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Article type : Original Article-Allergens

Accurate quantification of five German cockroach (GCr) allergens in complex extracts using multiple reaction monitoring mass spectrometry (MRM MS)

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Short Title: MRM assay for simultaneous quantification of German cockroach allergens

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cea.12986

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Abstract

Background: German cockroach (GCr) allergen extracts are complex and heterogeneous products, and methods to better assess their potency and composition are needed for adequate studies of their safety and efficacy.

Objective and Methods: The objective of this study was to develop an assay based on liquid chromatography and multiple reaction monitoring mass spectrometry (LC-MRM MS) for rapid, accurate, and reproducible quantification of five allergens (Bla g 1, Bla g 2, Bla g3, Bla g 4, and Bla g 5) in crude GCr allergen extracts.

Results: We first established a comprehensive peptide library of allergens from various commercial extracts as well as recombinant allergens. Peptide mapping was performed using high resolution MS and the peptide library was then used to identify prototypic and quantotypic peptides to proceed with MRM method development. Assay development included a systematic optimization of digestion conditions (buffer, digestion time, and trypsin concentration), chromatographic separation, and MS parameters. Robustness and suitability were assessed following ICH (Q2 (R1)) guidelines. The method is precise (RSD < 10%), linear over a wide range ($r > 0.99$, 0.01 -1384 fmol/ μ L), and sensitive (LLOD and LLOQ <1 fmol/ μ L). Having established the parameters for LC-MRM MS, we quantified allergens from various commercial GCr extracts and showed considerable variability that may impact clinical efficacy.

Conclusions and Clinical Relevance: Our data demonstrate that the LC-MRM MS method is valuable for absolute quantification of allergens in GCr extracts and likely has broader applicability to other complex allergen extracts. Definitive quantification provides a new standard for labeling of allergen extracts, which will inform patient care, enable personalized therapy, and enhance the efficacy of immunotherapy for environmental and food allergies.

Key words: German cockroach allergens, allergy, allergen standardization, immunotherapy, multiple reaction monitoring MS, LC-MRM MS,

1. Introduction

Cockroach allergens are widespread in homes and sensitization to these allergens can cause or exacerbate allergic disease, especially in inner city areas [1-3]. At present, management of cockroach-associated allergic disease involves avoidance, standard pharmacotherapy, and allergen immunotherapy [4]. Allergen avoidance is difficult and costly and of uncertain benefit [4-8]. Standard pharmacotherapy is often partially effective but inadequate to relieve symptoms of allergic rhinitis or impact the clinical course of allergic asthma. By contrast, immunotherapy for environmental allergens such as northern grasses or short ragweed may impact the natural history of allergic diseases [9-13]. Whether immunotherapy impacts the clinical course of cockroach allergy is unclear [4], in part because unlike grass and ragweed extracts, there are no established parameters to define biological potency and stability of cockroach allergen extracts. Cockroach extracts are complex mixtures of proteins, polysaccharides, lipids, and other compounds. More than 10 allergens have been identified to date, although none of them appear to be immunodominant [14, 15]. Standards of potency and stability facilitate informative scientific studies of efficacy, safety, and mechanisms of allergen immunotherapy [4, 8, 16]. Because of the importance of cockroach allergy to inner-city populations, it is a priority of Center for Biologics Evaluation and Research (CBER) research program to develop a potency assay to standardize GCr allergenic extract.

In the US, allergen standardization is based on a determination of *in vivo* biological potency and use of various *in vitro* assays as estimates of that potency. Results are compared to the performance of FDA-approved reference standards [17, 18]. In instances where there is a

single predominant allergen, as in short ragweed pollen or cat hair allergen extracts, a radial immunodiffusion assay (RID) is employed to measure specific major allergens [15, 18].

When there is no immunodominant allergen, competition ELISA using allergen-specific IgE in pooled sera from highly allergic donors is used to estimate overall potency. While these *in vitro* potency assays are valuable, they have inherent limitations. For example, the competition ELISA measures the overall binding activity to allergen specific IgE but cannot delineate compositional differences between preparations. Specific ELISAs can, in principle, provide specific compositional information, but for complex allergen extracts implementation is difficult, especially the challenges of raising high quality antibodies against a range of allergens. Multiplex immunoassays show promise, as demonstrated recently for GCr extract [15], but with similar operational drawbacks.

Multiple reaction monitoring mass spectrometry (MRM MS) offers the ability to definitively measure specific allergen content with great accuracy, sensitivity, and multiplexing capacity. Unlike immunological assays, MRM MS is not dependent on the availability of high-quality immunological reagents, such as monoclonal antibodies, scFv antibodies, or polyclonal human or animal sera. Instead, MRM MS quantifies peptides generated from allergens after tryptic hydrolysis; and thus requires specific reference peptides, which can be generated chemically at low cost. MRM experiments are usually carried out using a triple quadrupole mass spectrometer, with precursor mass selection at the first quadrupole analyzer (Q1) followed by fragmentation of selected precursor mass (q2) and then scanning fragment ions of interest (Q3). For quantification, the MRM fragment ion from an analyte peptide is compared to a reference peptide, usually an isotope-labeled peptide standard. The detailed workflow and method development of MRM MS is described elsewhere [19].

MRM MS has become an integral part of clinical research and biomarker validation efforts [20-22]. Various MS-based assays including MRM have also been used in allergen research

for identification and quantification of specific allergens [14, 23-27]. The application of this technique to GCr for characterization and comprehensive quantification is very limited. Expanding the use of LC-MS techniques in allergen extract research will enhance the characterization of these complex and variable biological products and may enhance the likelihood of allergen standardization in the US. For this work, we used high resolution MS (HRMS) to exhaustively identify allergenic proteins, isoforms, and variants in commercial allergen extracts. A library of peptides was then generated to identify peptide markers for use in the absolute quantification of allergens. These markers were prepared chemically as native and stable isotope-labeled standards (SIS) and used to establish LC-MRM MS for reproducible and accurate quantification of the allergens Bla g 1, Bla g 2, Bla g 3, Bla g 4 and Bla g 5. Towards consideration of this assay as a tool to define GCr potency; robustness, and suitability, of the assay was assessed following ICH Q2(R1) guidelines [28].

2. Materials and Methods

2.1 Materials

Glycerinated GCr allergen extracts were purchased from Stallergenes Greer (1:20), HollisterStier (1:10), Allermed (1:10), and ALK (1:20). In preliminary studies, trypsin from different sources was screened and optimum digestion was achieved using MS grade trypsin from Promega (Madison, WI). Recombinant allergens Bla g 1 isoform 1, Bla g 2, Bla g 4, and Bla g 5 were purchased from Indoor Biotechnologies. Recombinant Bla g 3 isoform 2 was expressed and purified from *E coli* as described previously [29]. Reference peptides were chemically synthesized in both unlabeled and isotopically labeled forms by Thermo Fisher Scientific (San Jose, CA). All solvents were LC-MS grade and were purchased from Fisher Scientific.

2.2 Peptide mapping using high resolution mass spectrometry

All allergen extracts contained 50% glycerol. Since glycerol causes ion suppression in downstream MS analysis, it was removed using an Amicon spin filter (3K MWCO, Sigma). Deglycerinated extracts were supplemented with RapiGest SF (Waters Corp; 0.1 % final concentration) and aliquoted into vials (LoBind, Eppendorf) containing 25 µg of total protein each for storage at -20 °C. RapiGest SF is a MS-compatible surfactant that improves solubility of proteins thereby facilitating enzymatic digestion. Protein content was measured by BCA (Thermo Fisher Scientific, USA). For peptide mapping, proteins were digested following 30 min reduction (10 mM dithiothreitol, DTT), 30 min alkylation (50 mM iodoacetamide), and 30 min quenching (10 mM DTT), all at 60 °C. Digests for LC-MRM MS analysis were prepared without alkylation and reduction. Recombinant GCr allergens were digested using the same protocol as extracts. In all instances, digestion was stopped by adding trifluoroacetic acid to pH 2 and hydrolyzed RapiGest SF was removed by centrifugation (10 000 g).

NanoAcquity UPLC system coupled with Synapt G2 MS (Waters Corp) was used for peptide mapping. Chromatographically separated peptides were infused into the MS system. Ions formed in the source compartment were guided into the inner chamber for fragmentation and further scanning. Ions were resolved both by their time-of-flight and drift and the system was operated in data-independent acquisition mode. Data were processed and searched against NCBI database containing *Blattella germanica* proteins (downloaded on July 2016) using Protein Lynx Global Server (PLGS) software. A one-time randomized target database was always used as decoy to control for the false discovery rate.

2.3 Selection of allergen-specific reference peptides

Markers were selected from the peptide library, which was established using Synapt G2 MS and *in silico* prediction (Skyline [30]). The selection criteria include the peptide's MS responsiveness and several other commonly used filter criteria. For example, reference peptides should be unique to specific allergens, should not be susceptible to missed cleavage, and should not contain residues susceptible for non-specific modification (e.g. methionine, cysteine) or post-translational modification (e.g. glycosylation NxxS/T). Peptides that are too hydrophilic or hydrophobic were excluded as they are unsuitable for chromatographic separation. Selected peptides were chemically synthesized in the FDA core facility and used for MRM method development.

