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**Food allergens: detection and immunogenic properties
as affected by processing and *in vitro* digestibility**

Porto

2020

PhD Thesis

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Thesis submitted to Faculdade de Farmácia do Universidade do Porto
for Doctor Degree in Sustainable Chemistry

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Porto

October 2020

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This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) under the Partnership Agreement UIDB 50006/2020, by the projects AlleRiskAssess—PTDC/BAA-AGR/31720/2017 and NORTE-01-0145-FEDER-00001 and by the PhD grant (PD/BD/114576/2016) financed by Programa Operacional Potencial Humano - Quadro de Referência Estratégico Nacional - Tipologia 4.1 - Formação Avançada (POPH-QREN) subsidised by Fundo Social Europeu (FSE) and national funds from Ministério da Ciência, Tecnologia e Ensino Superior (MCTES). In addition to the PhD Grant, the candidate also benefited from supplementary funding through COST Action FA1402 (ImpARAS) to perform a traineeship abroad (1 month in Italy) and to participate in international congresses, conferences and training schools.



The work presented in this thesis was mostly performed in the Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto. A part of the research was developed at the Institute of Sciences of Food Production, National Research Council (ISPA-CNR), Bari, Italy.

Acknowledgements

Science is my way to do magic...

But my magic wouldn't be possible without some incredible people, who I have to thank.

To Dr. Isabel Mafra, who has been my mentor, my inspiration and guide through all these years. I have to thank her for all the teaching, advices and help that she has given me, for her friendship and kindness. Her dedication and passion for science are traits of her personality for which I will always admire her so much.

To Dr. Joana Costa, one of the best scientists I ever met. For her perfection in everything she does, for her "sixth sense" that I always trusted, for becoming one of my best partners in travel and for her tireless work in always helping everyone! And because without her anything of this would not be possible.

To Dr. Beatriz Oliveira, for her co-supervision and for accepting me in her group at the beginning of my pathway in science.

To Fundação para a Ciência e a Tecnologia (FCT) by the PhD grant (PD/BD/114576/2016) financed by Programa Operacional Potencial Humano - Quadro de Referência Estratégico Nacional - Tipologia 4.1 - Formação Avançada (POPH-QREN) subsidised by Fundo Social Europeu (FSE) and national funds from Ministério da Ciência, Tecnologia e Ensino Superior (MCTES), and to project AlleRiskAssess – PTDC/BAA-AGR/31720/2017, which made possible all this work.

To ImpARAS (COST Action FA1402), for giving me the opportunity to participate in its conferences, meetings, short term scientific missions and training schools and for meeting and sharing experiences with young and senior researchers. Certainly, ImpARAS contributed significantly to my growth as a young scientist.

To Liliana Grazina, my fellow, my confident, one of my best friends. She was the person, who was always by my side in the most difficult times, being always there to support me. Together, we have also shared a lot of incredible moments of joy and complicity in this long journey of being a PhD student.

To Mónica Moura for the huge help in the lab during my experiments. Sharing two great passions with me, research and dance, every day you demonstrated to be a great person, interested, pro-active and I am very happy that you joined us to be part of our group. I am sure that nobody would be better than you!

Acknowledgements

To all my colleagues, professors, technicians and students at Laboratory of Bromatology and Hydrology of Faculty of Pharmacy University of Porto, for their great support in everything I needed to do my work, but also for the jokes, the laughs and the good stories that we share every day.

To Dr. Linda Monaci and her team, Simona, Elisabetta and Rosa, for their warm welcome in the short time that I have been at CNR-ISPA in Bari, Italy. I have to say that it was one of the best periods of my life, where I met incredible and amazing people in the most beautiful country of the world, Italy.

To my parents for their unconditional love, protection and support in every step of my life, because no matter how badly I could fail, I always knew that they would treat me like a winner. I grew up to be the best I can to make them proud of me. I hope I got it...

To Hélder, love of my life and my best friend, for his patience, for putting up with my mood swings and arrogance, for all the advices and “fights”. For walking along with me through all these years on the road that is our life and for helping me to continue in the right direction.

To my stars that are no longer with me, Zia Cristina, Nonni Anna e Alfio, Avós Francisco e Maria. Zia Cristina you will always be my wonder woman, my inspiration at work and in life, your strength and ambition, your will to make all your dreams come true were the pillars for the person I am today. To all my grandparents, who were taken for me along these four years, one by one, their light has been turned off. Each one of them taught me a lot since I was a child until I became an adult, and I was very lucky to have all of them with me so much time. But to my Nonno Alfio I am even more thankful because he taught me to love life and to enjoy every single moment of it. As he always said: “Viva la Vita!”

I am writing my acknowledgments at home because of the recent developments of Covid-19. I never thought, when I started my PhD, that a situation like this could ever happen...But here we are, far from each other and hoping that in a short time we will be able to come back to work, doing what we like most and stay with the people we love. Now, I am finishing to write my thesis, hoping that in a few months everything will be fine and the world will return to be what it was...

“If you can dream it, you can do it...”

Walt Disney

Abstract

Food allergy is an increasing health problem in western countries, with strict avoidance of the offending food(s) being the only available reliable treatment. However, accidental exposure can occur, rising the risk of developing adverse immunological reactions in sensitised individuals. Most (~90%) of the allergic reactions are due to specific classes of foods, namely milk, soybean, cereals containing gluten, eggs, fish, crustaceans, peanut and tree nuts. Many efforts have been employed to protect the allergic patients, namely through the establishment of several regulations regarding the mandatory labelling of pre-packaged foods containing allergenic ingredients. Nonetheless, sensitised/allergic consumers are forced to avoid these products due to the excessive use of precautionary labelling by the food industry, limiting their free and safe selection of processed foods. Recently, the development of hypoallergenic formulas has gained a great interest as a possible solution to improve quality of life of allergic consumers. The application of conventional and novel food processing technologies has revealed promising effects on the mitigation of the allergenicity of certain foods. However, understanding their induced structural and compositional alterations as related with the allergic potential of proteins is crucial, particularly using real food matrices, and further conjugating with gastro-duodenal digestion. Therefore, this work intended to address two main gaps in food allergen studies: (i) the need for highly sensitive and specific methods for the detection of allergenic foods; and (ii) increasing the knowledge on the effects of processing, food matrix and gastro-duodenal digestion on the structural features of food allergens. The first goal was focused on the development of specific and highly sensitive DNA-based methods to detect and quantify traces of milk and other milk protein substitutes used in processed foods, being lupine the case study. The second goal of this thesis was the evaluation of the effect of some food processing technologies (autoclaving, oven cooking and baking) and *in vitro* gastro-duodenal digestion on the final immunoreactivity of these allergens. For these purposes, protein-based methods, namely immunoblotting and mass spectrometry (MS) analysis were developed.

