency can be improved to a certain extent by some purification protocols, such as trichloroacetic acid–acetone extraction or phenol extraction. However, these purification procedures are time-consuming and laborious, and reproducibility can be difficult to achieve. In addition, target proteins may be lost during these procedures. By contrast, the internal standard method appears to be much simpler and more convenient for high-throughput analysis. Therefore, the experimental results demonstrated that the chosen isotope-labeled signature peptide (VVLV\*DNADFLK) and internal standard (DPDGWKVVLV\*DNADFLKELQ) could provide accurate and reproducible quantitative target protein results.

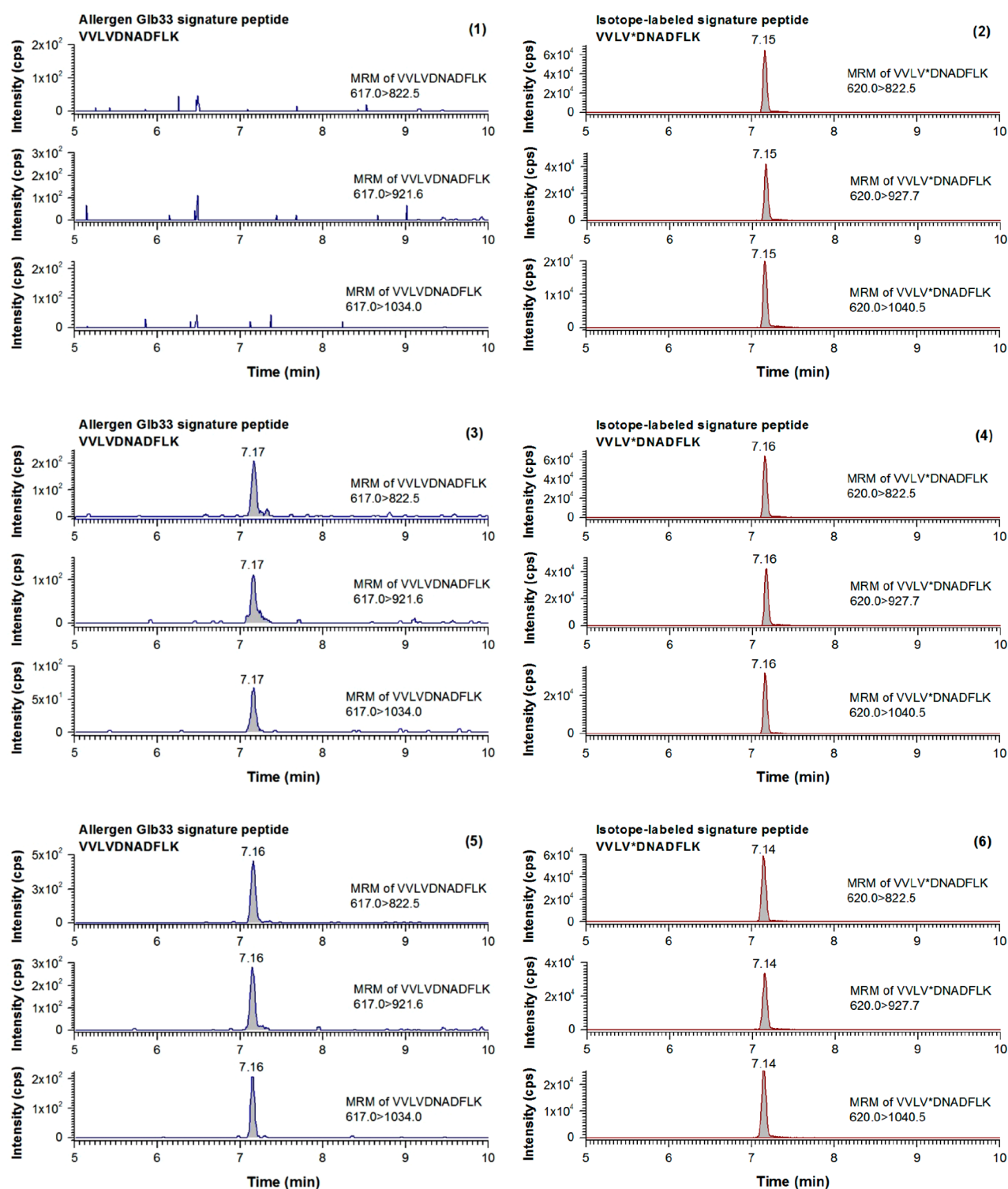


**Figure 3.** Multiple reaction monitoring chromatograms of allergen Glib33 signature peptide VVLVDNADFLK and its corresponding isotope-labeled signature peptide VVLV\*DNADFLK: (1, 2) blank samples; (3, 4) blank samples spiked at LOQ; and (5, 6) real samples (the detected concentrations were  $1.17 \pm 0.16$  mg/100 g for allergen Glib33).