2.4 LC-MRM MS method development

Reference peptides were reconstituted at 70 fmol/ μ L in 50% acetonitrile containing 0.1% formic acid, and infused at 5 μ L/min into a Xevo TQ-S tandem quadrupole MS (Waters, Milford, MA), which was operated by MassLynx software. For charge state scanning, positive ions of up to four charges per peptide were monitored and the most dominant charge states were selected. In a subsequent step, IntelliStart software (Waters) was used to automate the selection of optimum MRM operating conditions, including suitable transitions, cone voltages for ionization, collision energy, and dwell time.

For LC separation, we used Acquity I-Class UPLC system (Waters) equipped with an analytical column (Acquity UPLC BEH Shield RP C18 2.1 mm $\times$ 100 mm column with 1.7 μ m particle size, Waters) that was kept at 60°C. Optimum peptide separation was achieved when the mobile phases A (0.1% formic acid in H₂O) and B (0.1% formic acid in acetonitrile) varied according to the following plan: B increases from 1% to 27.7% in 13.90 min; increases to 95% in 1 min; remains at 95% for 2.60 min; and decreases to 1% in 1 min.

The assay runs for 25 min at 0.25 mL/min flow rate. To evaluate the overall performance of MRM parameters established by direct infusion, LC-MRM MS experiments were carried out by injecting reference peptide cocktails or digests prepared from various extracts. The following common MS setting was used throughout the acquisition: ESI spray voltage 3.0 kV, source temperature 150 °C, desolvation temperature 350 °C, cone gas flow 150 L/h, and desolvation gas flow 500 L/h. Processing and integration of raw files from LC-MRM MS runs was achieved using TargetLynx (Waters).

2.5 Optimizing digestion conditions

The influence of various factors on digestion was evaluated by subjecting allergens to various digestion conditions. These factors include enzyme concentration, duration of digestion, effect of denaturing agents, and enzyme sources. Proteolysis was evaluated by quantifying the peptides released into solution using LC-MRM MS.

2.6 Performance evaluation of the LC-MRM MS method

Various metrics were used to assess the LC-MRM MS method. When applicable, the ICH protocols were followed [28]. Linearity was assessed from independent calibration curves generated over several days using 16-point dilutions of reference peptide mixtures containing 50 fmol of labeled reference peptides. Regression analysis was performed and the acceptance criterion was set to $R > 0.99$. Lower limits of detection (LLOD) and quantification (LLOQ) were determined from the standard deviation (SD) and slope (m) of the calibration curve: $LLOD = 3 \times SD/m$, and $LLOQ = 3 \times LLOD$. Precision was assessed using the relative SD (RSD) of allergen quantity in extracts determined using multiple LC-MRM MS runs. Intra-assay precision was calculated from RSD of allergen quantity determined from multiple injection of the same extract digest. Inter-assay precision was calculated from RSD of at least three independent digests run over multiple days each in triplicate injections. Inter-

analyst precision was calculated from RSD of allergen quantity in digests prepared and run by two independent analysts over several days. Accuracy of the method was evaluated by comparing the quantity of recombinant allergens determined using LC-MRM MS with results of amino acid analysis or SDS PAGE.

3. Results and discussion

3.1 Allergenic peptide mapping and initial peptide selection

We used HRMS to establish a comprehensive library of tryptic peptides from allergens in various GCr extracts. Commercial allergen extracts contain 50% glycerol, which interferes with enzymatic digestion as well as downstream LC-MS analysis [31]. We used 3K MWCO spin filter to remove glycerol and exchange it with 50 mM ammonium bicarbonate buffer. As expected, based on the molecular mass of known GCr allergens, spin filtration did not affect the protein profile and band intensity of extracts (Fig S1). Using qualitative HRMS screening, we confirmed prior observations that commercial GCr extracts differ compositionally [8]. For example, while allergens Bla g 1 and Bla g 3 were detected in all extracts, Bla g 5 and Bla g 8 were variably identified among extracts. In addition to extract digests, we also performed peptide mapping of recombinant allergens (rBla g 1, rBla g 2, rBla g 3, rBla g 4, and rBla g 5) to confirm peptide identifications. Peptides were ranked based on their MS responses (Fig S2). We then used the peptide library, in conjunction with *in silico* prediction software (Skyline, University of Washington) and other commonly used filters, to select reference peptides for MRM assay development. Fourteen peptides representing five GCr allergens, with at least two peptides per allergen, were selected and chemically synthesized for MRM MS assay development (Table S1).

3.2 Generation of optimum LC and MRM parameters

Method development for MRM involves optimization of ionization and ion transmission conditions for the most accurate quantification. Electrospray generates ions with multiple charge states and the most dominant charge state of a peptide is usually selected [21]. Using a direct infusion technique, we determined that doubly-charged precursor ions were the most dominant for all peptides, and used doubly-charged precursor ions to optimize the cone voltage and fragmentation conditions for five selected transitions per peptide.

Once the MRM conditions were determined, LC-MRM MS experiments were performed using synthetic peptides as well as digests prepared from allergen extracts. The adopted LC conditions and linear gradient achieved an excellent separation of peptides with high reproducibility (Fig 1). Transitions were evaluated based on signal quality and strength, reproducibility, and presence of interference from co-eluting ions especially in runs acquired using extract digests. Using these parameters, the transition list was narrowed to three transitions per peptide for the final LC-MRM MS assay. To ensure the maximum specificity, the final LC-MRM MS assay was set up by defining at least three analyte-specific criteria: the precursor ion m/z , three transitions (one for quantification and two for peptide identity confirmation), and retention time (Table 1).

3.3 Evaluation of reference peptides

All peptides appear to be stable under normal working conditions (Fig S3). The reference peptide list was further narrowed based on additional desirable attributes including elution characteristics and signal quality. Chromatographic separation experiments revealed that some peptides required higher organic phase to elute from the column. For example, the

peptides LETSPDFLALYNAIK from Bla g 1 and IPSLPDVTFVINGR from Bla g 2 required at least 45% mobile phase B, which would require either a significant change in the gradient profile or overall run time; therefore these two peptides were excluded from further consideration. In repeated analyses of extract digests, peptides IGDTTVAPAGTQAIIDTSK from Bla g 2 and ANGGYLAAGK from Bla g 5 provided inconsistent MS responses and were likewise excluded. Finally, peptides TPYNPQLR from Bla g 3 and FTIDYNDK from Bla g 4 were eliminated because they generated consistently weak signals in digests. Altogether, 6 of the initial 14 peptides were removed from the final list based on these criteria. The remaining 8 peptides (Table 1) were chemically synthesized in both unlabeled and isotopically-labeled forms. The isotopically-labeled peptides have C-terminal Lys or Arg residues modified to contain ^{13}C and ^{15}N , which enabled mass-shifting of the entire y-ion series for selective detection by collision induced dissociation. The purity and quantity of these peptides were assessed by HPLC and amino acid analysis, respectively. The peptides were at least 97% pure.

3.4 Digestion conditions optimization

Incomplete or variable digestion of allergens can severely undermine the accuracy of MRM, which depends upon the quantitative recovery of tryptically generated peptides [32, 33]. We therefore evaluated the impact of some critical factors on digestion: enzyme concentration, duration of digestion, effect of denaturing agents, and enzyme sources. The extent of digestion was assessed by quantifying the released peptides using LC-MRM MS. Some GCr allergens required higher than usual trypsin amount (enzyme to protein ratio, 1:100 - 1:10) [32] to effect maximum digestion (Fig 2, Fig S4). Bla g 1 has several repeat helices that form a hydrophobic cavity in which long stretches of amino acids are buried [34], which may

contribute to its resistance to tryptic digestion. Comparatively, Bla g 2 and Bla g 3 were more susceptible to trypsin (Fig S4). The time course of digestion is shown in Fig 3. Maximum peptide concentrations were measured at 8 -16 h for all allergens and based on these results, we adopted a 16 h digestion for the assay. The effect of enzyme source on digestion efficiency was also investigated. Generally, porcine and recombinant trypsin digested GCr allergens with comparable and better efficiency than L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated bovine trypsin [35]; bovine trypsin that was not treated with TPCK performed even more poorly (Fig S5). Simultaneous use of trypsin and LyC had no obvious benefit (Fig S6). Lastly, protein denaturation prior to tryptic digestion was explored by exposing cockroach allergen to various concentration of RapiGest. The LC-MRM quantification shows that adding 0.1% RapiGest enhanced digestion efficiency, especially for Bla g 1 and Bla g 4 (Fig S7). Taken together, the sample preparation protocol adopted for digestion of allergen extracts used an overnight digestion (16 h) in 50 mM ammonium bicarbonate containing 0.1% RapiGest using sequencing grade porcine trypsin at enzyme to substrate ratio 1:1 (for quantification of Bla g 1 and Bla g 4) or 1: 5 (for Bla g 2, Bla g 3, and Bla g 5).

3.5 Evaluation of the effects of matrix components on LC-MRM MS assay

The matrix effect was assessed by adding equimolar isotopically-labeled reference peptides to buffer only or to digests prepared from various allergenic extracts. The average area under the curve (AUC) of quantifier transitions (Table S2) or the ion ratio (Table 2) was compared. Matrix interference was considered significant when the concentration measured by the two conditions differed by > 20% [21]. The calculated RSD for the ion ratio was <3.5% for all peptides. Similarly, the differences in AUC of quantifier transitions were <7.5% even when

the comparison was performed at multiple concentrations and over several days. This confirms that the background has minimal influence on the analyte quantification. Matrix influence in the present study is expectedly minimal because the UPLC has high resolving capacity that minimizes the number of co-eluting compounds [36]. Because the degree of interference is minimal for the most abundant species, our careful selection of the most abundant precursor and fragment ions (transitions) also limited the extent of the matrix effect in our assay [20].