The development of a robust and accurate real-time PCR method for the detection of milk ingredients in meat products included a careful *in silico* and experimental evaluation of several molecular markers (mitochondrial and allergen-encoding genes). The mitochondrial 12S rRNA gene revealed to be the most promising marker for the specific detection of cow's milk, being successfully used in the development of normalised calibration models, with sensitivities between 50-100 mg/kg of cow's milk protein in cooked-ham and autoclaved sausages. The preparation of model mixtures of hams and sausages submitted to oven

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cooking and autoclaving processing, respectively, enabled assessing the impact of such treatments on the analytical performance of the real-time PCR methods, which were successfully applied to analyse commercial foods. Similarly, for lupine detection, different model mixtures of breads prepared with wheat or rice flours and submitted to a baking process enabled assessing the effects of food matrix and thermal processing on the developed real-time PCR methods. Sensitivities down to 1 pg of *Lupinus albus* DNA and 5 mg/kg, 100 mg/kg and 500 mg/kg of lupine flour in rice flour, wheat flour and bread, respectively, were obtained, highlighting the clear negative influence of food matrix and baking on the analytical PCR performance.

As milk ingredients are often found in processed meat products, the evaluation of autoclaving (used in the manufacture of sausages) on the IgE-binding capacity of milk proteins followed by *in vitro* gastro-duodenal digestion was performed by immunoblotting with sera from allergic patients and by mass spectrometry (MS) analysis. The results showed a clear change of structural and immunogenic properties of whey proteins caused by autoclaving, with their complete degradation after digestion. On the contrary, caseins were more resistant to proteolytic digestion, resulting in the production of several digested peptides (21.3-25.1 kDa) whose IgE-binding capacity was only slightly affected by autoclaving, but completely eliminated after gastro-duodenal digestion. The combined effects of thermal processing and food matrix were also studied, assessing the immunoreactivity of lupine and soybean proteins used as ingredients in bakery and meat products, respectively. Generally, the immunoreactivity of target proteins (lupine gamma-conglutin and soybean trypsin inhibitor) was reduced by all the tested thermal treatments, though at a higher extent after autoclaving, being slightly altered by the food matrix. The immunochemical method additionally enabled the identification of target proteins in commercial products.

In summary, in the presented work, robust and accurate methods were successfully developed for the detection of milk and lupine at trace levels in processed foods. Considering the demonstrated applicability, the new proposed tools can be considered very useful to improve allergen management by the food industry. The present work also contributed with important achievements regarding the reduction of the immunoreactivity of milk and lupine by some conventional processing methods. These findings can help understanding and increase knowledge about the effect of food processing on the immunogenicity of cow's milk and lupine, aiming at developing hypoallergenic formulas to protect the health of sensitised individuals, although more in-depth studies are still needed.

Keywords: food allergies, real-time PCR, immunoreactivity, simulated digestion, food processing, MS analysis.

Resumo

As alergias alimentares constituem um crescente problema de saúde pública nos países ocidentais, cujo único tratamento confiável disponível é a completa eliminação do alimento de risco da dieta. No entanto, exposições acidentais aos alimentos alergénicos podem ocorrer, aumentando o risco de desenvolvimento de respostas imunológicas adversas em indivíduos sensibilizados. Cerca de 90% das reações alérgicas são devidas a grupos específicos de alimentos, tais como leite, soja, cereais contendo glúten, ovos, peixe, crustáceos, amendoim e frutos de casca rija. Tem havido um crescente e constante esforço em proteger os pacientes alérgicos através da adoção de vários regulamentos relativos à obrigatoriedade de rotulagem de alimentos pré-embalados contendo proteínas alergénicas. No entanto, os consumidores alérgicos continuam a ser forçados a evitar esses produtos devido à excessiva rotulagem de precaução, limitando a sua escolha livre e segura de alimentos processados. Recentemente, o desenvolvimento de alimentos/fórmulas hipoalergénicas ganhou grande interesse, sendo apresentada como uma possível solução para melhorar a qualidade de vida dos consumidores alérgicos. A aplicação de técnicas convencionais e inovadoras de processamento alimentar revelou ter efeitos promissores na mitigação da alergenicidade de certos alimentos. No entanto, a compreensão das alterações composicionais e estruturais relacionadas com o potencial alérgico das proteínas é crucial, particularmente usando matrizes alimentares reais, e posterior conjugação com a digestão gastro-duodenal. Deste modo, este trabalho teve como objetivo abordar duas lacunas principais nos estudos sobre alergénios alimentares: (i) a necessidade de métodos altamente sensíveis e específicos para a deteção de alimentos alergénicos; e (ii) aumentar o conhecimento sobre os efeitos do processamento, matriz alimentar e digestão gastro-duodenal sobre as características estruturais dos alergénios alimentares. O primeiro objetivo teve foco no desenvolvimento de técnicas específicas e altamente sensíveis baseadas na análise de DNA, a fim de detetar e quantificar vestígios de leite e outros substitutos proteicos do leite em alimentos processados, tendo como caso de estudo o tremço. O segundo objetivo desta tese é avaliar o efeito de algumas técnicas de processamento de alimentos (autoclavagem e cozedura) e da digestão *in vitro* na imunorreatividade final destes alergénios. Para tal, foram desenvolvidos métodos baseados na análise de proteínas, nomeadamente immunoblotting e análise por espectrometria de massa (MS).