**Method Validation.** Regarding specificity, the retention times of the synthetic peptide standards and selected signature peptides from tryptic samples were relatively stable at 7.15 min in the developed method. The maximum shift in retention time across the batch (more than 25 sample injections) compared with standards was between  $-0.8\%$  and  $1.1\%$  for both peptides. In samples without tryptic digestion, no peaks were observed

at this retention time. These results indicated no interference from matrix components on the retention time of target peptides. Moreover, specificity was also ensured by performing the analysis in MRM mode, providing three mass transitions, one for quantitation and two for confirmation. Thus, the method was specific for target quantification according to the criteria.<sup>30</sup>

**Table 3.** Measured Contents of Allergen Glb33 in 24 Different Rice Cultivars Using the Developed LC-MS/MS Method ( $n = 3$ )

| sample no. | cultivar name  | measured content (mg/100 g) | sample no. | cultivar name    | measured content (mg/100 g) |
|------------|----------------|-----------------------------|------------|------------------|-----------------------------|
| 1          | Zhongzao39     | 29.6 ± 2.7                  | 13         | Zhongzheyong 1   | 5.21 ± 0.18                 |
| 2          | Zhongjiazao17  | 18.0 ± 1.2                  | 14         | Shenliangyou5814 | 16.0 ± 0.9                  |
| 3          | Jinzao47       | 26.9 ± 3.0                  | 15         | Yongyou9         | 14.5 ± 1.5                  |
| 4          | Yongxian15     | 26.1 ± 0.2                  | 16         | Yongyou15        | 15.6 ± 0.7                  |
| 5          | Wen926         | 18.7 ± 0.3                  | 17         | Yongyou538       | 14.7 ± 0.9                  |
| 6          | Xiushui134     | 61.6 ± 6.4                  | 18         | Yongyou12        | 18.5 ± 0.5                  |
| 7          | Jia58          | 68.2 ± 4.9                  | 19         | Yongyou17        | 14.0 ± 0.3                  |
| 8          | Ning88         | 61.1 ± 5.1                  | 20         | Chunyou84        | 23.0 ± 1.5                  |
| 9          | Jiahe218       | 17.7 ± 0.5                  | 21         | Yongyou1540      | 14.2 ± 0.6                  |
| 10         | Ning84         | 31.0 ± 1.8                  | 22         | D-283            | 14.0 ± 0.8                  |
| 11         | Shaonuo9714    | 32.9 ± 2.4                  | 23         | D-287            | 14.7 ± 0.4                  |
| 12         | Zhongzheyong 8 | 12.1 ± 0.5                  | 24         | H-27             | 1.17 ± 0.16                 |

To assess matrix effects, each calibration curve was composed using the same five concentrations (5–200 nM) of signature peptide standard as used for the external standard method. Matrix effects were greater than −11.9% ( $n = 3$ ), indicating some interference from matrix components on the ion response of the signature peptide. Compared with the external standard method, calibration curves established using the internal method each contained 1–200 nM signature peptide, and each concentration contained 20 nM isotope-labeled signature peptide. Using this approach, matrix effects were as low as −3.7% ( $n = 3$ ). These results indicated that matrix effects could also be compensated by using an isotope-labeled signature peptide.

The method exhibited linearity between the peak area of analyte/internal standard ( $y$ ) and the concentration of the analyte/internal standard ( $x$ ) in the range of 1–200 nM. The typical linear regression equation was  $y = 0.843x - 0.006$  ( $n = 3$ ). Good linearity with coefficients of determination ( $R^2 \geq 0.9986$ ) over the investigated range were obtained, and a  $t$ -test performed on the intercept provided a  $p$ -value at the 95% confidence level  $>0.05$  ( $p = 0.124$ ), demonstrating that the calibration equation was in the form of  $y = 0.843x$  and thus confirming the absence of constant systematic errors. A relatively high sensitivity measurement was obtained with an LOD value of 0.29 nM and an LOQ of 0.97 nM (expressed as signature peptide concentration).