3.6 Evaluation of the performance of LC-MRM MS method

A. Linearity, LLOD, LLOQ, and reproducibility of linearity

To establish the linear boundaries of our LC-MRM MS assay, we prepared calibrants over a 138400-fold concentration range (0.01 – 1384 fmol/uL, 16 dilution points). We added 50 fmol/μL of isotope-labeled reference peptides to each dilution point. Linear regression analysis of the theoretical concentration against the absolute quantity determined with LC-MRM MS showed strong linearity ($r > 0.99$) in concentration ranging between 0.01 and 1384 fmol/μL with near unity slope for all peptides. Linearity was replicated over multiple days (stress-test for linearity) using freshly made solutions (Fig 4 and Table 3). LLOD and LLOQ were determined based on the calibration curve (Table 3) [28]; all allergens have LLOD and LLOQ in atto-mole range, consistent with prior reports for MRM [21].

B. Intra- and inter-assay precession of allergen quantification

Since multiple stages of sample handling and LC-MRM runs can increase sample variability, we assessed the precision of the assay using repeated quantification of allergens in digests prepared from extracts from commercial sources. Based on the RSD, the LC-MRM MS assay

is precise, with intra-day RSD < 5% for all allergens (Table S3), inter-day and between analysts RSD < 15% (Table 4). It is notable that Bla g 5 had higher RSD than the other allergens, perhaps due to its low quantity in these extracts.

C. Quantitative accuracy of the LC-MRM assay

Quantification accuracy was assessed using recombinant allergens by comparing MRM quantification to quantity determined using amino acid analysis or SDS PAGE. Recombinant Bla g 2, Bla g 4 and Bla g 5, 1µg each, were digested in multiple replicates and quantified using the optimized LC-MRM parameters. The results (Table 4) confirm the LC-MRM MS assay is accurate within 30% of the target quantity [28].

4. Summary and conclusion

Mass spectrometry has become an integral part of the arsenal to answer key biomedical questions in research and drug development. It is a versatile technique with potential benefits including sensitivity, speed, throughput, and specificity. Quantification of biologicals can be achieved in relative or in absolute quantities. LC-MRM MS is a mature platform for absolute quantification of biologicals including molecules in complex biological matrices such as plasma or urine. LC-MRM MS is selective, precise, reproducible, robust, and transferable across laboratories [22, 37]. We developed an LC-MRM MS assay for absolute quantification of five GCr allergens. We used an *in silico* prediction together with high resolution mass spectrometry for peptide mapping to choose the best representative peptides as surrogate for quantification. The resulting assay has attomole sensitivity and is highly specific. The assay is relatively rapid, requires minimal sample handling, and can be applied for a broad range of

samples with a wide linear dynamic range. The peptide panels we assembled represent five GCcr allergens and can easily be expanded to include peptides from all known GCcr allergens. With its high sensitivity and specificity, and with its potential for broad multiplexed analysis of complex biological mixtures, MRM would appear to fill an important niche in allergen characterization and standardization. Nearly all other potency assays in use or in development depend on antibody-based specificity, and these antibodies may be difficult to obtain, qualify, or replace. Other physicochemical assays used for allergen assessment are qualitative, and lack the exquisite sequence specificity of MRM.

At least three issues remain to be addressed before MRM can be implemented widely for allergen potency and stability studies. First, the biological significance of measured proteins needs to be determined independently. At present this information is derived from immune-prevalence surveys for allergen-specific IgE. Although informative, these data are at best a surrogate for determining which allergens are pathogenic, which can be best determined through specific avoidance and immunotherapy studies. Furthermore, even the best allergen sensitivity data can vary widely by region and HLA haplotype, so the determination of immunodominant allergens will require study in large numbers of individuals in multiple locations. Second, the sensitivity of MRM to product deterioration is limited to hydrolysis through the index peptides; conformational changes, and hydrolysis at other sites, are unlikely to be detected. Thus, the usefulness of MRM as a stability-indicating assay will have to be validated directly, and is not assured. Finally, in common with all multiplex assays, implementing MRM will challenge regulators to balance the requirements of generating an effective and safe product, against the reality of successfully producing a natural extract that meets multiple narrow specifications.

Conflict of interest

All authors declare no competing interests

Acknowledgments

We are grateful to Dr. Maryna Eichelberger, Division of Viral Products (DVP)/OVR/CBER, for allowing access to MS systems and for critically reading this manuscript. We also thank Dr. Philippa Hillyer for critically reading this manuscript.

Tables and figures legend

Table 1: Optimized LC and MRM parameters for allergen-specific peptides and their isotopic analogues from five GCr allergens.

Table 2: Comparison of ion ratio from isotope labeled peptides spiked in equimolar concentration into various background matrices. RSD is used to assess matrix effect.

Table 3: LC-MRM assay linearity determined based on unique tryptic peptides of five GCr allergens. Strong correlation exists between the theoretical and experimentally determined quantity of reference peptides over a wide concentration range (0.01 -1384 fmol/ μ L). From the calibration curve, the LLOD and LLOQ, and dynamic range were calculated.

Table 4: Precision of the LC-MRM MS assay was determined based on unique tryptic peptides of five GCr allergens. Extracts from different commercial sources were tryptically digested and the quantity of allergens was determined (fmol/ μ L) from triplicate LC-MRM MS runs. Percent RSD was calculated to evaluate precision of the assay. For accuracy assessment, the quantity of three recombinant allergens determined using LC-MRM MS was compared

with values from amino acid analysis or SDS PAGE. The quantity difference represents the accuracy of the assay.

- Fig 1: The elution profiles of allergen-specific peptides. The extracted ion chromatogram (intensity vs retention time) of the most intense transition from peptides representing five GCr allergens is shown.
- Fig 2: The effect of trypsin concentration on allergen digestion efficiency. LC-MRM MS data was acquired following an overnight tryptic digestion of Bla g 1 and Bla g 4. The integrated area under the curve of target peptides normalized using AUC of internal standard was compared. The error bars represent standard deviation.
- Fig 3: The effect of duration of exposure to trypsin on allergen digestion efficiency. LC-MRM MS data was acquired after an overnight tryptic digestion of Bla g 1, Bla g 2, Bla g 3, Bla g 4, and Bla g 5. The concentration of released peptides in solution was determined (fmol/ μ L) with error bars representing standard deviation.
- Fig 4: Representation of linearity of LC-MRM MS response with peptide concentration (log-log scale) and reproducibility over time. Shown is an overlay of LC-MRM MS calibration points for Bla g 1 across 0.01 -1384 fmol/ μ L concentration acquired over multiple days.

Table 1

Allergen	Peptide sequence	Retention time	Q1 (M+H) ⁺⁺	Quantifier		Qualifier 1		Qualifier 2	
				Q3 (M+H) ⁺	CE	Q3 (M+H) ⁺	CE	Q3 (M+H) ⁺	CE
Bla g 1	ALFNEK	9.81 ± 0.047	361.2	537.4	12	390.49	18	262.13	18
	ALFNE <u>K</u>	9.81 ± 0.047	365.2	545.4	12				
	IIE <u>L</u> IR	12.67 ± 0.023	378.8	530.44	12	643.61	14	401.35	16
	IIE <u>L</u> IR	12.67 ± 0.023	383.8	540.44	12				
Bla g 2	AIIVGPK	9.88 ± 0.046	349.2	513.48	10	400.38	14	257.25	12
	AIIVG <u>P</u> K	9.88 ± 0.046	353.2	521.48	10				
Bla g 3	LEDVPNVDIR	12.08 ± 0.023	585.3	713.61	20	358.19	22	457.38	16
	LEDVPNV <u>D</u> IR	12.08 ± 0.023	590.3	723.61	20				
	NYFVEIDR	12.37 ± 0.023	528.3	778.68	14	631.58	16	425.34	14
	NYFVEID <u>R</u>	12.37 ± 0.023	533.3	788.68	14				
Bla g 4	AIEEDLK	9.07 ± 0.048	409.2	633.31	12	615.53	16	356.18	20
	AIEEDL <u>K</u>	9.07 ± 0.048	413.2	641.31	12				
Bla g 5	TPVLEIDGK	11.49 ± 0.031	486.3	435.94	18	674.37	18	773.44	20
	TPVLEIDG <u>K</u>	11.49 ± 0.031	490.3	443.94	18				
	VLGLPAIK	13.77 ± 0.020	405.8	598.39	12	711.73	14	428.29	18
	VLGLPA <u>I</u> K	13.77 ± 0.020	409.8	606.39	12				

Amino acid residues underlined and in red font are stable isotope labeled. Q1 and Q3 refer to transition ions selected for quantification and identity confirmation, respectively. CE is collision energy for optimum dissociation of precursor ions.

