Elsevier Editorial System(tm) for Food Chemistry
Manuscript Draft

Manuscript Number: FOODCHEM-D-14-02165R1

Title: Novel liquid chromatography-mass spectrometry method for the sensitive determination of mustard allergen Sin a 1 in food.

Article Type: Research Article (max 7,500 words)

Keywords: Allergens, Hidden allergen, LC-MS, Mustard allergy, Quantification, Sin a 1, SRM.

Corresponding Author: Dr. Carlos Pastor-Vargas, PhD

Corresponding Author's Institution: Instituto de Investigacion Sanitaria Fundación Jiménez Díaz

First Author: Maria Posada, PhD

Order of Authors: Maria Posada, PhD; Gloria Alvarez-Llamas, PhD; Aroa S Maroto; Xavier Maes; Esther Muñoz-García, MD; Mayte Villalba, PhD; Rosalia Rodriguez, PhD; Marina Perez-Gordo, PhD; Fernando Vivanco, PhD; Carlos Pastor-Vargas, PhD; Javier Cuesta-Herranz, MD, PhD

Abstract: Mustard is a condiment added to a variety of foodstuffs and a frequent cause of food allergy. A new strategy for the detection of mustard allergen in food products is presented. The methodology is based on the analysis by liquid chromatography coupled to mass spectrometry. Mustard allergen Sin a 1 was purified from yellow mustard seeds. Sin a 1 was detected with a total of five peptides showing a linear response (lowest LOD was 5 ng). Sin a 1 was detected in mustard sauces and salty biscuit (19±3mg/Kg) where mustard content is not specified. Sin a 1, as an internal standard, allowed quantification of this mustard allergen in foods. A novel LC/MS/MS SRM-based method has been developed to detect and quantify the presence of mustard, a principal allergen in foods. This method could help to detect mustard allergen Sin a 1 in processed foods and protect mustard allergic consumers.

Dear editor,

I send you the revised version of our manuscript "**Novel liquid chromatography-mass spectrometry method for the sensitive determination of mustard allergen Sin a 1 in food.**"

We have corrected the text according to the reviewers recommendations and added additional data requested by the reviewers. Below you can find the response, point by point, to the issues indicated by the reviewers. All the modifications are included in the revised version. We expect that the revised version is now suitable for publication in Food Chemistry Journal.

Looking forward to hearing from you,

Carlos Pastor-Vargas

Reviewer #1

The manuscript "Novel high-throughput methodology for the sensitive and quantification of mustard allergens in foods" presents an interesting study because it covers an interesting issue within food safety "allergens" and develop an analytical method to determine these allergens that uses currently used techniques. The study is very innovative Importantly, the study offers solutions for the problem of those food that do not declare in the label the addition of mustard. Because all these reasons, the manuscript is clearly worthy of publication in Food Chemistry.

From the experimental point of view, the design of the experiments has been properly performed in order to ensure the reliability of the results as well as to avoid possible interferences and to eliminate false negatives. From the formal point of view, however, the manuscript raises some concerns:

(i) the excessive use of the first plural person (try to be more impersonal)

The sentences with the use of the first plural person have been changed as suggested by the reviewer

(ii) the inappropriate use of some chromatographic terminology (see recommendations below).

Following your suggestions, chromatographic terminology has been changed

(iii) lack of discussion in some parts.

New paragraphs in discussion section have been added as suggested by the reviewer

Then, the manuscript should be revised. Some comments and questions that need to be addressed are:

1.-The title is little descriptive and does not give information on the analytical techniques used. A more appropriate one would be:

High throughput liquid chromatography-mass spectrometry method for the sensitive determination of mustard allergens in food.

Following your suggestion and reviewer 2 suggestion, the title has been changed by:
"Novel liquid chromatography-mass spectrometry method for the sensitive determination of mustard allergen Sin a 1 in food."

2.-In the abstract, some quantitative data are required to better summarize the results. Please, include limits of detection and the range of allergens concentrations detected in samples.

Following your suggestion, data have been added in the abstract.

3.-The highlights can be simplified. The third highlight can be rewritten as "Mustard principal allergen was determined in several foods"

Following your suggestion, the third highlight has been changed.

4.-Introduction
L52-55 needs some clarifications. Some text should be missed and the meaning of the sentence is unclear.
Sentence has been changed.

In general when several previous articles are cited together, they should be separated by "," or ";" but not by parenthesis.

Bibliography has been formatted, as suggested by the reviewer.

L97-104 would be better if they were moved after L69.

Following your suggestions, the L97-104 has been moved after L69.

5. Materials and methods
The section titled "Selected Reaction Monitoring (SRM) analysis of extract" would be better as "Novel liquid Chromatography-Mass Spectrometry". The authors over-dimension the interest of working in Selected Reaction Monitoring (SRM), which is just a working mode of the triple quadrupole.

Following your suggestions, the section title has been changed.

L142, replace "SRM" with "LC-MS"

"SRM" has been replaced by "LC-MS", as suggested by the reviewer

L150, delete "in SRM mode"

"in SRM mode" has been deleted, as suggested by the reviewer

Most information on the samples analyzed in this study is required. How many were the commercial sauces analyzed? How many of each class? Could you present as supplementary material the characteristics of these sauces and salted biscuit?
All sauces analyzed were commercial as we mentioned in lines 131-133 (original manuscript) "Commercial sauces (mustard sauce, garlic mayonnaise, barbecue sauce, honey-mustard sauce, ketchup and mayonnaise) and salted biscuit samples used were purchased from local market (Madrid, Spain)". A new table as supplementary material (Supplementary table I) has been added as suggested by the reviewer.

Results and discussion
L194-L196, please delete because they are repetitive with L164-167.

L194-L196 has been deleted, as suggested by the reviewer

Fig. 1 is not the most interesting one that you could provide for this manuscript. It would be much more interesting to shown some chromatograms of the real samples in the manuscript and move the standard to the supplementary material. Furthermore, the chromatograms of the samples can be extracted and presented in a more graphical way. As presented, the chromatograms and the MS information are not acceptable.

Old Fig. 1 has been moved to Supplementary Material (Supplementary fig 1). A new figure (Figure 1) has been added to the manuscript as suggested. Different Sin a 1 transitions, monitored in several real samples, are shown.

The chromatogram shown in Fig.1 is not the Total Ion Chromatogram (TIC) but the eXtracted Ion Chromatogram (XIC) of the different transitions monitored.
Change has been made in Supplementary material Fig 1.

L210, the "R" value is quite unacceptable. Could you discuss this value and why it does not reach the common value of 0.99?
We thank you the reviewer for this comment. The mentioned value ($r \geq 0.985$) refers to the lowest r value among all obtained for all monitored transitions (see Supplementary Table 2 in the new version). The average value is, in fact, 0.992. We have revised the value and to avoid misleading we have changed the text accordingly.

A table with the quantitative data obtained in the real samples is needed. Instead, Table 2 can be moved to the supplementary material because those data are interesting but not so important as those reported in Table 3.
As requested, Table 2 has been moved to supplementary material and Table 3 is Table 2 in the new manuscript. A new table 3 with quantitative data obtained in salted biscuit has been added

A paragraph in the discussion section on electivity and specificity as well as issues as blank contamination, etc.. should be added.
Following your suggestion, new paragraphs have been added in discussion section.

Reviewer #2: To some extent, this work is novel. However, there are many serious problems in most section of the manuscript addressed as follow:

1. Highlights
In this work, only Sin a 1 was detected, while not all the mustard allergens. It is better to specify the allergen.
Following your suggestion, the highlights have been changed to specify the allergen.