O desenvolvimento de um método de PCR em tempo real robusto e eficaz para a deteção de ingredientes lácteos em produtos cárneos envolveu uma avaliação bioinformática e experimental de vários marcadores moleculares (genes mitocondriais e

genes codificadores de alergénios). O gene mitocondrial 12S rRNA revelou-se o marcador mais promissor para a deteção específica de leite bovino, sendo utilizado com sucesso no desenvolvimento de vários modelos de calibração normalizados, com sensibilidades entre 50-100 mg/kg de proteínas do leite em fiambres e salsichas autoclavadas. A preparação de misturas modelo de fiambre e salsichas submetidas a cozedura no forno e autoclavagem, respetivamente, permitiu avaliar o efeito destes processamentos alimentares no desempenho dos métodos de PCR em tempo real, tendo sido posteriormente aplicados com sucesso em amostras comerciais. Da mesma forma, para a deteção de tremço, diferentes misturas modelo de pães preparados com farinhas de trigo ou arroz e submetidas a um processo de cozimento permitiram avaliar o efeito da matriz alimentar e do processamento térmico nos métodos de PCR em tempo real desenvolvidos. Sensibilidades de 1 pg de DNA de *Lupinus albus* e de 5 mg/kg, 100 mg/kg e 500 mg/kg de concentrado proteico de tremço em farinha de arroz, farinha de trigo e pão, respetivamente, foram obtidas pelo método ΔC_t , com um claro efeito negativo no desempenho da PCR causado pela matriz alimentar e pelo processamento.

Dado os ingredientes lácteos serem frequentemente encontrados em produtos cárneos processados, a avaliação da autoclavagem (usada no fabrico de salsichas) na imunorreatividade das proteínas do leite seguida da digestão gastro-duodenal *in vitro* foi realizada por immunoblotting com soro de pacientes alérgicos e por análise de MS. Os resultados mostraram um claro efeito da autoclavagem nas propriedades estruturais e imunogénicas das proteínas do soro de leite com a sua completa degradação após digestão. No entanto, as caseínas revelaram ser mais resistentes através da produção de vários péptidos após a digestão (21.3-25.1 KkDa), sendo a sua imunorreatividade ligeiramente afetada pela autoclavagem, mas completamente eliminada depois da digestão. Os efeitos combinados do processamento térmico e da matriz alimentar também foram avaliados na imunorreatividade das proteínas de tremço e soja utilizadas como ingredientes tecnológicos em produtos de panificação e cárneos, respetivamente. Geralmente, a imunorreatividade das proteínas alvo (gama-conglutinas de tremço e inibidor de tripsina da soja) foi reduzida por todos os tratamentos térmicos testados, embora em maior grau após autoclavagem, sendo ligeiramente afetada pela matriz alimentar. Adicionalmente, o método imunoquímico permitiu a identificação de proteínas alvo em produtos comerciais.

Resumidamente, neste trabalho foram desenvolvidas técnicas robustas e eficazes para deteção de ingredientes tecnológicos do leite e do tremço em alimentos processados. Considerando a aplicabilidade demonstrada, as ferramentas propostas podem ser consideradas de elevada utilidade para a indústria alimentar na gestão de alergénios. O presente trabalho, também contribuiu com resultados interessantes na redução da

imunorreatividade do leite e tremço após aplicação de alguns métodos de processamento convencionais. Estes resultados podem contribuir para melhorar e aumentar o conhecimento sobre o efeito do processamento de alimentos na imunogenicidade do leite e do tremço, tendo como objetivo o desenvolvimento de fórmulas hipoalergénicas, essenciais para a qualidade de vida de indivíduos sensibilizados/alérgicos, apesar de ser essencial realizar estudos mais aprofundados.

Palavras-chave: alergias alimentares, PCR em tempo real, imunorreatividade, digestão gastro-duodenal simulada, processamento alimentar, análise por MS.

List of publications and communications

(Within the scope of this PhD)

Publications in international peer-review Journals

1. Bovine Milk Allergens: A Comprehensive Review.
Caterina Villa, Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra.
Comprehensive Reviews in Food Science and Food Safety, **2018**, 17(1): 137-164.
DOI:10.1111/1541-4337.12318.
2. Effect of food matrix and thermal processing on the performance of a normalised quantitative real-time PCR approach for lupine (*Lupinus albus*) detection as a potential food allergen.
Caterina Villa, Cristina Gondar, Joana Costa, M. Beatriz P. P. Oliveira, Isabel Mafra.
Food Chemistry, **2018**, 262: 251-259.
DOI: 10.1016/j.foodchem.2018.04.079
3. Development of DNA-based methods for the identification of pistachio nut in foods.
Isa Silva, Joana Costa, Caterina Villa, M. Beatriz P. P. Oliveira, Isabel Mafra.
Annals of Medicine, **2018**, 50(sup 1): S114.
DOI: 10.1080/07853890.2018.1427445.
4. Detection and quantification of milk ingredients as hidden allergens in meat products by a novel specific real-time PCR method.
Caterina Villa, Joana Costa, Isabel Mafra.
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DOI: doi.org/10.3390/biom9120804
5. Cow's milk allergens: screening gene markers for the detection of milk ingredients in complex meat products.
Caterina Villa, Joana Costa, Isabel Mafra.
Food Control, **2020**, 108: 106823.
DOI: 10.1016/j.foodcont.2019.106928
6. Immunoreactivity of lupine and soybean allergens in foods as affected by thermal processing.
Caterina Villa, Mónica B. M. V. Moura, Joana Costa, Isabel Mafra.

Foods, **2020**, 9: 254

DOI: 10.3390/foods9030254

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Ricardo N. Pereira, Joana Costa, Rui M. Rodrigues, Caterina Villa, Luís Machado, Isabel Mafra, António Vicente.