Table 2:

Allergen	Peptide	Transitions (m/z)	Ion ratio					
			Buffer	Allermed	HollisterStier	ALK	Greer	%RSD
Bla g 1	ALFNEK	545 and 398	17.45	18.36	18.51	17.4	18.76	3.5
Bla g 2	AIIVGPK	521 and 408	4.71	4.74	4.66	4.73	4.78	0.93
Bla g 3	NYFVEIDR	788 and 641	1.13	1.17	1.16	1.09	1.11	2.96
Bla g 4	AIEEDLK	641 and 268	2.33	2.37	2.42	2.3	2.38	1.96
Bla g 5	VLGLPAIK	606 and 463	1.93	1.97	1.94	1.96	1.94	0.84

Table 3:

Linearity						
Allergen	Peptide		Day 1	Day 3	Day 5	Day 7
Bla g 1	ALFNEK	R*	0.9966	0.9996	0.9923	0.9984
		Slope	0.9672	0.9775	0.8798	0.9947
	IIEILIR	R	0.9948	0.9964	0.9828	0.9831
		Slope	0.9535	0.9918	0.8397	0.8977
Bla g 2	AIIVGPK	R	0.9921	0.9992	0.9994	0.9991
		Slope	0.9362	0.9764	0.9932	1.009
Bla g 3	LEDVPNVDIR	R	0.9966	0.9992	0.9932	0.9926
		Slope	0.9947	1.001	0.9937	0.991
	NYFVEIDR	R	0.9862	0.9986	0.9828	0.9820
		Slope	1.01	1.024	0.9899	1.003
Bla g 4	AIEEDLK	R	0.9987	0.9928	0.9997	0.9987
		Slope	0.9878	0.9983	1.012	0.9983
Bla g 5	TPVLEIDGK	R	0.9995	0.9981	0.9977	0.9961
		Slope	0.9837	0.9549	0.9895	0.9818
	VLGLPAIK	R	0.9938	0.9993	0.9960	0.9758
		Slope	1.03	0.9924	0.9514	1.061
LLOD, LLOQ, and linear range						
Allergen	Average slope	SD (at 0.01 fmol/μL)	LLOD (fmol/μL)	LLOQ (fmol/μL)	Linear range	
Bla g 1	0.9892	0.053	0.161	0.483	7500	
Bla g 2	0.9787	0.019	0.058	0.174	22000	
Bla g 3	0.9956	0.022	0.066	0.198	19000	
Bla g 4	0.9991	0.008	0.024	0.072	51000	
Bla g 5	1.0080	0.007	0.021	0.063	58000	

- R Correlation coefficient

Table 4:

Inter-day precision											
Allergen		Allermed	Hollister Stier	ALK	Greer	Allergen		Allermed	Hollister Stier	ALK	Greer
Bla g 1	Day 1	181.12	85.91	104.83	129.85	Bla g 4	Day 1	44.04	20.02	36.45	15.69
	Day 2	200.11	97.66	126.38	152.23		Day 2	46.35	22.55	41.65	18.15
	Day 3	160.94	94.03	100.13	131.35		Day 3	36.28	19.60	30.74	13.78
	%RSD	11	7	13	9		%RSD	12	8	15	14
Bla g 2	Day 1	66.93	29.46	51.97	27.30	Bla g 5	Day 1	2.83	5.14	0.90	0.45
	Day 2	64.42	28.73	55.08	27.60		Day 2	1.91	5.60	1.08	0.85
	Day 3	60.37	24.83	42.64	23.22		Day 3	1.48	3.04	0.77	0.60
	%RSD	5	9	13	9		%RSD	33	30	17	31
Bla g 3	Day 1	614.76	371.39	121.64	422.86						
	Day 2	503.68	340.16	116.88	408.24						
	Day 3	539.76	328.91	100.44	372.96						
	%RSD	10	6	10	6						
Inter-analyst precision											
		Allermed		HollisterStier		ALK		Greer			
Allergen		Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2
Bla g 1	Rep1	181.12	198.04	85.91	97.55	104.83	119.04	129.85	138.96		
	Rep2	200.11	197.71	97.66	100.85	126.38	117.19	152.23	137.41		
	Rep3	160.94	196.31	94.03	97.83	100.13	119.63	131.35	137.10		
	%RSD	8		5		9		6			
Bla g 2	Rep1	66.93	64.10	29.46	28.25	51.97	51.78	27.30	26.42		
	Rep2	64.42	64.20	28.73	27.97	55.08	51.87	27.60	27.13		
	Rep3	60.37	64.33	24.83	27.85	42.64	50.87	23.22	26.60		
	%RSD	3		6		8		6			
Bla g 3	Rep1	614.76	458.62	371.39	305.76	121.64	109.63	422.86	355.30		
	Rep2	503.68	463.87	340.16	295.01	116.88	106.78	408.24	363.01		
	Rep3	539.76	468.44	328.91	300.32	100.44	105.18	372.96	354.87		
	%RSD	12		9		7		8			

Bla g 4	Rep1	44.04	47.73	20.02	23.70	36.45	41.75	15.69	17.70
	Rep2	46.35	47.71	22.55	22.78	41.65	40.08	18.15	17.58
	Rep3	36.28	47.95	19.60	23.16	30.74	41.62	13.78	17.37
	%RSD	10		8		11		10	
Bla g 5	Rep1	2.83	2.03	5.14	5.26	0.90	1.20	0.45	0.95
	Rep2	1.91	2.07	5.60	5.38	1.08	1.22	0.85	0.95
	Rep3	1.48	1.95	3.04	5.46	0.77	1.18	0.60	0.95
	%RSD	22		19		17		27	

Accuracy of LC-MRM MS

Recombinant allergen	Replicate 1 (µg)	Replicate 2 (µg)	Replicate 3 (µg)	Average	% difference
Bla g 2	1.19	1.27	1.23	1.23	23
Bla g 4	1.09	1.01	1.05	1.05	5
Bla g 5	1.05	1.07	1.06	1.06	6

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Table S1: Peptides synthesized for preliminary screening and initial MRM method development

Allergen type	Peptide sequence	M/z (MALDI)	theretical m/z	(M+H) ⁺⁺
Bla g 1.0101	LETSPDFLALYNAIR	1722.83	1722.9061	847.9536
	ALFNEK	719.83	721.3879	361.1976
	IIEIIR	754.74	756.4978	378.7525
Bla g 2.0101	IGDTTVAPAGTQAIIDTSK	1857.69	1858.9756	
	AIIVGPK	700.59	697.4606	349.2340
	IPSLPDVTFVINGR	1526.9	1527.8529	764.4301
Bla g 3.0101	LEDVPNVDIR	1168.12	1169.616	585.3117
	TPYNPQLR	987.56	988.521	494.7642
	NYFVEIDR	1054.73	1055.5156	528.2615
Bla g 4.0101	FTIDYNDK	1014.75	1015.4731	508.2402
	AIEEDLK	818.69	817.4301	409.2187
Bla g 5.0101	TPVLEIDGK	970.87	971.5407	486.274
	VLGLPAIK	809.25	810.5447	405.776
	ANGGYLAAGK	921.6	921.4788	461.2431

Table S2: Assessment of matrix effect: Labeled peptides were spiked in equimolar concentration to buffer only or different matrices made from digests of allergen extracts. The quantifier ion area is compared between different background matrices

Allergen	Peptide	Transition (m/z)	AUC in buffer	AUC in AM	AUC in HS	AUC in ALK	AUC in Gr	CV
Bla g 1	ALFNEK	545.4	388150	350448	346123	355890	342806	5.1
Bla g 2	AIIVGPK	521.4	684417	581574	583214	596235	583891	7.3
Bla g 3	NYFVEIDR	788.6	9436	10619	10085	10804	10674	5.5
Bla g 4	AIEEDLK	641.3	130873	120887	118457	120368	119169	4.2
Bla g 5	VLGLPAIK	606.4	599298	600165	575840	612998	581974	2.5

Table S3: Precision (intra-, inter-day, and inter-analyst) of the LC/ MRM MS assay