2. Title and running title
(1) All the results cannot support the "term" of high-throughput.
(2) It is better to specify the detected allergen.

Following your suggestion and reviewer 1 suggestion, the title has been changed by:
Novel liquid chromatography-mass spectrometry method for the sensitive determination of mustard allergen Sin a 1 in food.
Running title has been change by:
Quantification of mustard allergen Sin a 1 by mass spectrometry.

3. Abstract and conclusion

The results could only support a specific mustard allergen of Sin a 1 because of four major allergens in mustard

Following your suggestion, the abstract and the conclusion have been changed

4. Discussion

(1) The limitation of this method should be discussed.

Following your suggestion, new paragraphs have been added in discussion section.

(2) Since a total of 16 transitions were in the work of linearity response, it shoud be mentioned and discussed in this section.

Following your suggestion, a new paragraph has been added: "Only three peptides and their transitions (IYQTATHLPK, EFQQAQHLR and EFQQAQHLR) showed good reproducibility to be used in quantification. The peptides QQLGQQGQQGPHLQHVISR and QVSVCPFK showed poor reproducibility, thus they could be used to detect mustard traces in foods but not with quantification purposes".

(3) Protein can be broken down by processing, especially a strong treatment, for example, irradiation and fermentation. Actually, the fragment sits of the treated protein is not just out of the three peptides. So, a deep discussing should extend for the possible absence of the three peptides in the processed food containing mustard allergen.

Following your suggestion, a new paragraph has been added " Proteins can be broken down by processing steps such as heat treatment or fermentation, although many food allergens are characterized by their high stability after strong treatment (heat, low pH, etc.) and their ability to survive food processing. In the case of protein allergens, mass spectrometric methods are performed at the peptide scale, and the proteotypic peptide chosen to be monitored can still be valuable after food processing, thus making the quantification independent of the structure of the allergen. In this sense, peptides described in this paper have been detected in foods with different processing forms (sauces and salted biscuit)."

5. Additionally

Grammar errors and typing errors still existed in the manuscript. Quantification of food allergens is still a challenge work. This work has set up a new method to quantify mustard allergens Sin a 1 in food based on LC/MS/MS SRM. A three peptides were successfully confirmed to be used as the detected marker of the processed mustard allergen Sin a 1. The creactivity is obvious, making contribution to food allergens quantification. However, the presentation is not good, especially the conclusion is not logical.

Grammar errors and typing errors have been corrected. Following your suggestion, we have changed the presentation and the conclusion.

RECEIVING EDITOR'S COMMENTS:

- **Address the issues raised by the reviewers.**

The manuscript has been changed following reviewers suggestions

- **Actually read the Guide to Authors (as compared with just ticking yes on the submission form) and apply with respect to references in the text and source of reagents (e.g. substance, supplier [city, country]) - the editor has better things to do!**

References in the text and source of reagents have been changed following the Guide to Authors

- **use mol/ l or mol -1 and parts thereof rather than Molar (M).**

Molar (M) has been changes by mol/l as suggested by the reviewer.

- **There are significant issues with the English that must be resolved before the paper can be considered for publication - seek the assistance of a native speaker or use an English language editing service, such as that provided by Elsevier http://webshop.elsevier.com/languageservices/languageediting/**
This method allowed detection and quantification of mustard, a principal allergen in foods This method could help to detect mustard in processed food and protect mustard allergic consumers against accidental exposure.

25 Mustard is a condiment and added to a variety of foodstuffs for flavour.
30 was detected with a total of five peptides showing a linear response.
31 in mustard sauces and foods where mustard content was not specified. Sin a 1, as an internal standard, allowed quantification of this mustard allergen in foods. A novel
33 LC/MS/MS SRM-based method has been developed to detect and quantify the presence of mustard, a principal allergen in foods. This method could help to detect mustard in processed foods and protect mustard allergic consumers.
50 Mustard is a condiment made from the seeds of a mustard plant that belongs to the Brassicaceae family, which includes other vegetables such as radish, rutabaga, cabbage, broccoli, turnip, watercress, horseradish, castor oil plant, and rapeseed.
55 Mustard seed is added to a large assortment of foodstuffs such as pickled
Changes have been made

57 improve flavor and nutritional values. - what nutritional value?
Reviewer was right. "nutritional value" has been deleted.

57 But, mustard is also frequently included as a hidden component in sauces, flavoring powders and salad dressings.
Change has been made

59 reported as a frequent cause of food allergy. - reference
References for mustard allergy are mentioned below: Caballero et al. 2002.

62 Therefore, mustard was included in the European Union Directive 1169/2011/EC making declaration of mustard mandatory on food labels.
Change has been made

65 mustard allergy has been increasingly reported (Figueroa et al. 2005) - 2005 reference can hardly be considered current, especially in this field.
The correct references are mentioned after this sentence: (Vereda, Sirvent, Villalba, Rodriguez, Cuesta-Herranz & Palomares 2011; Sirvent et al., 2012). The reference Figueroa et al. 2005 has been deleted.

66 IgE cross-reactivity
68 … is, currently, the only solution to avoid an allergic reaction. However, mustard allergens can be introduced to food products accidentally, by means of deficient processing (e.g. improper handling, cross-contamination or incomplete cleaning, Jackson et al. 2008) or labeling errors. - re: labelling (British English) - the rest of the manuscript uses US English, decide and be consistent
75 the determination of mustard content in foods is very important to protect allergic consumers.
82 Methodology based on mass spectrometry (MS) have advanced significantly in terms of sensitivity and specificity, having improved the identification, characterization and determination of food allergens (…). - mass spectrometry is not a proper noun and does not require capital letters
85 et al. is an abbreviation and requires a full stop after al. on all occasions
86 coupled to MS analysis has become an important detection tool in the identification of allergens in
88 LC-MS/MS has superior 89 characteristics, improved reproducibility, recovery, sensitivity, dynamic range, and 90 selected reaction monitoring (SRM) - just because the abbreviation has capitals, it does not mean the terms are proper nouns and also require them!
94 A proteotypic peptide is defined as a peptide that identifies a protein uniquely: thus, the protein can be specifically quantified by measurement of the peptide.
101 Sin a 3 and Sin a 4 were identified as non-specific lipid transfer
107 The development of such a method would allow the implementation of reliable and accurate mustard allergens detection. The new method could be applied to other allergens and create an essential tool for food industry and regulatory agencies in the control of food allergens and better serve the food-allergic consumer.
Changes have been made

source of reagents (e.g. substance, supplier [city, country])
Changes have been made

117 The pellet was extracted twice with borate buffer and the supernatants were combined and lyophilized.
118 The remaining plant material was then extracted three times with 10% (w/v) acetone.
Changes have been made

123 lyophilized seeds extract - which one sodium borate or acetone extract or both?
Acetone was used to wash sodium borate extract. This sentence has been change to highlight this item: "The remaining plant material was then three times washed with 10% (w/v) acetone."
127 fraction containing protein - identified how?

A new sentence has been added: "(identified by an specific rabbit polyclonal antibody against Sin a 1)"

132 sauce, ketchup and mayonnaise) and salted biscuit samples were purchased from

Change has been made

137 fraction containing protein - identified how?

A new sentence has been added: "(identified by OD at 280 nm)"

154 on a ProtID Zorbax …

Change has been made

164 (…) (…) and manually inspected for co-elution at the retention time for transitions from the same precursor ion. - if not correct, please clarify as the sentence did not make sense as presented.