Food and Function, **2020**, 11: 4002-4013

DOI: 10.1039/C9FO02834J

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Caterina Villa, Joana Costa, Isabel Mafra.

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DOI: 10.1111/1541-4337.12646

9. Are physicochemical properties shaping the allergenic potency of plant allergens?

Joana Costa, Simona Lucia Bavaro, Sara Benedé, Araceli Diaz, Cristina Bueno-Diaz, Eva Gelencser, Julia Klueber, Colette Larré, Daniel Lozano-Ojalvo, Roberta Lupi, Isabel Mafra, Gabriel Mazzucchelli, Elena Molina, Linda Monaci, Laura Martín-Pedraza, Cristian Piras, Pedro M. Rodrigues, Paola Roncada, Denise Schrama, Tanja Cirkovic-Velickovic, Kitty Verhoeckx, Caterina Villa, Annette Kuehn, Karin Hoffmann-Sommergruber, Thomas Holzhauser.

Clinical Reviews in Allergy and Immunology, **2020**.

DOI: 10.1007/s12016-020-08810-9

10. Effect of autoclaving and *in vitro* gastroduodenal digestion on the modulation of IgE binding capacity of milk proteins incurred in sausage model food.

Caterina Villa, Simona Bavaro, Elisabetta de Angelis, Rosa Pilolli, Joana Costa, Simona Barni, Elio Novembre, Isabel Mafra, Linda Monaci.

Nutrients (submitted).

11. Are physicochemical properties shaping the allergenic potency of animal allergens?

Joana Costa, Caterina Villa, Kitty Verhoeckx, Tanja Cirkovic-Velickovic, Denise Schrama, Paola Roncada, Pedro M. Rodrigues, Cristian Piras, Laura Martín-Pedraza, Linda Monaci, Elena Molina, Gabriel Mazzucchelli, Isabel Mafra, Roberta Lupi, Daniel Lozano-Ojalvo, Colette Larré, Julia Klueber, Eva Gelencser, Cristina Bueno-Diaz, Araceli Diaz-Perales, Sara Benedé, Simona Lucia Bavaro, Annette Kuehn, Karin Hoffmann-Sommergruber, Thomas Holzhauser.

Clinical Reviews in Allergy and Immunology (submitted).

Book chapters

1. Advances in food allergen analysis.
Joana Costa, Telmo J. R. Fernandes, Caterina Villa, M. Beatriz P. P. Oliveira, Isabel Mafra.
In U.G. Spizzirri, G. Cirillo (Eds.) *Food Safety: Innovative Analytical Tools for Safety Assessment*, **2017**, chapter 9, pp. 305-360, John Wiley and Sons and Scrivener Publishing LLC, Beverly, MA.
ISBN: 9781119160557.
2. Peanut allergy: clinical relevance and allergen characterisation.
Joana Costa, Caterina Villa, Telmo J. R. Fernandes, M. Beatriz P. P. Oliveira, Isabel Mafra.
In A.A. Rahman (Ed.) *Food Allergy - Methods of detection and clinical studies*, **2017**, chapter 3, pp. 35-56, CRC Press/ Taylor & Francis Group.
ISBN: 9781498743570.
3. Cow's milk allergens (accepted).
Caterina Villa, Joana Costa, Isabel Mafra.
In F Toldrá and L Nollet (Eds.) *Handbook of Dairy Foods Analysis* 2nd ed., Boca Raton, CRC Press.

Awards

- Travel grant for the participation in the Training School – “The use of Proteomics and Mass Spectrometry analysis to improve allergenicity risk assessment strategies” in November 2017, Liège, Belgium, attributed by the COST Action FA1402 – ImpARAS – “Improving Allergy Risk Assessment Strategy for New Food Proteins”.
- 3rd Prize Flash Presentation attributed in the 3rd International ImpARAS conference, 10th-12th October 2017, Helsingør, Denmark.
- Travel grant for the participation in the Training School – “Animal models of food allergy” in June 2018, Vienna, Austria, attributed by the COST Action FA1402 – ImpARAS – “Improving Allergy Risk Assessment Strategy for New Food Proteins”.
- Travel grant for the participation in a Short-Term Scientific Mission (STSM) at Institute of Sciences of Food Production (ISPA), Bari, Italy, entitled: “Effect of processing and simulated in vitro digestion on the immunoreactivity of milk protein concentrates used as technological aids in meat products” from 1st of July to 1st of August 2018.

- FOODS Travel award attributed by FOODS journal for the participation in the 9th International Symposium on Recent Advances in Food Analysis in November 2019, Prague, Czech Republic and for a peer-reviewed free of charge publication in FOODS journal.

Oral communications in Scientific Meetings

1. *In silico* and experimental analysis of allergen encoding genes as potential DNA markers for pistachio identification in foods.
Isa Silva, Caterina Villa, Joana Costa, Maria B. P. P. Oliveira, Isabel Mafra.
10th Meeting of Young Researchers of University of Porto (IJUP 2017), book of abstracts, p. 12429.
8-10 February 2017, Porto, Portugal.
2. Lupine allergens in food products: a new real-time PCR approach to its detection and quantification.
Caterina Villa, Joana Costa, Cristina Gondar, Maria B. P. P. Oliveira, Isabel Mafra.
3rd ImpARAS conference, book of abstracts, p. 61.
10-12 October 2017, Helsingør, Denmark.
3. Milk proteins in food products as affected by thermal processing assessed by immunochemical and DNA-based methods.
Caterina Villa, Joana Costa, Maria B. P. P. Oliveira, Isabel Mafra.
4th ImpARAS conference, book of abstracts, p.56.
19-21 June 2018, Naples, Italy.
4. Profile of soybean allergenic proteins as affected by thermal processing and food matrix.
Mónica Moura, Joana Costa, Caterina Villa, Maria B. P. P. Oliveira, Isabel Mafra.
12th Meeting of Young Researchers of University of Porto (IJUP 2019), book of abstracts, p. 15223.
13-15 February 2019, Porto, Portugal.
5. Assessment of electrical effects of ohmic heating on structural and immunoreactivity properties of bovine beta-lactoglobulin.
Ricardo Pereira, Joana Costa, Rui Rodrigues, Caterina Villa, Jorge Teixeira, Isabel Mafra, António Vicente.
8th International Symposium on "Delivery of Functionality in Complex Food Systems", book of abstracts, p. 42.