	Holister Stier					ALK					Greer				
	Analyst 1 (A1)			Analyst 2		Analyst 1 (A1)			Analyst 2		Analyst 1 (A1)			Analyst 2	
Bla g 1	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2
R1	83.57	99.31	93.82	96.91	98.20	102.59	128.92	101.71	115.83	122.24	128.37	150.94	133.94	140.03	137.89
R2	86.23	97.67	94.77	100.33	101.36	107.15	125.09	98.78	113.49	120.89	132.83	153.83	130.78	138.29	136.54
R3	87.92	95.99	93.50	97.59	98.07	104.77	125.13	99.90	116.50	122.76	128.36	151.93	129.34	135.16	139.04
Average	85.91	97.66	94.03	98.27	99.21	104.83	126.38	100.13	115.27	121.96	129.85	152.23	131.35	137.83	137.82
SD	2.20	1.66	0.66	1.81	1.86	2.28	2.20	1.48	1.58	0.97	2.58	1.47	2.35	2.47	1.25
CV	2.56	1.70	0.70	1.85	1.88	2.17	1.74	1.48	1.37	0.79	1.99	0.96	1.79	1.79	0.91
	Between days (A1)			Between Analysts		Between days (A1)			Between Analysts		Between days (A1)			Between Analysts	
Average	92.53			95.01		Average 110.45			113.71		Average 137.81			137.82	
SD	5.40			5.26		SD 12.25			10.40		SD 11.00			8.38	
CV	5.84			5.53		CV 11.09			9.14		CV 7.98			6.08	
Bla g 2	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2
R1	29.77	29.90	25.10	28.57	27.93	52.66	55.57	43.58	51.59	51.97	26.99	27.21	23.17	27.24	25.60
R2	29.38	28.34	24.62	28.58	27.35	51.38	53.60	41.15	52.39	51.35	27.63	27.71	23.25	27.49	26.77
R3	29.24	27.94	24.78	28.59	27.11	51.88	56.06	43.19	51.00	50.74	27.29	27.87	23.25	26.80	26.40
Average	29.46	28.73	24.83	28.58	27.46	51.97	55.08	42.64	51.66	51.35	27.30	27.60	23.22	27.18	26.26
SD	0.27	1.04	0.24	0.01	0.42	0.65	1.30	1.31	0.70	0.62	0.32	0.34	0.05	0.35	0.60
CV	0.93	3.61	0.98	0.03	1.54	1.24	2.36	3.06	1.35	1.20	1.17	1.25	0.20	1.29	2.28
	Between days (A1)			Between Analysts		Between days (A1)			Between Analysts		Between days (A1)			Between Analysts	
Average	27.67			27.81		Average 49.90			50.54		Average 26.04			26.31	
SD	2.22			1.74		SD 5.69			4.39		SD 2.13			1.69	
CV	8.03			6.24		CV 11.40			8.69		CV 8.18			6.44	
Bla g 3	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2
R1	365.33	335.23	318.98	316.42	295.10	123.46	122.51	102.38	110.67	108.59	421.62	408.44	372.91	360.82	349.78
R2	370.53	340.62	325.65	295.52	294.51	121.29	114.56	99.17	106.72	106.85	416.94	413.10	370.27	364.43	361.60
R3	378.31	344.63	342.10	314.77	285.86	120.19	113.58	99.79	104.40	105.95	430.03	403.18	375.71	364.13	345.61
Average	371.39	340.16	328.91	308.90	291.82	121.64	116.88	100.44	107.26	107.13	422.86	408.24	372.96	363.12	352.33
SD	6.53	4.72	11.90	11.62	5.17	1.66	4.90	1.70	3.17	1.34	6.63	4.96	2.72	2.00	8.30
CV	1.76	1.39	3.62	3.76	1.77	1.37	4.19	1.70	2.96	1.25	1.57	1.22	0.73	0.55	2.35
	Between days (A1)			Between Analysts		Between days (A1)			Between Analysts		Between days (A1)			Between Analysts	
Average	346.82			328.24		Average 112.99			110.67		Average 401.35			383.90	
SD	20.37			29.10		SD 10.01			8.22		SD 22.64			28.38	
CV	5.87			8.86		CV 8.86			7.43		CV 5.64			7.39	

	Holister Stier					ALK					Greer				
	Analyst 1 (A1)			Analyst 2		Analyst 1 (A1)			Analyst 2		Analyst 1 (A1)			Analyst 2	
Bla g 4	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2
R1	19.42	22.58	19.32	23.87	23.53	35.58	42.43	41.41	42.28	41.22	15.49	18.55	16.88	17.49	17.91
R2	21.10	22.20	19.67	22.34	23.22	36.87	40.77	40.09	39.20	40.96	15.62	17.89	16.87	17.44	17.71
R3	19.55	22.86	19.80	23.22	23.10	36.91	41.75	40.74	40.88	42.35	15.96	18.02	17.06	17.48	17.26
Average	20.02	22.55	19.60	23.14	23.28	36.45	41.65	40.75	40.79	41.51	15.69	18.15	16.94	17.47	17.63
SD	0.93	0.33	0.25	0.77	0.22	0.76	0.83	0.66	1.54	0.74	0.24	0.35	0.11	0.03	0.33
CV	4.67	1.47	1.27	3.32	0.95	2.08	2.00	1.62	3.78	1.78	1.55	1.93	0.63	0.15	1.89
	Between days (A1)			Between Analysts		Between days (A1)			Between Analysts		Between days (A1)			Between Analysts	
	Average	20.72		21.72		Average	39.62		40.23		Average	16.93		17.18	
	SD	1.47		1.71		SD	2.49		2.15		SD	1.09		0.89	
	CV	7.11		7.88		CV	6.29		5.35		CV	6.43		5.19	
Bla g 5	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2	Day 1	Day 5	Day 9	BR1	BR2
R1	5.16	5.72	3.08	5.49	5.03	0.87	1.10	0.80	1.21	1.19	0.48	0.84	0.62	0.97	0.93
R2	5.16	5.51	3.08	5.37	5.40	0.91	1.08	0.71	1.21	1.22	0.43	0.85	0.60	0.97	0.94
R3	5.09	5.56	2.98	5.63	5.29	0.94	1.06	0.80	1.23	1.12	0.45	0.85	0.60	0.95	0.96
Average	5.14	5.60	3.04	5.49	5.24	0.90	1.08	0.77	1.22	1.18	0.45	0.85	0.60	0.96	0.94
SD	0.04	0.11	0.06	0.13	0.19	0.04	0.02	0.05	0.01	0.05	0.03	0.01	0.01	0.01	0.02
CV	0.76	1.91	1.90	2.37	3.58	3.89	1.63	6.58	1.09	4.32	6.10	0.59	1.73	1.20	1.60
	Between days (A1)			Between Analysts		Between days (A1)			Between Analysts		Between days (A1)			Between Analysts	
	Average	4.59		4.90		Average	0.92		1.03		Average	0.63		0.76	
	SD	1.18		0.98		SD	0.14		0.18		SD	0.17		0.21	
	CV	25.71		20.04		CV	15.11		17.27		CV	27.23		27.21	

Fig S1: The SDS PAGE profiles of cockroach allergen extracts before and after glycerol removal using Amicon spin filter (3K MWCO).

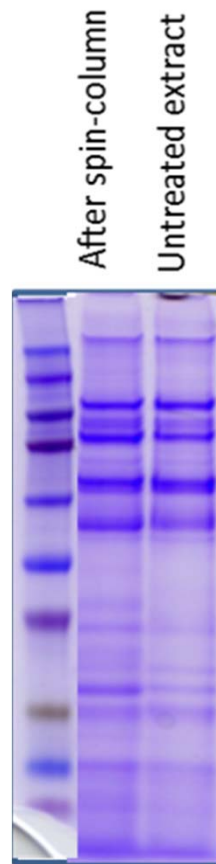
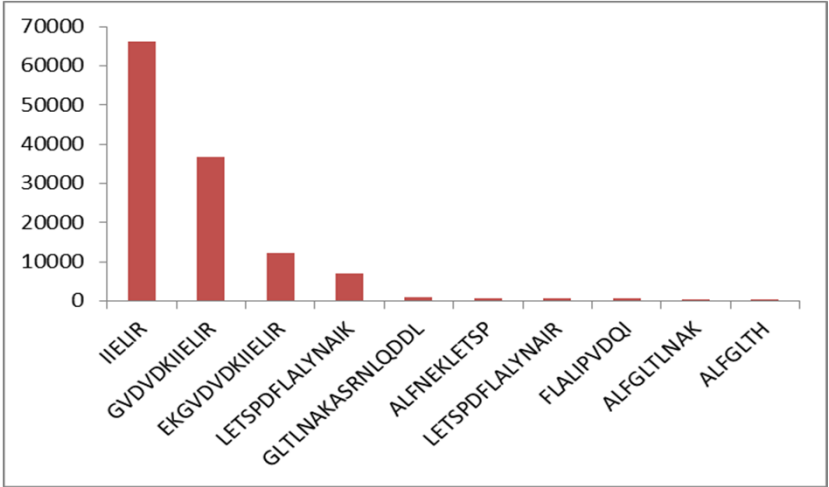
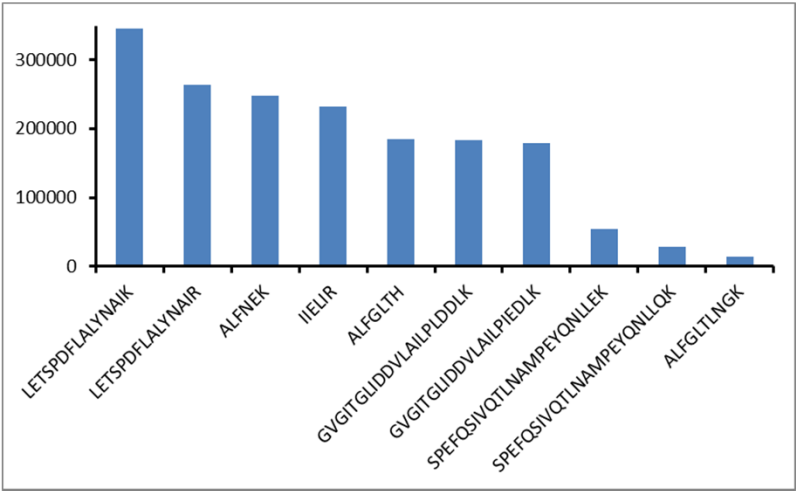
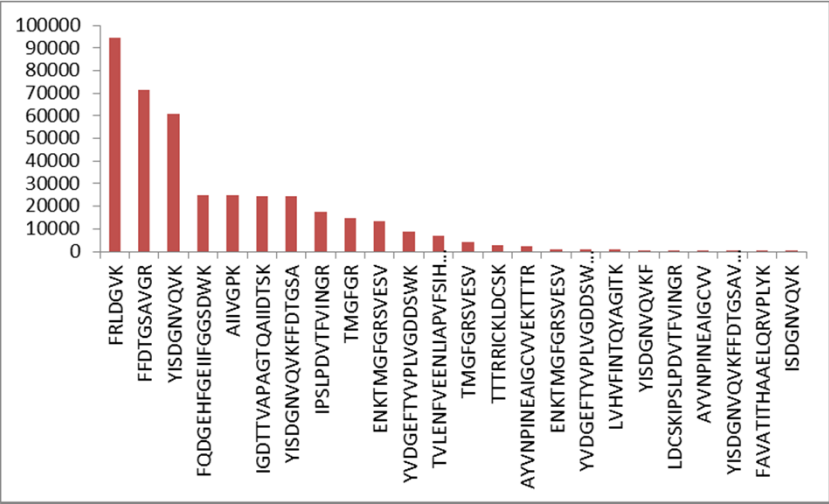
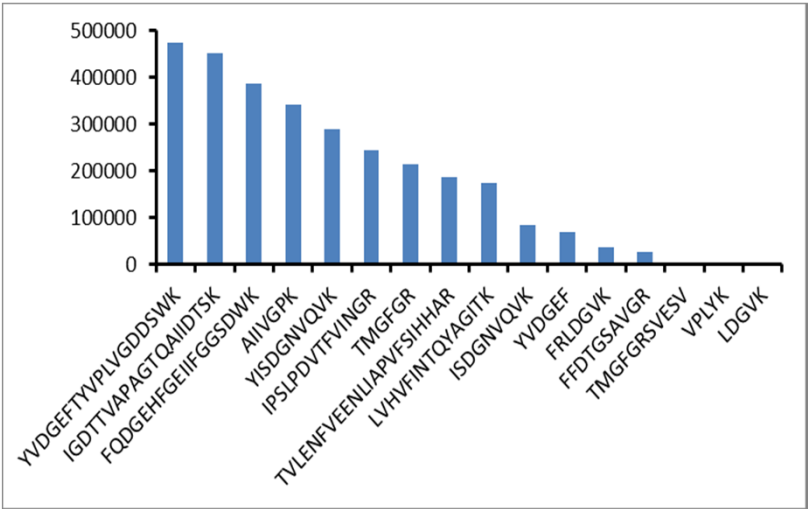