We agree with the reviewer. The sentence is confuse. A new sentence has been added: "Theoretical SRM transitions were designed using Skyline (v.1.1.0.2905) (MacLean et al., 2010) and monitored by LC/MS/MS to finally set the best performing ones. Transitions from the same precursor ion should co-elute in retention time. Otherwise, they were not further considered"

171 amino acid sequence of the peptide would be a change in mass and, therefore, would not be selected in the first quadrupole. Also, peptides selected did not match with any other protein (with 100% identity).

185 The amount of Sin a 1 was calculated by standard addition: …

191 For Sin a 1, all selected peptides had a 100% match with the 2S seed storage proteins family from Brassiceae, also called the mustard family.

195 were chosen based on in-silico data analysis using Skyline software, …

196 All those showing an appropriate signal to noise ratio (…) were selected for further examination, independent of ion type.

198 five

199 seven and three - numbers less than 10 should be written in full unless in a method (e.g. 2 g) - 10 or greater as digits

201 reproducibility; thus, the other three peptides were used in further studies.

205 10

207 linear response to this protein in mustard sauce.

209 at every concentration assayed. ... Sin a 1 showed a linear response in the range 0.1 to 10-6 <mu>g (r≥0.985).

212 values were not more than 28% …

216 In a further step, we evaluated the sensitivity and reproducibility of the method using the standard addition method. One mustard sauce extract was selected and …

Changes have been made

221 quantification obtained for every transition measured for Sin a 1 are shown in Table 3.

222 For the quantitation, the whole group of transitions described was analyzed to ensure the sample size was sufficient to achieve statistical significance.

228 extract was calculated from transition data satisfying the linearity criteria r≥0.98.

Changes have been made

231 Sin a 1 was also analyzed blind in a series of food extracts. As expected Sin a 1 was detected in both mustard and honey-mustard sauces, and salty biscuit, which contained mustard according to the label, as well as barbecue, mayonnaise, garlic mayonnaise and ketchup sauces, which contained spices (according to label) but did not specify mustard (…). To confirm the method described in this paper was valid for quantification of processed food, we analyzed Sin a 1content in a salty biscuit extract by the standard addition method. Using transitions 535.8 > 839.4, 535.8 > 924.4, 578.8 > 624.4 and 578.8 > 880.5, we obtained an average value for Sin a 1 in the extract of 20±4mg per kg of salty biscuit (17%RSD). - which was good, bad or indifferent? Put into context.
The sentence has been changed according reviewer suggestion.

244 The presence of hidden allergens is a serious health hazard, affecting people with food allergy. Therefore, the detection of allergenic components is a priority in food safety in order to protect allergic consumers. At present, the development of mass spectrometry-based methodologies has advanced detection of proteins, peptides, metabolites, and small molecules in general.
249 In this study, the selected peptides, used as markers of Sin a 1, satisfy various criteria including: …
254 five peptides satisfied these criteria (Table 1). These selected peptides were checked using BLAST searches and resulted specific for mustard proteins.
257 applying a selective exclusion chromatographic cleaning up system and, thus, increasing sensitivity and reducing background. Extracts from different foods containing
259 mustard (mustard presence was detailed on the label) were analyzed. Mustard allergens could be detected not only in those food extracts, in which the presence of mustard was declared, but also in foods where the label only stated spices.
263 In order to test whether mustard allergens could be used as markers to quantify mustard in different foods, we carried out an assay to evaluate the linearity response for purified Sin a 1 transitions. Sin a 1 produced a linear response (Table 2) and, thus, further quantification studies were based on this protein.
272 (…). This technology has allowed the quantification of allergens in soybean (…), …
274 In a different approach, an intact protein has been used, as an internal standard, to quantify allergens in peanut …
278 In this way, we avoided potential problems associated with the use of
281 … 2) incomplete protein digestion, which may cause peptide behavior not comparable with the intact protein.
283 LOD and LOQ for this SRM-based methodology were as low as 0.25 ppm and
284 These values represent a substantial improvement compared with those obtained for ELISA-based methods (…), including current commercial ELISAs or PCR-based methods (…).
290 quantify the presence of the main mustard allergens in foods. This method will help to detect mustard in processed foods and protect consumers with food allergy, specifically those with mustard allergy.

Changes have been made

***Highlights (for review)**

- We developed a new strategy for the detection of mustard allergen Sin a 1 in food products.
- The methodology is based on the application of liquid chromatography coupled to mass spectrometry analysis in selected reaction monitoring (SRM) mode.
- Mustard principal allergen Sin a 1was determined in several foods.
- This method will help to detect mustard in processed food and protect mustard allergic consumers.

1 **TITLE**

2 **Novel liquid chromatography-mass spectrometry method for the sensitive**
3 **determination of mustard allergen Sin a 1 in food.**

4 **RUNNING TITLE**

5 **Quantification of mustard allergen Sin a 1 by mass spectrometry.**

6 Maria Posada-Ayala[a]*, Gloria Alvarez-Llamas[a]*, Aroa S. Maroto[a], Xavier Maes[b],
7 Esther Muñoz-Garcia[a], Mayte Villalba[c], Rosalía Rodríguez, Marina Perez-Gordo[d],
8 Fernando Vivanco[a,c], Carlos Pastor-Vargas[a]*, Javier Cuesta-Herranz[e]*.

9 [a] Department of Immunology, IIS- Fundación Jiménez Díaz, Madrid.

10 [b] Agilent Technologies GmbH, Waldbronn, Germany.

11 [c] Department of Biochemistry and Molecular Biology I, Chemistry Faculty, Universidad
12 Complutense de Madrid.

13 [d] Institute of Molecular Applied Medicine (IMMA), and San Pablo CEU University
14 School of Medicine of Madrid.

15 [e] Department of Allergy, IIS-Fundación Jiménez Díaz, Madrid.

16 * Both authors and seniors contributed equally to this work.

17 **Corresponding author**

18 Dr. Carlos Pastor Vargas, Department of Immunology, Hospital Fundación Jimenez
19 Diaz, Avda. Reyes Católicos 2, 28040 - Madrid, Spain

20 E-mail: cpastor@fjd.es

21 Tel: +34915504800 ext 2202

22 Fax: +34915448246

23

ABSTRACT

Mustard is a condiment added to a variety of foodstuffs and a frequent cause of food allergy. A new strategy for the detection of mustard allergen in food products is presented. The methodology is based on the analysis by liquid chromatography coupled to mass spectrometry. Mustard allergen Sin a 1 was purified from yellow mustard seeds. Sin a 1 was detected with a total of five peptides showing a linear response (lowest LOD was 5 ng). Sin a 1 was detected in mustard sauces and salty biscuit (19±3mg/Kg) where mustard content is not specified. Sin a 1, as an internal standard, allowed quantification of this mustard allergen in foods. A novel LC/MS/MS SRM-based method has been developed to detect and quantify the presence of mustard, a principal allergen in foods. This method could help to detect mustard allergen Sin a 1 in processed foods and protect mustard allergic consumers.

Abbreviations used

LC-MS: liquid chromatography coupled to mass spectrometry.

LC-MS/MS: liquid chromatography coupled to mass spectrometry in tandem.

LOD: limit of detection

LOQ: limit of quantification

SRM: Selected Reaction Monitoring.

MS: mass spectrometry.

QQQ: triple quadrupole mass spectrometer

OD: Optical density

Key words: Allergens, Hidden allergen, LC-MS, Mustard allergy, Quantification, Sin a 1, SRM.