7-10 July 2019, Porto, Portugal.

6. Effect of thermal processing and food matrix in the immunoreactivity of soybean and lupine proteins.

Caterina Villa, Mónica Moura, Joana Costa, Isabel Mafra.

13th Meeting of Young Researchers of University of Porto (IJUP 2020), book of abstracts, p. 15223.

12-14 February 2020, Porto, Portugal.

7. Multicopy versus unicopy genes as potential molecular markers for the detection of sesame as an allergenic food.

Daniela Mendes, Joana Costa, Caterina Villa, Liliana Grazina, Isabel Mafra.

13th Meeting of Young Researchers of University of Porto (IJUP 2020), book of abstracts, p. 15223.

12-14 February 2020, Porto, Portugal.

Poster communications in Scientific Meetings

1. Selection of DNA markers for the identification of Anacardiaceae family members (*Anacardium occidentale* and *Pistacia vera*).

Joana Costa, Isa Silva, Cintia Mendes, Caterina Villa, Maria B. P. P. Oliveira, Isabel Mafra.

Food Integrity 2017 Conference, book of abstracts, p. 194.

10-11 May 2017, Parma, Italy.

2. A new quantitative real-time PCR approach for the detection of lupine allergens in food products.

Caterina Villa, Joana Costa, Cristina Gondar, Maria B. P. P. Oliveira, Isabel Mafra.

Food Integrity 2017 Conference, book of abstracts, p. 145.

10-11 May 2017, Parma, Italy.

3. Development of DNA-based methods for the identification of pistachio nut in foods.

Isa Silva, Joana Costa, Caterina Villa, Maria B. P. P. Oliveira, Isabel Mafra.

2nd International Congress of CiiEM, Annals of Medicine, 50 (suppl 1), S114

11-13 June 2017, Caparica, Portugal.

4. Selection of mitochondrial DNA markers for cow's milk allergen detection.

Caterina Villa, Joana Costa, Maria B. P. P. Oliveira, Isabel Mafra.

14^o Encontro de Química dos Alimentos, book of abstracts, p. 161.

6-9 November 2018, Viana do Castelo, Portugal.

List of Publications and Communications

5. Mitochondrial gene screening to identify cow's DNA markers for meat authentication and milk allergen detection.
Caterina Villa, Joana Costa, Maria B. P. P. Oliveira, Isabel Mafra.
Food Integrity 2018 Conference, book of abstracts, p. 208.
14-15 November 2018, Nantes, France.
6. Evaluation of the thermal processing and food matrix on the soybean allergenic proteins.
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XXIV Encontro Luso-Galego de Química, book of abstracts, p. 198.
21-23 November 2018, Porto, Portugal.
7. Milk allergen detection in food products by DNA-based methods: exploiting mitochondrial and nuclear gene markers from cow.
Caterina Villa, Joana Costa, Maria B. P. P. Oliveira, Isabel Mafra.
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1-3 April 2019, Amsterdam, Netherlands.
8. Mitochondrial vs allergen-encoding genes for milk detection in processed foods.
Caterina Villa, Joana Costa, Maria B. P. P. Oliveira, Isabel Mafra.
EAACI congress 2019, poster discussion session, PD0554.
1-5 June 2019, Lisbon, Portugal.
9. Lupine allergens in food products: effect of processing and food matrix on their detection and immunoreactivity.
Caterina Villa, Joana Costa, Isabel Mafra.
9th International Symposium on Recent Advances in Food Analysis (RAFA), book of abstracts, p. 229.
5-8 November 2019, Prague, Czech Republic.

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CHAPTER 1. General introduction

1.1. Food allergy

Food allergy is a substantial public health problem in industrialised/westernised countries, which has been drastically increasing in the last 2 to 3 decades, affecting more commonly children than adults. Today, Australia has the highest prevalence of IgE-mediated food allergy (10% of infants), followed by Europe and United States with a prevalence around 1-5% [1]. Food allergies are typically IgE-mediated reactions, but they can also be triggered by non-IgE (eosinophilic oesophagitis, allergic proctocolitis and food protein-induced enterocolitis) or mixed IgE cellular-mediated [2]. In IgE-mediated allergy, food allergens – minute fractions of total proteins to which humans are exposed – are responsible for the production of specific IgE and the occurrence of adverse reactions in sensitised/allergic individuals. More than 170 food proteins have been described as allergens, being peanut, tree nuts, fish, shellfish, egg, milk, wheat and soybean responsible for 90% of food allergy cases [2, 3]. The first stage of an allergy, called sensitisation, occurs after the ingestion of the offending food, whereas several events are triggered ultimately priming the B-cells to produce specific IgE, which will posteriorly recognise the target allergen(s). After a second exposure, an allergic reaction can arise by the activation of mast cells and the release of substances (histamine, prostaglandins and cytokines, among others), being responsible for the elicitation of clinical symptoms. According to the degree of exposure, clinical symptoms can vary in intensity, from disturbances to the skin, respiratory and gastrointestinal tract, as well as cardiovascular disorders, to more severe cases known as anaphylaxis [4]. A schematic representation of the development of an allergic reaction is showed in Figure 1.