Fig S2: Summary of tryptic peptides identified from recombinant cockroach allergens (Blue bars) and allergen extracts (red bars) using high resolution LC/MS. The y-axis represent the precursor mass ion intensity used to rank the peptides

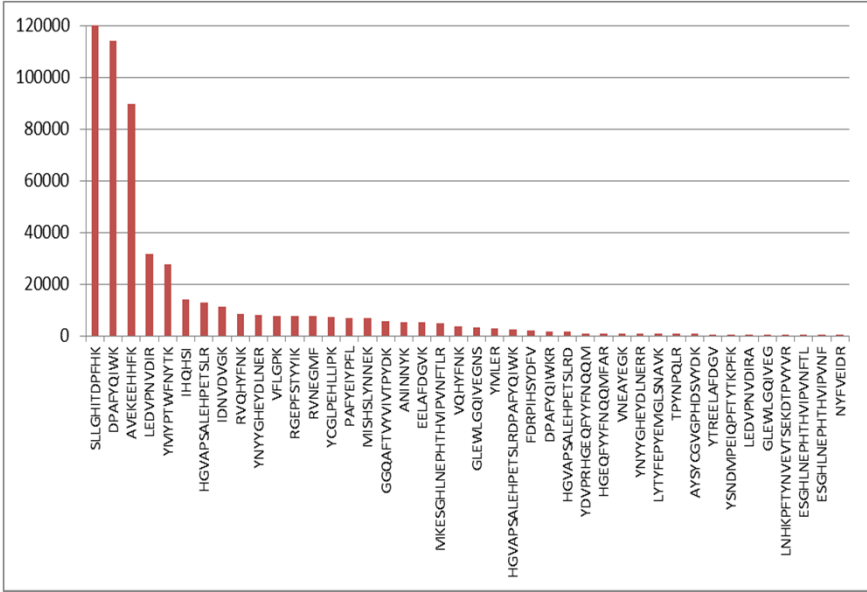
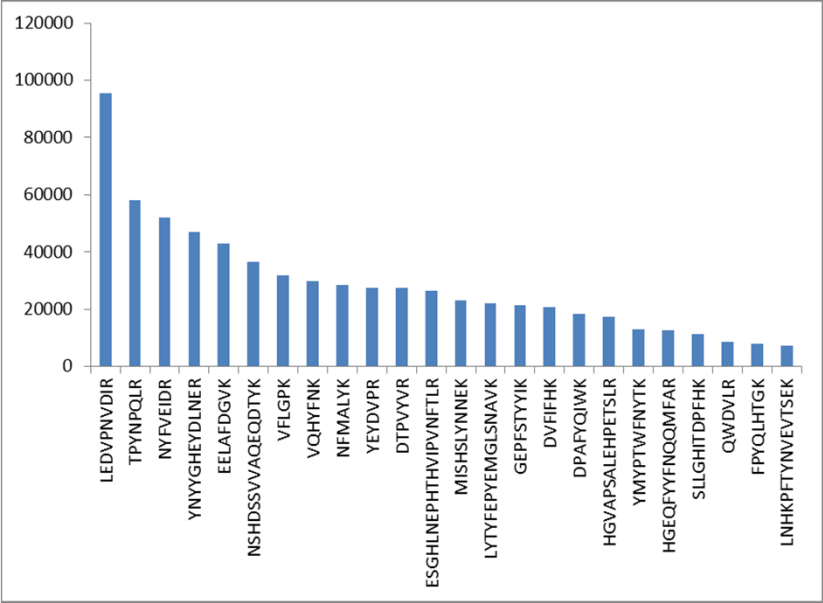
Bla g 1



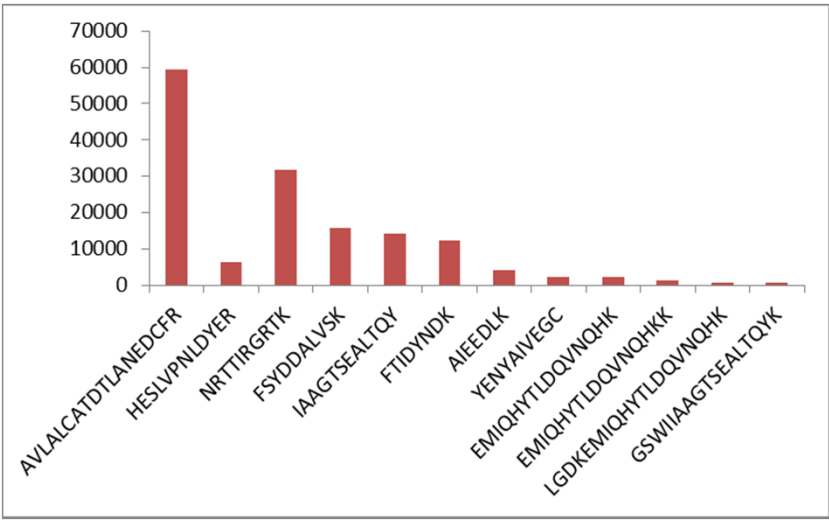
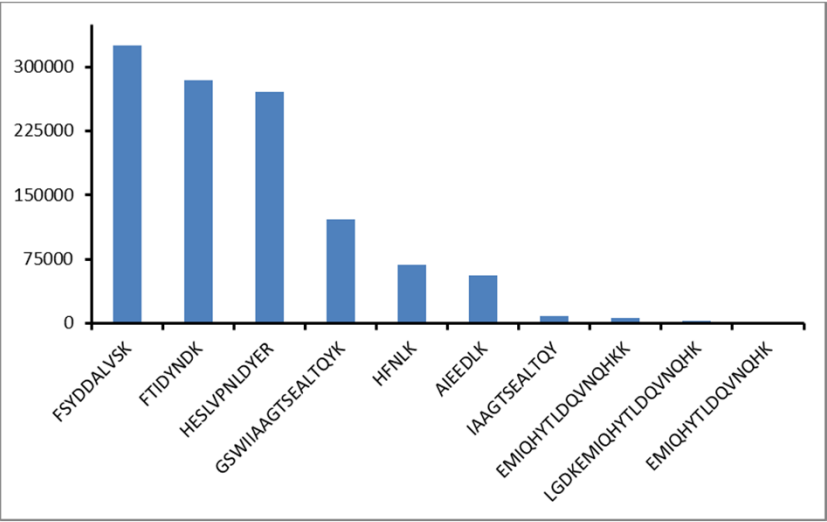
Bla g 2