INTRODUCTION

Mustard is a condiment made from the seeds of a mustard plant that belongs to the Brassicaceae family, which includes other vegetables as radish, rutabaga, cabbage, broccoli, turnip, watercress, horseradish, castor oil plant, and rapeseed. There are various mustard varieties, being the most important varieties: 1) yellow mustard (*Sinapis alba*), the most commonly used in Europe, and 2) oriental mustard (*Brassica juncea*), used in United States and Asia. Mustard seed is added to a large assortment of foodstuffs such as pickled products, processed meats, seasoning blends, salad dressings, sauces and condiments to improve flavor. But, mustard is also frequently included as a hidden component in sauces, flavoring powders or salad dressings. Mustard has been reported as a frequent cause of food allergy. Symptoms in allergic reactions to mustard range from oral allergy syndrome to immediate skin reactions, angioedema and severe reactions, such as anaphylactic shock in hypersensitive patients (Caballero et al. 2002). Therefore, mustard was included in the European Union Directive 1169/2011/EC making declaration of mustard mandatory on food labels.. The prevalence of mustard allergy has been increasingly reported in the last years and IgE cross-reactivity has been described with other plant-derived foods (Vereda, Sirvent, Villalba, Rodriguez, Cuesta-Herranz & Palomares 2011; Sirvent et al., 2012), such as nuts, legumes, or Rosaceae fruits. No treatment has been described at present, and strict avoidance of the mustard-containing foods is, currently, the only treatment to avoid allergic reactions. In yellow mustard seeds, four allergens have been identified and characterized. Sin a 1 and Sin a 2 are specific seed-storage proteins and major allergens in mustard. Sin a 1 is a 2S albumin; a protein family highly resistant to proteolysis and stable under heat treatments (Menendez-Arias, Moneo, Dominguez & Rodriguez, 1988). Sin a 2 belongs to the 11S

74 globulin family (Palomares, Vereda, Cuesta-Herranz, Villalba & Rodriguez, 2007). Sin
75 a 3 and Sin a 4 were identified as non-specific lipid transfer protein (nsLTP) and
76 profilin, respectively (Sirvent, Palomares, Vereda, Villalba, Cuesta-Herranz &
77 Rodriguez, 2009). In addition, mustard allergens Sin a 3 and Sin a 4 are involved in IgE
78 cross-reactivity with fruits such as peach or melon, respectively.
79 The mustard allergens can be introduced in food products accidentally, by means of a
80 deficient processing (e.g. improper handling, cross-contamination or incomplete
81 cleaning, Jackson et al., 2008) or labeling errors. Also, in some countries, the presence
82 of mustard in foods is not specifically mentioned in the label, but it is included in the
83 generic term "spices". Therefore, the development of sensitive analytical methods for
84 the determination of mustard content in foods is very important to protect allergic
85 consumers.
86 Several ELISA methods have been described to detect mustard traces (Koppelman et al.
87 2007; Shim & Wanasundara 2008; Lee, Niemann, Lambrecht, Nordlee & Taylor, 2009).
88 These tests have decreased the limit of proteins quantification up to 1 ppm, and they are
89 capable of detecting mustard proteins from different species, including yellow and
90 oriental mustard (Cuhra, Gabrovska, Rysova, Hanak & Stumr, 2011).
91 Methodology based on mass spectrometry (MS) have advanced significantly in terms of
92 sensitivity and specificity, having improved the identification, characterization and
93 determination of food allergens (Faeste, Ronning, Christians & Granum, 2011). Since
94 the first study by Shefcheck and Musser (2004), liquid chromatography (LC) coupled to
95 MS analysis has become an important detection tool to identify allergens in food (Careri
96 et al., 2007; Abdel Rahman, Kamath, Lopata, Robinson & Helleur, 2011; Heick,
97 Fischer & Popping, 2011b; Mattarozzi, Bignardi, Elviri & Careri, 2012; Abdel Rahman,
98 Kamath, Gagne, Lopata & Helleur, 2013). LC-MS/MS has superior characteristics,

improved reproducibility, recovery, sensitivity, dynamic range, and quantifiability (Heick, Fischer, Kerbach, Tamm & Popping, 2011a). In this sense, selected reaction monitoring (SRM) is a highly specific and quantitative methodology, based on the measurement of specific masses of selected proteotypic peptides (peptide precursor and precursor fragments masses) corresponding to the protein of interest in a triple quadrupole (QQQ) mass spectrometer (Picotti, Bodenmiller & Aebersold, 2013). A proteotypic peptide is defined as a peptide that identifies a protein uniquely: thus, this protein can be specifically quantified by measurement of the peptide.

The aim of this study was to develop a sensitive and specific method based on LC-MS/MS measurements of allergens for the detection of mustard traces in food products. The development of such a method would allow the implementation of a reliable and accurate mustard allergens detection. The new method could be applied to other allergens and create an essential tool for food industry and regulatory agencies in the control of food allergens and better serve the food-allergic consumer.

MATERIAL AND METHODS

Yellow mustard seed extract. Sin a 1 purification

Mustard powder obtained from seeds of *Sinapis alba* L. was suspended in 0.15 mol/ l sodium borate buffer, pH 8, at a 10% (w/v) ratio, gently stirred for 1 h and centrifuged at 4ºC. The pellet was extracted twice with borate buffer and the supernatants were combined and lyophilized. The remaining plant material was then three times washed with 10% (w/v) acetone. The pellets were air-desiccated and dissolved in 0.15 mol/ l ammonium bicarbonate, pH 8.0. After centrifugation at 8700g, at 4ºC, supernatants were lyophilized and stored at -20ºC.

Sin a 1 was purified as previously described (Menendez-Arias et al. 1988). Briefly, lyophilized seeds extract was dissolved in 0.15 mol/ l ammonium bicarbonate buffer, pH 8.0, and applied onto a Sephadex G-50 *Fine* column. Fractions containing 2S albumin protein (identified by an specific rabbit polyclonal antibody against Sin a 1) were pooled and resolved by ion-exchange chromatography on a SP-Sephadex C-25 column equilibrated in 3 mmol/ l pyrophosphate buffer, pH 9.0. Proteins were eluted with a gradient from 3 to 100 mM pyrophosphate buffer, pH 9.0. Fractions containing protein were pooled and resolved by ion-exchange chromatography in CM-Cellulose. After a NaCl gradient from 0.15 to 0.3 mol/ l, fractions containing Sin a 1 were pooled.

Commercial samples protein extraction

Commercial sauces (mustard sauce, garlic mayonnaise, barbecue sauce, honey-mustard sauce, ketchup and mayonnaise) and salty biscuit samples were purchased from local market (Madrid, Spain)(Supplementary table I). 0.5 g of each sample was suspended in 50 mmol/ l ammonium bicarbonate buffer pH 8.0, at a 10% (w/v) ratio, gently stirred for 48 h at 4ºC and centrifuged at 20000 x g, at 4ºC. After centrifugation, supernatants

were lyophilized, resuspended in 0.5 ml PBS and loaded into a Superdex 25 column (GE Healthcare, Uppsala, Sweden) equilibrated in 50 mmol/ l ammonium bicarbonate buffer pH 8.0. Fractions containing proteins (identified by OD at 280 nm) were lyophilized and resuspended in 1ml 50 mol/ lM ammonium bicarbonate buffer pH 8.0. Total protein content was determined by Coomasie Plus (Bradford) Assay ,Pierce (Rockford, IL, USA) and 50µg of total protein was taken for digestion.