Food allergens can be classified as Class I when the triggered symptoms are caused by a secondary contact, after a primary sensitisation with the same ingested allergen. In Class II food allergens, the symptoms are elicited by primary sensitisation to inhalant allergens and subsequent IgE cross-reaction to homologous proteins in food. The majority of food allergens are assigned to a small number of protein families/superfamilies [5], such as prolamins that include cereal prolamins (glutenins and gliadins), the non-specific lipid transfer proteins (nsLTP) and the 2S albumins. Cupins encompass important families of allergens, namely 7/8S globulins (vicilins) and the 11S globulins (legumins), which are seed-storage proteins. Profilins and pathogenesis-related (PR)-10 proteins (Bet v 1-like proteins, known as birch pollen-related food allergens) are other two important families of allergenic proteins, mainly related to the defence mechanisms of plants. In animals, the most common food allergens are tropomyosins and parvalbumins from shellfish and fish, respectively, and the alpha/beta caseins from cow's, sheep's and goat's milk [6].

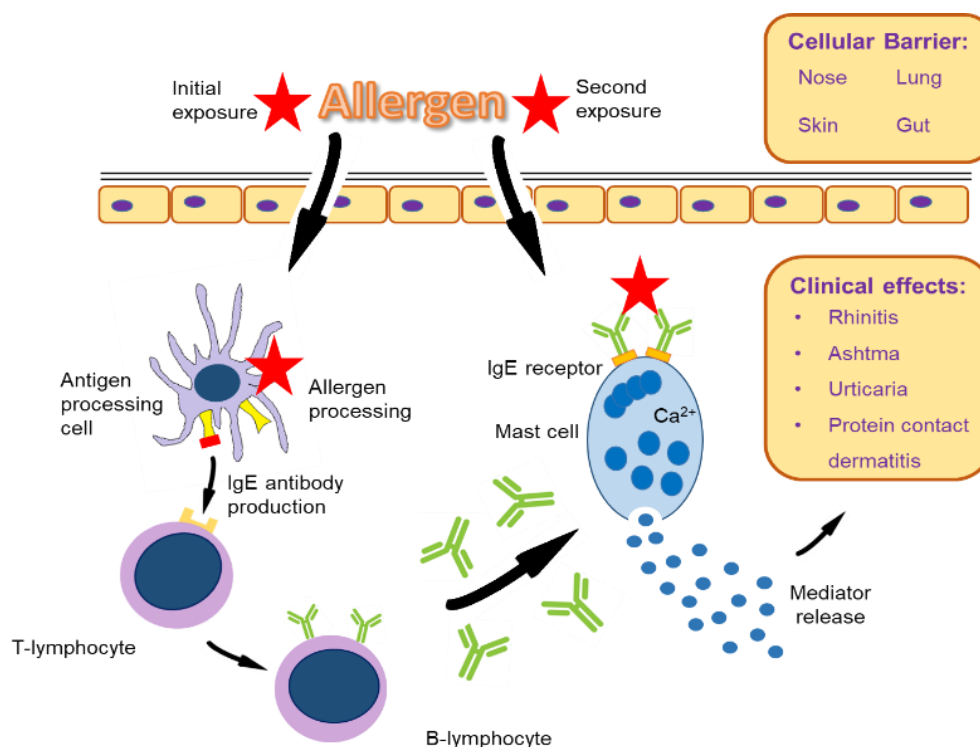


Figure 1. Schematic representation of an IgE-mediated allergic reaction. Adapted from [7].

The diagnosis of IgE-mediated food allergies is based on the definition of a clear clinical history and supported by different *in vivo* and *in vitro* tests. The latter include skin prick tests (SPT), which provide rapid methods to screen patients for the presence of food specific IgE bound to cutaneous mast cells. The quantification of food-specific and food-component protein-specific IgE levels in patient sera, such as ImmunoCAP (ThermoFisher), Immulite (Siemens AG) and HYTEC-288 (Hycor), are also commonly used [1]. However, they are not truly predictive of allergy since they are merely considered as indicators of sensitisation [2]. The double-blind placebo-controlled food challenge (DBPCFC), an *in vivo* test, is considered the gold standard for allergy diagnosis. This food challenge test is similar to a clinical trial, in which increasing quantities of the offending food are administrated to the patient in a matrix indistinguishable from the placebo, until objective symptoms are developed or the full challenge ingestion dose is achieved [1].

The strict avoidance of the known or suspected food(s) that causes the allergic reactions is the only effective ‘therapy’ for food allergy. However, this does not avoid the unintended exposure to food allergens, with main consequences to individuals allergic to multiple foods, particularly plant foods (high probability for cross-reactivity among different vegetables and pollen) [2]. Self-administered injectable treatments, such as adrenaline (epinephrine), must be carried by patients at risk of developing severe and systemic allergic reactions (e.g. anaphylaxis) [8]. Emerging therapies, including modified proteins and peptides, DNA

vaccines and food allergen immunotherapies, are now being developed as promising strategies to treat food allergy. Food allergen immunotherapies aim at creating a desensitisation state (or sustained unresponsiveness) in patients, through the daily exposure to increasing doses of food allergens, thus reducing or avoiding the life-threatening risk associated with accidental ingestion and improving their quality of life [2, 9]. Food allergen immunotherapies are currently under clinical trials, but with the recent approval by the Food and Drug Administration (FDA) of the palforzia (AR101) for peanut allergy oral immunotherapy [10], novel allergen immunotherapies are expected in the near future. Still, food-allergic patients are most likely to carry on with permanent maintenance doses of the allergenic food in order to preserve some unresponsiveness towards accidental exposures of the culprit food [9]. However, it is also important to refer that a major obstacle for the wide implementation of immunotherapies regards the unpredictability of the level of desensitisation, once the immunologic changes are often transient. The absence of biomarkers to distinguish a temporary desensitisation state from a permanent tolerance (as in natural nonallergic individuals) is also a major drawback in defining immunotherapies as effective food allergy treatments [9, 11].