Bla g 3



Bla g 4



Bla g 4

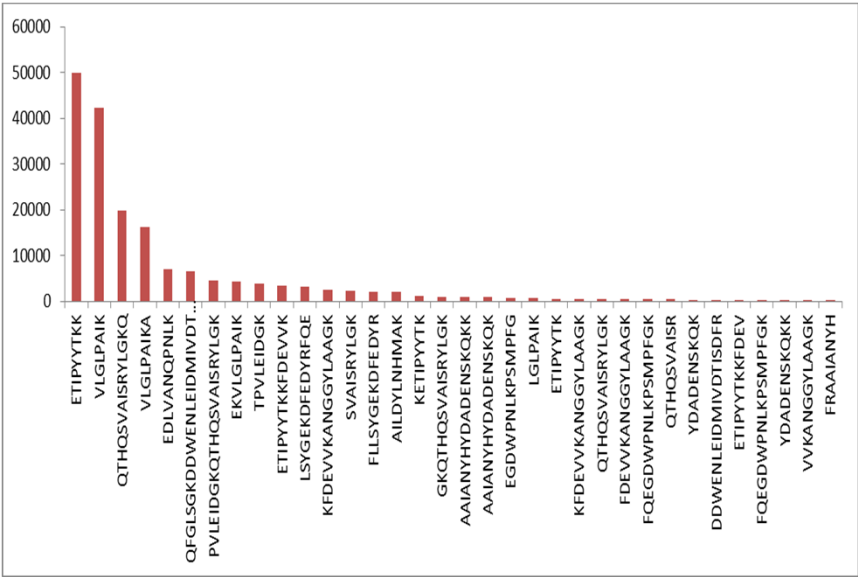
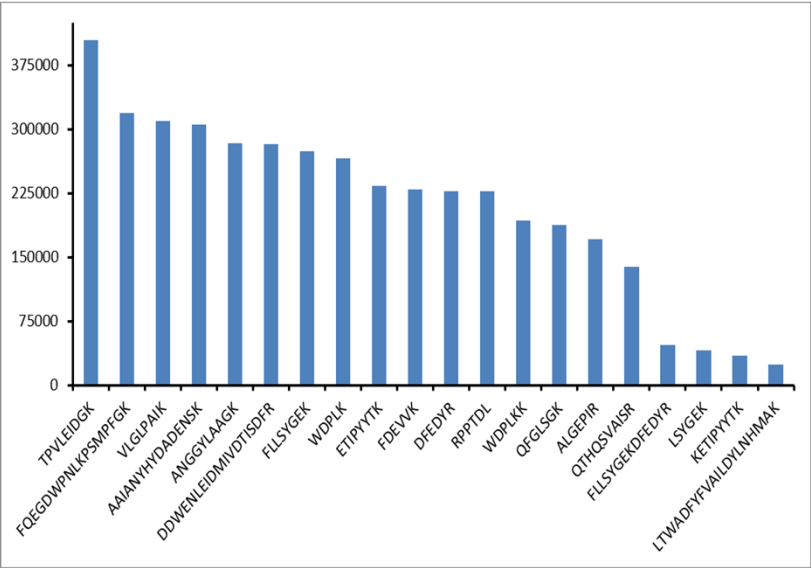


Fig S3: Stability of representative allergen-specific peptides in 0.1% formic acid buffer stored at different conditions
The integrated area under the curve of target peptides normalized using AUC of internal standard was compared.

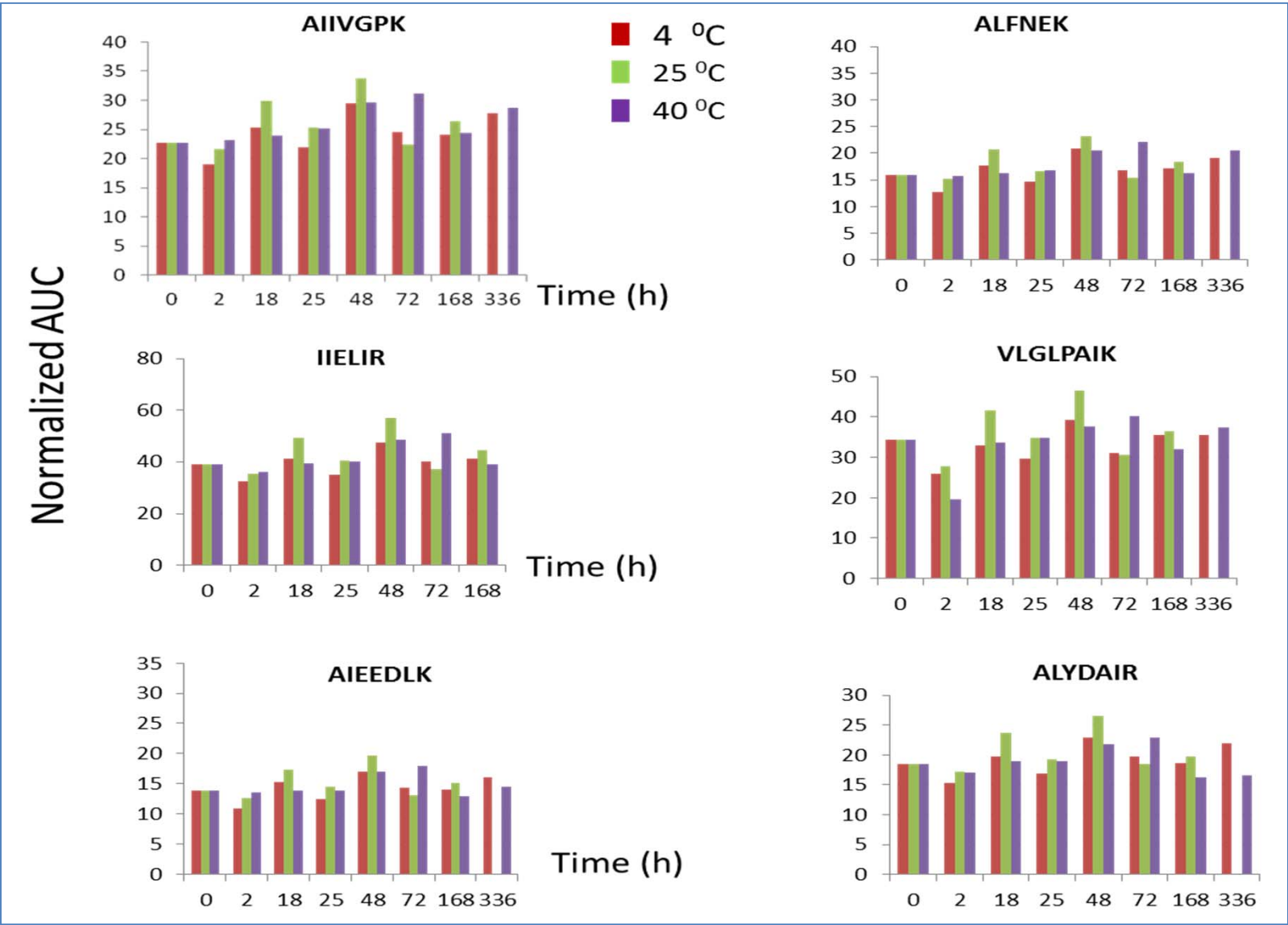


Fig S4: Effect of trypsin concentration on allergen digestion efficiency. LC-MRM MS data was acquired following an overnight tryptic digestion of five GCr allergens. The integrated area under the curve of target peptides normalized using AUC of internal standard was compared. The error bars represent standard deviation.