Liquid Chromatography-Mass Spectrometry

For LC-MS analysis, protein samples (purified protein or extracts obtained from commercial samples) were reduced with dithiothreitol (DTT), Bio-Rad (Hercules, CA, USA) and alkylated with iodoacetamide (IAA), Bio-Rad (Hercules, CA, USA). Digestion was performed with sequencing grade modified bovine trypsin, Roche (Branford, CT, USA), at a final concentration of 1:50 (trypsin:protein) for commercial extracts (50µg total protein digested) or by adding 0.2µg trypsin to all standard solutions of purified Sin a 1 used in the calibration curve. Tryptic peptides solutions were cleaned with C18 spin columns, Protea Biosciences (Morgantown, WV, USA) according to manufacturer's instructions, and diluted 1:1 with mobile phase A (0.1% formic acid, Sigma-Aldrich (St. Louis, MO, USA) containing 5% acetonitrile, Merck (Darmstadt, Germany)). Samples were analyzed using a 6460 Triple Quadrupole LC/MS/MS on-line connected to a HPLC-Chip Cube interface, Agilent Technologies (Palo Alto, CA, USA) and 1200 Series LC Modules, Agilent Technologies (Palo Alto, CA, USA) provided with a pre-cooled nano LC autosampler. Peptides separation was carried out on a ProtID Zorbax 300B-C18-5µm chip with 43 x 0.075 mm analytical column and 40 nl enrichment column, Agilent Technologies (Palo Alto, CA, USA). One microlitre of sample was injected at 3 µl/min and separation took place at 0.4 µl/min in a continuous acetonitrile gradient as follows: 5% for 1 min, 5-30%B for 1 min, 30-60%

B for 8 min, up to 95% B in 1 min and 95% B for 2 min. The system was controlled by Mass Hunter LC-MS Acquisition Software v4.01 Agilent Technologies (Palo Alto, CA, USA). The mass spectrometer was operated in positive ion mode with capillary voltage of 1950V, 325ºC source gas temperature and 5 L/min source gas flow. Fragmentor potential was set to 130V, dwell time to 50 ms, delta electron multiplier voltage (EMV) to 600 V and collision energy was optimized for each SRM transition. Theoretical SRM transitions were designed using Skyline (v.1.1.0.2905) (MacLean et al., 2010) and monitored by LC/MS/MS to finally set the best performing ones. Transitions from the same precursor ion should co-elute in retention time. Otherwise, they were not further considered. A transition refers to the measurement of a specific peptide fragment, coming from a known and previously selected peptide precursor. Protein specificity was confirmed using Basic Local Alignment Search Tool (BLAST) with the following parameters: organism Viridiplantae; database non-redundant protein sequences; algorithm blast (matrix BLOSUM62). Only peptides with 100% identity were selected since a change in the amino acid sequence of the peptide would be a change in mass and, therefore, would not be selected in the first quadrupole. Also, selected peptides did not match with any other protein (with 100% identity). Then, proteotypic peptides were selected, that is, only those specifically representing the protein of interest. Individual transition signals were normalized based on TIC (total ion current) according to the mathematical equation: Normalized Area = (Peak Area / Total Area) x 100.

Quantification and method validation

To aliquots containing 30µg of total protein in 20 µl, increasing amounts of Sin a 1 (0, 2, 4, 6 or 8µg) were added and digestion was performed as previously described. Limits of detection (LOD) and quantification (LOQ) were calculated as 3 or 10 times the

standard deviation of a blank (ten replicates measured) divided by the slope of the calibration curve, respectively. The amount of Sin a 1 originally present in the mustard extract was calculated from data of all transitions measured for this protein to test the reliability of the method and select the best SRM conditions for this allergen quantification. The amount of Sin a 1 was calculated by standard addition: Sin a 1 (µg) = intercept / slope. The final content of Sin a 1 in ng was referred to 30 µg total protein content.

RESULTS

Analysis of purified Sin a 1.

For Sin a 1, all selected peptides had a 100% match with 2S seed storage proteins family from Brassiceae family, also called mustard family. Table 1 shows 22 transitions for Sin a 1 measured in a first approach, together with peptide sequences, precursor and fragment masses (products) and collision energy applied in each case. All those showing an appropriate signal to noise ratio (ionization) were selected for further experiments, independent of ion type. Sin a 1 could be detected with a total of five peptides. In particular, peptide R.ACQQWLHK.Q could be measured with seven transitions, R.IYQTATHLPK.V with three transitions and K.EFQQAQHLR.A with six transitions. The peptides R.QQLGQQGQQGPHLQHVISR.I and R.QVSVCPFK.K showed poor reproducibility; thus, the other three peptides were used in further studies. Supplementary material Fig 1 shows typical eXtracted Ion Chromatogram (XIC) for all transitions detected for Sin a 1.

Linearity response for purified Sin a 1 transitions

As a proof of concept, 10 transitions were tested from the three best performing peptides in SRM (R.ACQQWLHK.Q, R.IYQTATHLPK.V and K.EFQQAQHLR) to check for a linear response for this protein. Calibration curves were obtained with purified proteins standards, including a total of three technical replicates at every concentration assayed. Data are shown in Supplentary Table 2. Sin a 1 showed a linear response in the range of 0.1 to 10^{-6} µg (average r=0.992), for a total of 7 assayed concentrations. Relative standard deviation (%RSD) was calculated in the range from

10^{-4} to 1µg and values were not more than 28% for Sin a 1, except for the transition 357.511->500.245.

Sensitivity and Selectivity of the LC/MS/MS developed method for Sin a 1 analysis in mustard extracts

In a further step we evaluated the sensitivity and reproducibility of the method using the standard addition method. One mustard sauce extract was selected and increasing amounts of purified Sin a 1 were added (from 0 to 8µg). Five technical replicates were performed both at the lowest and the highest calibration points to evaluate reproducibility (%RSD). Calibration curves, limits of detection and limits of quantification obtained for every transition measured for Sin a 1 are shown in Table 2. For the quantitation, the whole group of transitions described was analyzed to ensure the sample size was sufficient to achieve statistical significance.. Finally, the sixteen transitions shown were used. The lowest LOD and LOQ were obtained for the peptides: R.ACQQWLHK.Q (SRM transitions 357.511->500.245 and 357.511->420.229) and K.EFQQAQHLR.A (386.2->440.741 and 386.2->514.275). %RSD was in the range 2-14% in all cases. The amount of Sin a 1 originally present in the mustard extract was calculated from transition data satisfying the linearity criteria of $r \geq 0.98$. The average value was 87±7ng of Sin a 1 per µg of total protein in the extract (8% RSD).

Sin a 1 analysis in food extracts from commercial sauces and salty biscuits

Sin a 1 was also analyzed blind in a series of food extracts. As expected, Sin a 1 was detected in both mustard sauce and honey-mustard sauce, and salty biscuit which contains mustard according to label, as well as barbecue, mayonnaise, garlic mayonnaise and ketchup sauces which contained spices (according to label) but did not

specify mustard (see Supplementary Fig 2). To confirm the method described in this paper was valid for quantification of processed food, we analyzed Sin a 1 content in a salty biscuit extract by addition standard method. Using transitions 535.8 > 839.4, 535.8 > 924.4, 578.8 > 624.4 and 578.8 > 880.5, an average value of 19±3mg Sin a 1 per kg of salty biscuit was obtained(15%RSD)(Table 3), showing the utility of the proposed methodology to quantify main mustard allergen in foods other than mustard.

DISCUSSION

The presence of hidden allergens is a serious health hazard affecting people with food allergy. Therefore, the detection of allergenic components is a priority in food safety in order to protect allergic consumers. At present, the development of mass spectrometry-based methodologies has advanced detection of proteins, peptides, metabolites, and small molecules in general.