1.2. Technological aids as hidden allergens in food products

Food industry commonly uses ingredients from plant and animal sources in order to improve some technological characteristics of processed foods. For example, meat products, besides fat and water, also contain a wide range of other ingredients, including salt, phosphates, vegetable proteins (wheat, soybean and lupine), animal proteins (milk, egg white and blood plasma), hydrocolloids, fibre, transglutaminase and spices. These substances are normally added to foods to improve their technological properties (condensation, gelation, emulsification, etc), nutritional value or flavour. However, they can also be added to reduce production costs, or unintentionally, when manufacturers fail to comply with the procedures of good manufacturing practices [12]. The bakery industry uses flours from alternative sources, such as some legumes (e.g. soybean and lupine), in order to increase the protein content, protein nutritional quality and dietary fibre content of wheat-based foods, like bread, muffins, cookies and brownies, instant noodles and pasta, and in gluten-free foods [13-15]. Unfortunately, some of these ingredients are allergenic foods (e.g. soybean, wheat, lupine), which can represent a health risk if ingested by allergic/sensitised individuals.

Milk proteins are included into the group of products that are most often responsible for the occurrence of allergic reactions, but they are also frequently added to a wide variety of food products as technological aids. Enriched milk proteins, namely milk protein

concentrates (40 to 90% of total protein) and milk protein isolates (>90% of total protein), are typical dairy ingredients used in the production of meat products in order to improve their juiciness and texture, increase their heat stability, solubility and gel-forming capacity, influencing the flavour profile of the finished product [12, 16]. These concentrated forms of milk proteins contain caseins and whey proteins in the same proportions as the whole milk (80% and 20%, respectively) [17]. According to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) list of allergens, the major allergenic milk components are caseins (Bos d 8), alpha-lactalbumin (Bos d 4), beta-lactoglobulin (Bos d 5) and bovine serum albumin (Bos d 6), which are responsible for the occurrence of allergic reactions in approximately 50% of allergic patients to milk [18]

As previously referred, legumes are other important group used as ingredients by the food industry, particularly in bakery and meat products, which can cause allergic reactions. Soybean, belonging to the Fabaceae family, is one of the eight most important allergenic foods, being a source of “hidden allergens” when undeclared in the final packed foods. Soybean protein isolates and concentrates and soybean hydrolysates are ingredients that can be often used in the production of infant formulas, milk replacers and creamers, bakery products and pasta, low (beverages) and high (sauces, mayonnaise, desserts) viscosity products and confectionary [14]. In meat products, soybean proteins stabilise the ingredients of meat, contributing to a better binding of water and fat in product due to their emulsifying properties. Additionally, the gelling capacity leads to an improvement of the consistency and structure of finished products. Besides the cost reduction and quality improvements, these technological aids also allow increasing the consumers’ options for new dietary meat products with enhanced content of total protein [14].

Similarly, lupine can be used as a technological aid in a wide variety of products, but it is also considered an allergenic food. Lupine has a high nutritional value, with high contents of protein and dietary fibre, being regarded as a suitable substitute for cow’s milk and wheat in individuals with milk intolerance, milk allergy, wheat allergy or celiac disease because it does not contain lactose or gluten [19, 20]. Lupine can be utilized to fortify the protein content of pasta, biscuits, bread, sausages and hamburgers. In addition, lupine flours or lupine protein isolates/concentrates have been used in the formulation of baked, meat and dairy products due to their important technological properties (great emulsifying and foaming capacities, and high water binding ability) [13, 21]. Despite the important nutritional and technological values of legumes, they contain several allergenic proteins, mostly belonging to the storage protein families (including two different super families: cupins and prolamins), which are resistant to proteases and heat. Profilins and PR proteins are other relevant allergens found in legumes, the latter stable in acidic conditions and resistant to proteolytic degradation [15, 22].

1.3. How does food processing affect food allergens?

Foods can be subjected to different processing treatments to improve functional, nutritional and sensory attributes, as well as for detoxification and preservation. Thermal treatments, high pressure, high intensity ultrasound, radiation and biotechnological approaches are some of the conventional and novel processing technologies currently applied to foods (Figure 2) [23]. Such treatments may alter the biochemical characteristics of proteins, inducing structural modifications such as unfolding and aggregation, loss of secondary and tertiary structures, formation of intra and/or inter-molecular covalent and non-covalent interactions or generate chemical reactions within the food matrix components (oxidation or glycation – Maillard reactions) [23, 24]. Such modifications can alter the allergenic potential of proteins, depending on the applied treatment, processing conditions, time and environment, as well as on the type of food, among others [25]. Food ingredients used as technological aids and considered as allergens can, therefore, be subjected to all kinds of food processing, which can somehow affect their allergenic potential.