The accuracy of the developed method was evaluated by spiking samples at four different concentrations (1, 5, 25, and 100 mg/100 g standard protein). All experiments were repeated six times. As listed in Table 2, the average recoveries obtained for the four levels ranged from 82.7%–98.1% at all spiking levels in intraday experiments and 75.1%–107.4% in interday experiments. Regarding the overall precision, good results were obtained both in terms of intra- and interday precision, with RSDs below 8.5% and 14.6%, respectively (see Table 2). Typical LC-MS/MS chromatograms of the blank sample and the spiked sample at LOQ are shown in Figure 3. These results demonstrated that the developed method fully satisfied the requirements for the quantification of allergen Glb33 in various rice samples.

**Application of the LC-MS/MS Method to Rice Samples.** To verify the applicability of the validated method, 24 rice samples (from 24 different rice varieties) were subjected to analysis of allergen Glb33. All rice varieties were grown in the same field site and included *indica*, *japonica*, and *glutinous* varieties. In addition, samples spiked at a level of 25

mg/100 g were used as quality controls and were included in each batch of samples.

The results of LC-MS/MS analyses showed that the selected signature peptide from allergen Glb33 and its corresponding isotope-labeled signature peptide from the spiked internal standard were successfully identified and detected in the tryptic cleavage products of all samples. Both the synthetic peptide standards and the selected signature peptide exhibited sharp symmetric peaks at 7.16 or 7.14 min, and excellent sensitivity and specificity for the detection of target analytes were achieved, although the detected concentrations were as low as  $1.17 \pm 0.16$  mg/100 g for allergen Glb33 (the LC-MS/MS chromatograms are shown in Figure 3). As listed in Table 3, there were extreme differences in the content of allergen Glb33 among different rice cultivars, varying from 1.17–68.2 mg/100 g. Moreover, the recoveries from samples used for quality control were between 89.8% and 95.4%, demonstrating reliable quantification over the entire batch of samples.

Food allergy prevalence is rising worldwide and is recognized as a significant public health issue. Currently, the most effective strategy is to avoid exposure to allergens.<sup>31</sup> To reduce the risk of allergic reactions, food allergen labeling law should be reinforced by allergic thresholds based on clinical reactivity. To our knowledge, this is the first absolute quantification analysis of allergen Glb33 in rice by LC-MS/MS. The developed method may prove useful for improving the labeling of rice allergens.

## ■ ASSOCIATED CONTENT

### § Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b06738.

(Figure S1) SDS-PAGE and Western blot analyses of Glb33 and (Table S1) information related to peptides from tryptic allergen Glb33 identified by NanoLC-LTQ-Orbitrap analysis verified by sequence database searches (PDF)

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## Author Contributions

<sup>§</sup>M.-X.C. and H.Y. contributed equally to this work. F.-M.C., Z.-Y.C., and M.-X.C. designed the study. Both H.Y. and M.-X.C. carried out the laboratory work and analyzed data. Y.-N.M. and R.-X.M. interpreted the data. M.-X.C. and H.Y. wrote the manuscript. Z.-W.Z. provided technical support and conceptual advice.

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## Notes

The authors declare no competing financial interest.

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# **Supporting Information**

## **Absolute Quantification of Allergen Glb33 in Rice by Liquid Chromatography-Mass Spectrometry using Double Isotope-Labelled Standard Peptides**

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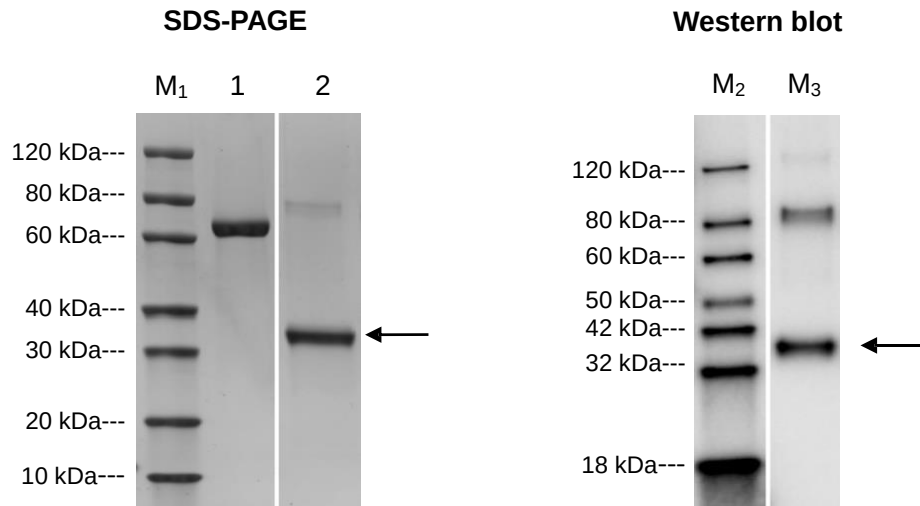