In this study, the selected peptides, used as markers of Sin a 1, satisfy various criteria including: 1) sequence specificity: proteotypic peptides specific for mustard allergens; 2) ESI sensitivity: peptides are highly ionized resulting in stable and sufficient signal intensity for detection, and 3) more than two transitions are detectable per peptide (and more than three peptides per protein could be analyzed). In particular, five peptides satisfied these criteria (Table 1). These selected peptides were checked using BLAST searches and resulted specific for mustard proteins. Only three peptides and their transitions (IYQTATHLPK, EFQQAQHLR and EFQQAQHLR) showed good reproducibility to be used in quantification. The peptides QQLGQQGQQGPHLQHVISR and QVSVCPFK showed poor reproducibility, thus they could be used to detect mustard traces in foods but not with quantification purposes.

This method allows detection of proteins easily extracted from complex matrix, by applying a selective exclusion chromatographic cleaning up system and, thus, increasing sensitivity and reducing background. Analysis in SRM mode favors specificity and minimizes potential blank interferences, as only those proteotypic peptides (i.e. those uniquely present in the protein sequence of interest) are analyzed. Extracts from different foods containing mustard (mustard presence is detailed on the

label) were analyzed. Mustard allergens could be detected not only in those food extracts, in which the presence of mustard was declared, but also in foods where the label only stated spices.

In order to test whether mustard allergens could be used as markers to quantify mustard in different foods, we carried out an assay to evaluate the linearity response for purified Sin a 1 transitions. Sin a 1 produced a linear response (Supplementary Table 2) and, thus, further quantification studies were based on this protein.

The analytical characteristics of the proposed method were evaluated by addition of the purified mustard allergen Sin a 1 as internal standard to a mustard sauce extract. Other methodologies to quantify proteins in biological matrices by LC-MS at peptide level include an isotopically labeled synthetic peptide from the tryptic digestion as internal standard. This methodology has been successfully used to quantify biomarkers (Gillette and Carr, 2013). This technology has allowed the quantification of allergens in soybean (Houston et al., 2011), snow crab (Abdel Rahman et al., 2011) and shrimp (Abdel Rahman et al., 2013). In a different approach, an intact protein has been used, as internal standard, to quantify allergens in peanut (Shefcheck and Musser 2004), milk (Czerwenka, Maier, Potocnik, Pittner & Lindner, 2007) and lupin (Brambilla, Resta, Isak, Zanotti & Arnoldi, 2009). In this work, method evaluation was carried out by addition of purified intact Sin a 1 to mustard sauce extracts. In this way, we avoid potential problems associated with the use of peptides as internal standards, i.e.: 1) a different behavior of internal standard peptides compared to that of the intact protein during the steps of the analytical workflow that take place prior to digestion, or 2) incompleted protein digestion, which may cause peptide behavior not comparable with the intact protein.

Proteins can be broken down by processing steps such as heat treatment or fermentation, although many food allergens are characterized by their high stability after strong treatment (heat, low pH, etc.) and their ability to survive food processing. In the case of protein allergens, mass spectrometric methods are performed at the peptide scale, and the proteotypic peptide chosen to be monitored can still be valuable after food processing, thus making the quantification independent of the structure of the allergen. In this sense, peptides described in this paper have been detected in foods with different processing forms (sauces and salty biscuit).

LOD and LOQ obtained for this SRM-based methodology were as low as 0.25 ppm and 0.75 ppm, respectively. These values represent a substantial improvement compared with those obtained for ELISA-based methods (Lee, Hefle & Taylor, 2008), including current commercial ELISAs or PCR-based methods (Fuchs, Cichna-Markl & Hochegger, 2010). Additionally, the variability observed for quantification data obtained from all transitions measured proved the robustness and feasibility of the developed method.

Insufficient sensitivity may be a limitation in foods where mustard content is not clearly detailed. Particular care has to be taken prior to inform about a negative result regarding mustard presence when dealing with a potential allergy case.

In conclusion, a novel LC/MS/MS SRM-based method have been developed to detect and quantify the presence of the main mustard allergen Sin a 1 in foods. This method will help to detect mustard in processed foods and protect consumers with food allergy, specifically those with mustard allergy.

Acknowledgements

RETIC Red de Investigación de Reacciones Adversas a Alérgenos y Fármacos (RD12/0013/0013 and RD12/0013/0015), Fondo de Investigación Sanitaria (PI10/00974, PI11/01401, PI13/01873, CP09/00229 and IF08/3667-1), Ministerio de Ciencia e Innovación (SAF2011-26716) and Fundación Conchita Rábago de Jiménez Díaz supported this study. Proteomics Facility UCM-PCM, a member of the ProteoRed Network, for support with SRM experiments.

Conflict of interest

The authors declare that no conflict of interest exist.

REFERENCES

Abdel Rahman, A.M., Kamath, S.D., Gagne, S., Lopata, A.L. and Helleur, R. (2013) Comprehensive proteomics approach in characterizing and quantifying allergenic proteins from northern shrimp: toward better occupational asthma prevention. J. Proteome. Res. 12, 647-656.

Abdel Rahman, A.M., Kamath, S.D., Lopata, A.L., Robinson, J.J. and Helleur, R.J. (2011) Biomolecular characterization of allergenic proteins in snow crab (Chionoecetes opilio) and de novo sequencing of the second allergen arginine kinase using tandem mass spectrometry. J. Proteomics. 74, 231-241.

Brambilla, F., Resta, D., Isak, I., Zanotti, M. and Arnoldi, A. (2009) A label-free internal standard method for the differential analysis of bioactive lupin proteins using nano HPLC-Chip coupled with Ion Trap mass spectrometry. Proteomics. 9, 272-286.

Caballero, T., San-Martin, M.S., Padial, M.A., Contreras, J., Cabanas, R., Barranco, P. and Lopez-Serrano, M.C. (2002) Clinical characteristics of patients with mustard hypersensitivity. Ann. Allergy Asthma Immunol. 89, 166-171.

Careri, M., Costa, A., Elviri, L., Lagos, J.B., Mangia, A., Terenghi, M., Cereti, A. and Garoffo, L.P. (2007) Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara h 3/4 for food control by liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 389, 1901-1907.

Cuhra, P., Gabrovska, D., Rysova, J., Hanak, P. and Stumr, F. (2011) ELISA kit for mustard protein determination: interlaboratory study. J. AOAC Int. 94, 605-610.

350 Czerwenka, C., Maier, I., Potocnik, N., Pittner, F. and Lindner, W. (2007) Absolute
351 quantitation of beta-lactoglobulin by protein liquid chromatography-mass spectrometry
352 and its application to different milk products. Anal. Chem. 79, 5165-5172.

353 Faeste, C.K., Ronning, H.T., Christians, U. and Granum, P.E. (2011) Liquid
354 chromatography and mass spectrometry in food allergen detection. J. Food Prot. 74,
355 316-345.

356

357 Fuchs, M., Cichna-Markl, M. and Hochegger, R. (2010) Development and Validation of
358 a Real-Time PCR Method for the Detection of White Mustard (Sinapis alba) in Foods.
359 J. Agric. Food Chem.

360 Gillette, M.A. and Carr, S.A. (2013) Quantitative analysis of peptides and proteins in
361 biomedicine by targeted mass spectrometry. Nat. Methods 10, 28-34.

362 Heick, J., Fischer, M., Kerbach, S., Tamm, U. and Popping, B. (2011a) Application of a
363 liquid chromatography tandem mass spectrometry method for the simultaneous
364 detection of seven allergenic foods in flour and bread and comparison of the method
365 with commercially available ELISA test kits. J. AOAC Int. 94, 1060-1068.