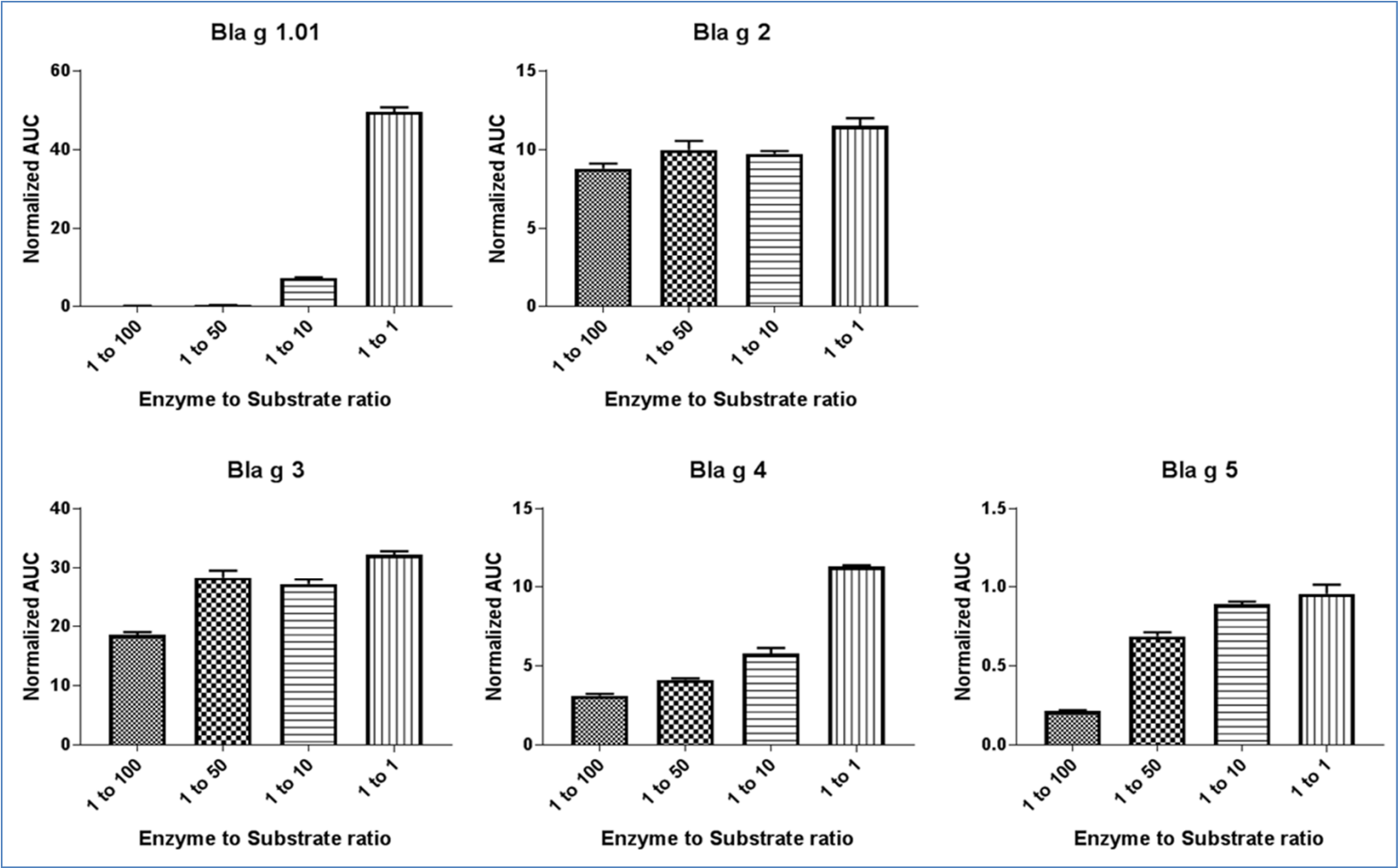
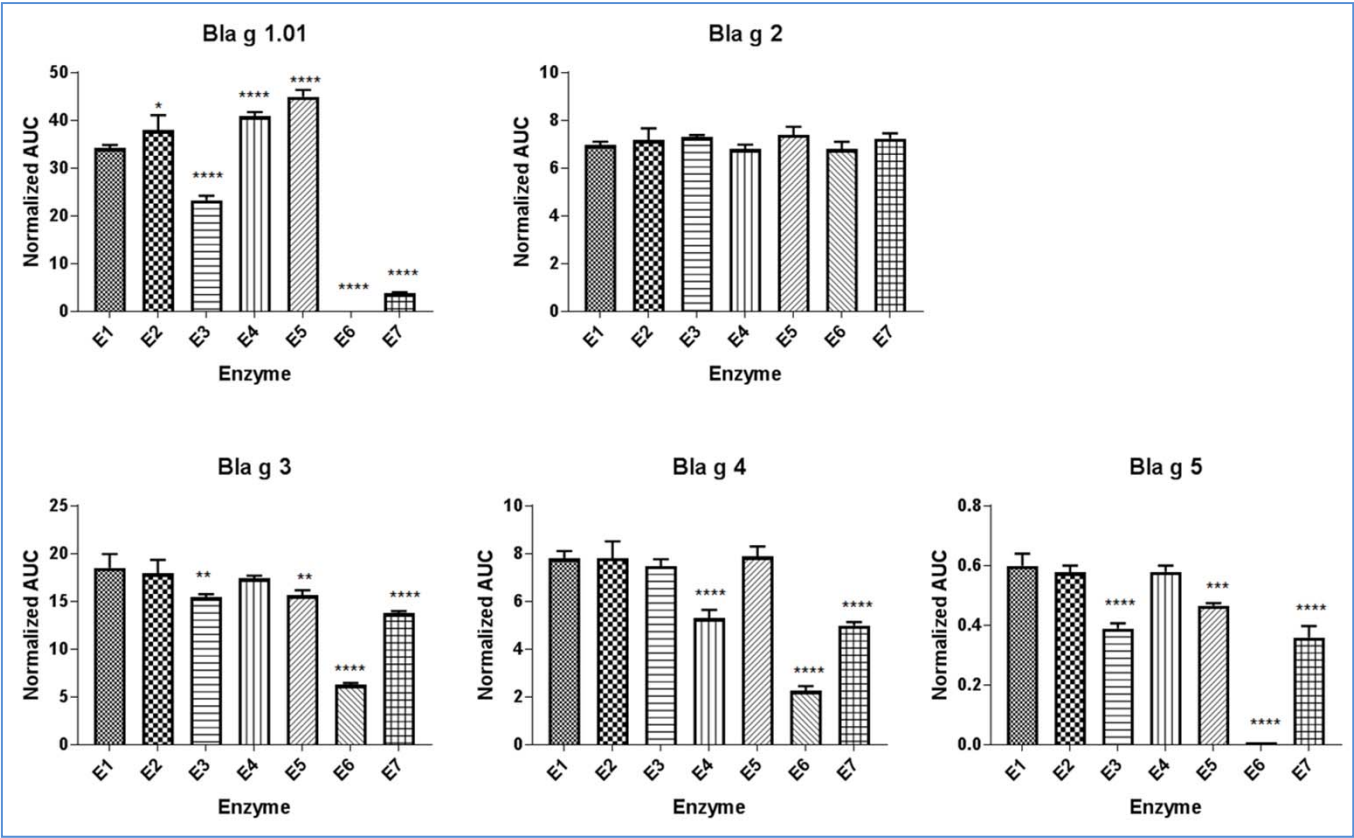


Fig S5: Effect of trypsin source on digestion of GCr allergens. The efficiency of trypsin from bovine, porcine, and recombinantly prepared were compared. The integrated area under the curve of target peptides normalized using AUC of internal standard was compared.



Legend			
Enzyme name	Symbol	Source	Modification
Modified trypsin, Promega	E1	Porcine	Methylated, TPCK treated
Trypsin Gold, Promega	E2	Porcine	Methylated, TPCK treated
Trypsin-Ultra, Biolabs	E3	Bovine	Acetylated, TPCK treated
Pierce™ Trypsin Protease, MS Grade	E4	Porcine	Methylated, TPCK treated
Trypsin recombinant, Roche	E5	Recomb	NA
Trypsin from bovine pancreas, Sigma	E6	Bovine	NA
Trypsin Sequencing Grade, Roche	E7	Bovine	NA

Fig S6: Effect of combining trypsin and LyC at 1:1 ratio on digestion of GCr allergens. The results show LyC did not improve digestion efficiency. The integrated area under the curve of target peptides normalized using AUC of internal standard was compared.

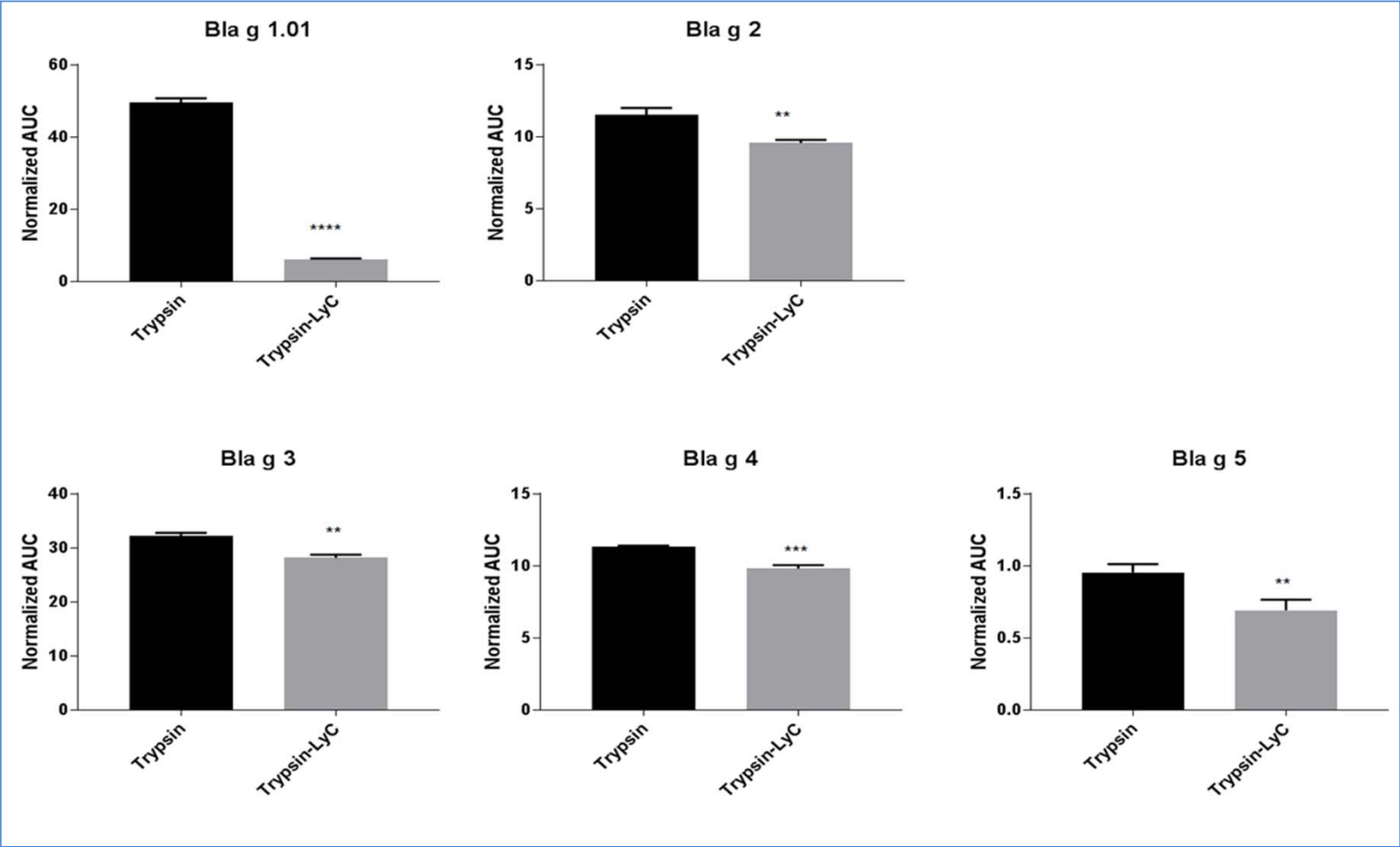


Fig S7: Effect RapiGest on digestion efficiency of GCr allergens. Results show that 0.1% RapiGest seems to benefit digestion of most allergens.