**Figure S1.** SDS-PAGE and Western blot analysis of Glb33. Lane M<sub>1</sub>, Lane M<sub>2</sub>, protein marker; Lane 1, BSA (2.00  $\mu$ g); Lane 2, Glb33 (Reducing conditions, 2.00  $\mu$ g); Lane 3, Glb33 (Reducing conditions); Primary antibody, mouse-anti-His mAb.

**Table S1**

**Information related to peptides from tryptic allergen Glb33 identified by NanoLC-LTQ-Orbitrap analysis was verified by sequence database searches.**

| Sequence in Protein                | Positions in Protein | Quality PEP <sup>a</sup> | Protein Groups | Proteins | PSMs <sup>b</sup> | Master Protein Accessions | Missed Cleavages | Theo. MH+ (Da) |
|------------------------------------|----------------------|--------------------------|----------------|----------|-------------------|---------------------------|------------------|----------------|
| R.GPTPEPLCQVMLR.V                  | [150-162]            | 0.235                    | 1              | 4        | 6                 | Q948T6                    | 0                | 1497.75523     |
| K.YTNAFLGFGPEDTNFALELTYNYGVDK.Y    | [61-87]              | 0.00422                  | 1              | 3        | 4                 | Q948T6                    | 0                | 3059.43091     |
| K.YTIAMLGYADEDKTTVIELTYNYGVTEYTK.G | [192-221]            | 0.161                    | 1              | 2        | 4                 | Q948T6                    | 1                | 3465.6658      |
| K.YTIAMLGYADEDK.T                  | [192-204]            | 0.0796                   | 1              | 2        | 2                 | Q948T6                    | 0                | 1489.68792     |
| K.YDIGAGFGHFIAIATEDVYK.L           | [88-106]             | 0.00543                  | 1              | 3        | 7                 | Q948T6                    | 0                | 2073.99163     |
| K.VVLVDNADFLKELQ.                  | [278-291]            | 0.0806                   | 1              | 1        | 3                 | Q948T6                    | 1                | 1602.87375     |
| K.VVLVDNADFLK.E                    | [278-288]            | 0.0593                   | 1              | 1        | 4                 | Q948T6                    | 0                | 1232.68851     |
| K.TTVIELTYNYGVTEYTK.G              | [205-221]            | 0.0248                   | 1              | 2        | 5                 | Q948T6                    | 0                | 1994.99571     |
| K.SPEVVLEWPKK.D                    | [10-20]              | 0.345                    | 1              | 3        | 2                 | Q948T6                    | 1                | 1311.73071     |
| K.SPEVVLEWPK.K                     | [10-19]              | 0.118                    | 1              | 3        | 4                 | Q948T6                    | 0                | 1183.63575     |
| K.SAEAVELVTK.E                     | [238-247]            | 0.177                    | 1              | 2        | 4                 | Q948T6                    | 0                | 1046.57282     |
| K.ILRQPGPLPGLNTK.I                 | [253-266]            | 0.671                    | 1              | 2        | 1                 | Q948T6                    | 1                | 1503.90057     |
| K.IASFLDPDGWK.V                    | [267-277]            | 0.128                    | 1              | 2        | 2                 | Q948T6                    | 0                | 1248.62591     |
| K.GNAYAQVAIGTEDVYK.S               | [222-237]            | 0.00799                  | 1              | 2        | 7                 | Q948T6                    | 0                | 1698.83334     |
| K.GGSTVIAFAQDPDGYMFELIQR.G         | [128-149]            | 0.000619                 | 1              | 3        | 12                | Q948T6                    | 0                | 2415.16492     |

<sup>a</sup> posterior error probability for the identified peptide group.

<sup>b</sup> the total number of peptide-spectrum matches identified from all included searches.