366 Heick, J., Fischer, M. and Popping, B. (2011b) First screening method for the
367 simultaneous detection of seven allergens by liquid chromatography mass spectrometry.
368 J. Chromatogr. A 1218, 938-943.

369 Houston, N.L., Lee, D.G., Stevenson, S.E., Ladics, G.S., Bannon, G.A., McClain, S.,
370 Privalle, L., Stagg, N., Herouet-Guicheney, C., MacIntosh, S.C. and Thelen, J.J. (2011)

371 Quantitation of soybean allergens using tandem mass spectrometry. J. Proteome. Res.
372 10, 763-773.

373 Jackson, L.S., Al-Taher, F.M., Moorman, M., DeVries, J.W., Tippett, R., Swanson,
374 K.M., Fu, T.J., Salter, R., Dunaif, G., Estes, S., Albillos, S. and Gendel, S.M. (2008)
375 Cleaning and other control and validation strategies to prevent allergen cross-contact in
376 food-processing operations. J. Food Prot. 71, 445-458.

377 Koppelman SJ, Vlooswijk R, Bottger G, Van Duijn G, Van der Schaft P, Dekker J, Van
378 Bergen H. (2007) Development of an enzyme-linked immunosorbent assay method to
379 detect mustard protein in mustard seed oil. J. Food Prot. 70, 179-183.

380 Lee, P.W., Hefle, S.L. and Taylor, S.L. (2008) Sandwich enzyme-linked
381 immunosorbent assay (ELISA) for detection of mustard in foods. J. Food Sci. 73, T62-
382 T68.

383 Lee, P.W., Niemann, L.M., Lambrecht, D.M., Nordlee, J.A. and Taylor, S.L. (2009)
384 Detection of mustard, egg, milk, and gluten in salad dressing using enzyme-linked
385 immunosorbent assays (ELISAs). J. Food Sci. 74, T46-T50.

386 MacLean, B., Tomazela, D.M., Shulman, N., Chambers, M., Finney, G.L., Frewen, B.,
387 Kern, R., Tabb, D.L., Liebler, D.C. and MacCoss, M.J. (2010) Skyline: an open source
388 document editor for creating and analyzing targeted proteomics experiments.
389 Bioinformatics. 26, 966-968.

390 Mattarozzi, M., Bignardi, C., Elviri, L. and Careri, M. (2012) Rapid Shotgun Proteomic
391 Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry-Based

392 Method for the Lupin (Lupinus albus L.) Multi-allergen Determination in Foods. J.
393 Agric. Food Chem.

394 Menendez-Arias, L., Moneo, I., Dominguez, J. and Rodriguez, R. (1988) Primary
395 structure of the major allergen of yellow mustard (Sinapis alba L.) seed, Sin a I. Eur. J.
396 Biochem. 177, 159-166.

397 Palomares, O., Vereda, A., Cuesta-Herranz, J., Villalba, M. and Rodriguez, R. (2007)
398 Cloning, sequencing, and recombinant production of Sin a 2, an allergenic 11S globulin
399 from yellow mustard seeds. J. Allergy Clin. Immunol. 119, 1189-1196.

400 Picotti, P., Bodenmiller, B. and Aebersold, R. (2013) Proteomics meets the scientific
401 method. Nat. Methods 10, 24-27.

402 Shefcheck, K.J. and Musser, S.M. (2004) Confirmation of the allergenic peanut protein,
403 Ara h 1, in a model food matrix using liquid chromatography/tandem mass spectrometry
404 (LC/MS/MS). J. Agric. Food Chem. 52, 2785-2790.

405 Shim, Y.Y. and Wanasundara, J.P. (2008) Quantitative detection of allergenic protein
406 Sin a 1 from yellow mustard (Sinapis alba L.) seeds using enzyme-linked
407 immunosorbent assay. J. Agric. Food Chem. 56, 1184-1192.

408 Sirvent, S., Akotenou, M., Cuesta-Herranz, J., Vereda, A., Rodriguez, R., Villalba, M.
409 and Palomares, O. (2012) The 11S globulin Sin a 2 from yellow mustard seeds shows
410 IgE cross-reactivity with homologous counterparts from tree nuts and peanut. Clin
411 Transl. Allergy 2, 23.

412 Sirvent, S., Palomares, O., Vereda, A., Villalba, M., Cuesta-Herranz, J. and Rodriguez,
413 R. (2009) nsLTP and profilin are allergens in mustard seeds: cloning, sequencing and
414 recombinant production of Sin a 3 and Sin a 4. Clin. Exp. Allergy 39, 1929-1936.

415 Vereda, A., Sirvent, S., Villalba, M., Rodriguez, R., Cuesta-Herranz, J. and Palomares,
416 O. (2011) Improvement of mustard (Sinapis alba) allergy diagnosis and management by
417 linking clinical features and component-resolved approaches. J. Allergy Clin Immunol.
418 127, 1304-1307.

419

420

421

422

FIGURE LEGENDS

Figure 1: eXtracted Ion Chromatogram (XIC) of the different transitions monitored of food extracts. E1: Mustard sauce: E2: Garlic mayonnaise; E3: Barbecue sauce; E4: Honey-mustard; E5: Ketchup; E6: Mayonnaise; E7: Salty biscuit. X axis: Retention time. Y axis. E1. Precursor mass 357.511. Fragment masses 397.255 (green), 420.229 (blue) and 500.245 (orange). E2. Precursor mass 386.2 and fragment mass 440.741 (purple). Precursor mass 357.511 and fragment mass 397.255 (green). E3. Precursor mass 578.796. Fragment masses 624.357 (blue) and 880.474 (red). E4. Precursor mass 357.511. Fragment masses 397.255 (green), 420.229 (blue) and 500.245 (orange). E6. Precursor mass 535.763. Fragment masses 583.335 (green), 711.393 (blue), 839.422 (orange) and 924.414 (pink). E7. Precursor mass 535.763. Fragment masses 583.335 (green), 711.393 (blue) and 839.422 (orange).

Supplementary Material:

Figure 1. SRM optimized transition analysis of Sin a 1. The figure shows 5 peptides from Sin a 1 and. Each chromatogram has its corresponding transitions. X axis: Retention time. Y axis: Signal intensity.

Figure 2: Chromatograms of food extracts from commercial sauces. E represents total ion current (TIC) chromatograms for all the food extracts tested: E1: Mustard sauce: E2: Garlic mayonnaise; E3: Barbecue sauce; E4: Honey-mustard; E5: Ketchup; E6: Mayonnaise; E7: Salty biscuit. X axis: Retention time. Y axis: TIC (signal intensity).

Table(s)

Table 1

Protein (UniProt ID)	Peptide sequence	Retention time (min)	m/z precursor (charge state)	m/z product	Collision energy (V)
Sin a 1 (P15322)	R.ACQQWLHK.Q	4.66±0.15	535.763 (+2)	924.414(b7)(+)/839.422(y6)(+) /711.393(y5)(+)/583.335(y4)(+)	12
			357.511 (+3)	500.245(y7)(2+)/420.229(y6)(2+)/397.255(y3)(+)	5
	R.IYQTATHLPK.V	4.70±0.1	391.22 (+3)	506.26(b4)(+)/408.206(b7)(+)/405.213(b3)(+)	5
	K.EFQQAQHLR.A	4.50±0.1	578.796 (+2)	880.474(y7)(+)/752.416(y6)(+)/624.357(y5)(+)	14
			386.2 (+3)	514.275(y8)(2+)/440.741(y7)(2+)/425.261(y3)(+)	5
	R.QQLGQQGQQGPHLQHVISR.I	5.00±0.15	1070.061 (+2)	1287.618(b12)(+)/543.812(y9)(2+)	39
	R.QVSVCPFK.K	4.67±0.15	322.168 (+3)	369.166(y6)(2+)/336.162(b6)(2+)/325.67(y5)(2+)/287.636(b5)(2+)	2

Table 1. Sin a 1 optimized SRM transitions for the different peptides. Values of m/z ratios for precursor and fragment ions, collision energy (V) applied and retention time (min) are shown.

Table 2

Peptide sequence	Transition	LOD(µg)	LOQ(µg)	Calibration curve y=b ± mx	r	ng Sin a 1 /µg total protein in the extract
R.IYQTATHLPK.V	391.22->405.213	0.105	0.297	y = 8996 ± 3079 x	0.9709	97,390
	391.22->408.206	0.166	0.468	y = 70002.6 ± 1952.4 x	0.9707	119,553
	391.22->506.26	0.101	0.286	y = 9193.3 ± 3192.1 x	0.981	96,000
K.EFQQAQHLR.A	578.796->880.474	0.055	0.155	y = 12978 ± 5873.3 x	0.9894	73,653
	578.796->752.416	0.032	0.090	y = 27993 ± 1013.4 x	0.9852	92,073
	578.796->624.357	0.034	0.096	y = 26473 ± 9468.5 x	0.9877	93,197
	386.2->425.261	0.038	0.108	y = 19766 ± 8477.1 x	0.9812	77,720
	386.2->440.741	0.007	0.022	y = 114584 ± 41613 x	0.9752	91,783
	386.2->514.275	0.010	0.028	y = 86409 ± 31755 x	0.9784	90,703
R.ACQQWLHK.Q	357.511->397.255	0.038	0.109	y = 38208 ± 8342.8 x	0.9722	152,657
	357.511->420.229	0.011	0.031	y = 118144 ± 28978 x	0.9551	135,897
	357.511->500.245	0.005	0.015	y = 278404 ± 59713 x	0.9658	155,410
	535.763->839.422	0.067	0.190	y = 12442 ± 4805.4 x	0.9913	86,303
	535.763->924.414	0.235	0.662	y = 3702.7 ± 1382.3 x	0.985	89,280
	535.763->583.335	0.151	0.426	y = 5385.6 ± 2187.1 x	0.993	86,273
	535.763->711.393	0.166	0.459	y = 5446.8 ± 1951.6 x	0.9919	90,300

Table 2. Addition standard method curves of Sin a 1 for its quantification in mustard sauce. *LOD: limit of detection. LOQ: limit of quantification. r: Pearson correlation coefficient. RSD: Relative standard deviation. %RSD is in the range 2-14% in all cases.

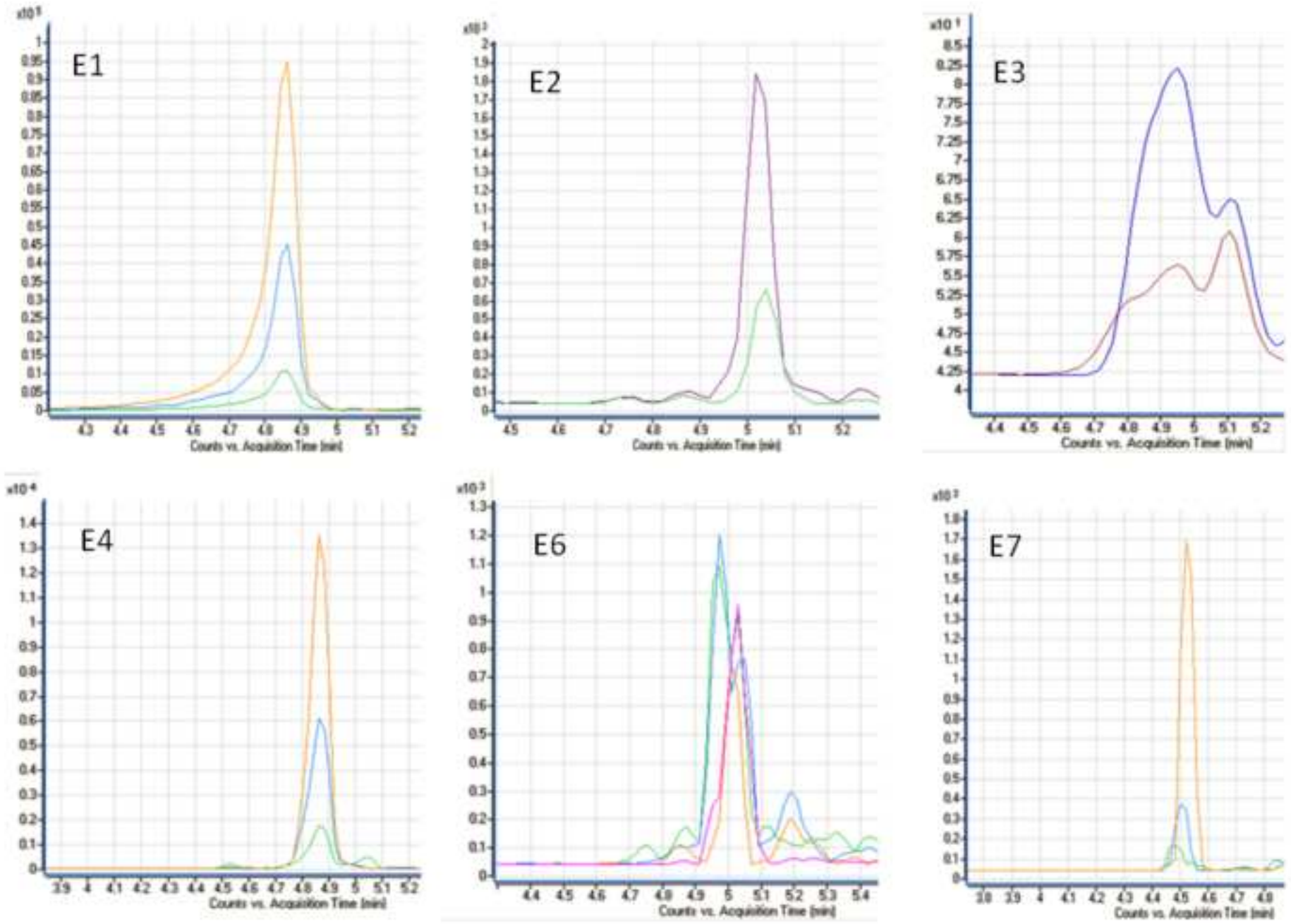
Table 3

Peptide sequence	Transition	Calibration curve y=b ± mx	ng Sin a 1 /µg total protein in the extract
K.EFQQAQHLR.A	578.796->880.474	y = 4138,2 + 1048,4x	131,57
	578.796->624.357	y = 9031,4 + 2582x	116,59
R.ACQQWLHK.Q	535.763->839.422	y = 3178 + 1066,7x	99,37
	535.763->924.414	y = 1305,2 + 306,4x	142.16

Table 3. Addition standard method curves of Sin a 1 for its quantification in salty biscuit.

Figure 1

Click here to download high resolution image



Marked up version

Click here to download Supplementary Material: Manuscript Mustard marked up version.doc

Table 1

Click here to download Supplementary Material: Supplementary table I.doc

Table 2

Click here to download Supplementary Material: Supplementary table II.doc

Fig 1

Click here to download Supplementary Material: Fig 1 Supplementary.tiff

Fig 2

Click here to download Supplementary Material: Fig 2 Suplementary.tif