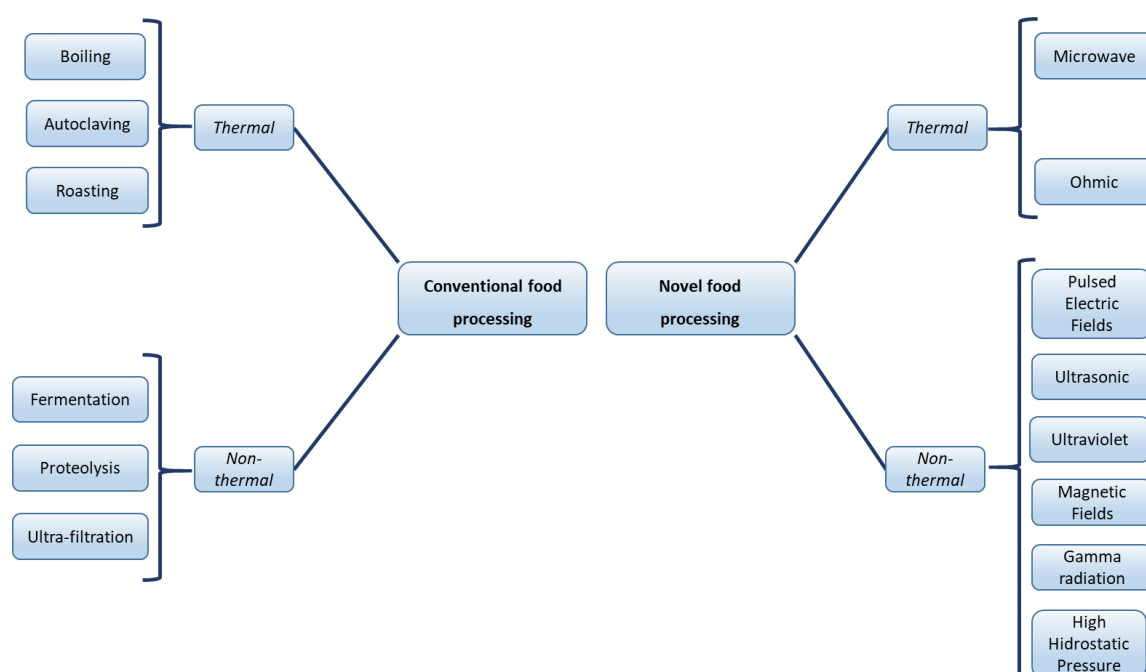


Figure 2. Examples of conventional and novel food processing methods used by food industry.

An allergic reaction relies on the binding of a small linear stretch of amino acids or a specific three-dimensional structure in a protein to IgE, known as linear or conformational epitopes, respectively. Depending on the type of processing, protein allergenicity can be directly influenced by the disruption of conformational or linear epitopes or by the formation of neoallergens [26]. Sequential (linear) epitopes can be affected by enzymatic or acidic hydrolysis and by extreme Maillard reactions, while conformational epitopes can be

exposed or hidden by unfolding or aggregation of proteins, respectively [25]. Conformational epitopes are associated to tertiary and secondary protein structures, being easily disrupted by harsh conditions (especially when involving high temperature or extreme pH) caused by food processing. For example, Bet v 1-homologue food allergens have been shown to be affected by thermal processing because of potentially losing IgE-binding conformational epitopes upon specific treatments. On the contrary, other allergens, such as those belonging to the prolamin superfamily (2S albumin and LTP) are characterised by their high resistance to denaturation and enzymatic degradation due to their conserved structure stabilised by disulphide bonds or by the presence of intra or inter molecular cystines (disulphide bridges) [23, 27]. Therefore, protein-induced modifications during food processing can lead, not only to the disruption of a certain epitope, but also to its alteration, masking, or unmasking it, with consequent increase or decrease of protein allergenicity [24]. Biochemical reactions that may occur during food processing among allergens and other components within the food matrix (proteins, sugars, fats, water, etc.) have also the potential to affect allergenicity. A common example is Maillard reaction, which is a non-enzymatic condensation of N ϵ -group of amino acid residues (lysine) in protein with the carbonyl group of reducing sugars to form glycosamine [23, 24]. Lastly, the physicochemical changes of proteins induced by processing can be further influenced by gastrointestinal digestibility, absorbance kinetics through mucosa, as well as their presentation to the immune system, consequently influencing their allergenicity [23].

Recently, many studies have been performed in order to understand the impact of food processing on allergenicity as strategies to create hypoallergenic formulas. Despite the total elimination of the allergenic potential of proteins is unlikely to occur by food processing, the minimisation of the elicitation threshold could be achieved with the application of specific conditions [26]. As an example, Lozano-Ojalvo et al. showed that high hydrostatic pressure produced, in minutes, a hypoallergenic hydrolysate of whey proteins, which retained sufficiently immunogenicity to stimulate Th2 responses, but not enough to generate specific IgE or IgG1 that could mediate systemic anaphylaxis [28]. Similarly, as suggested by Guillamón et al., lupine allergenicity could be decreased by the application of a novel processing technique called instantaneous controlled pressure drop (DIC) with a complete elimination of the IgE-binding capacity of lupine proteins after treatment with DIC at 6 bar for 3 min [29].

1.4. Strategies to evaluate the allergenicity

Understanding the potential allergenicity of proteins, naturally present or added during food transformation, and their changes upon application of several food processing

technologies is crucial to ensure public health protection. Several *in vitro* and *in vivo* models have been developed to address the factors and mechanisms involved in the sensitisation phenomena [30]. The methodologies used to characterise the potential immunogenicity of a protein are based on the classical immunochemical methods and, more recently, on mass spectrometry (MS). These approaches rely on protein chemistry and *in vitro* and *in silico* methods to characterise native and altered food proteins upon processing, which may explain why certain food proteins induce sensitisation of the immune system, while others are tolerated. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunochemical assays, namely western blot or enzyme-linked immunosorbent assay (ELISA) provide information on the molecular weight, stability to heat and the immunoreactivity/IgE-binding capacity of proteins by means of specific antibodies or sera from food-allergic patients. Digestibility tests may also be performed to assess the effect of gastrointestinal proteases on the IgE-binding capacity of proteins [31]. Proteomic methods employing MS allow investigating protein-protein interactions, yielding precise information on critical linear epitopes, their degree of sequence homology with known allergens, detecting compositional/structural modifications induced by food processing or profiling complex mixtures of proteins in food ingredients (relying on already existing libraries of protein sequences) [31].

Immunological methods based on *in vitro* (*ex vivo*) cell degranulation models (mediator release assays) are also important to understand the allergenicity of food proteins. Food allergy implicates the release of mediators, such as histamine, cytokines and interleukins, which are produced by the degranulation of basophils or mast cells after the crosslinking of IgE to the corresponding allergen. Mast cells, human basophils and rat basophilic leukemia cells (e.g. RBL-2H3) are *ex vivo* cell models that measure the release of therein inflammatory mediators when activated by the sera from food-allergic individuals, enabling their use as a quick methods to screen food allergenicity [32].

In vivo food allergy models are required to elucidate the mechanisms underlying food allergen sensitisation [30]. Animal models are useful means of avoiding life-threatening and ethical limitations of clinical studies. Mouse strains, namely BALB/c, C3H/HeJ, C57BL/6 and A/J, are the most commonly used animal models for food allergy because they present similar gastrointestinal systems to humans, producing human-like IgE and similar allergic reactions [32]. Mice models are submitted to several *in vivo* tests to assess the elicitation of an allergic response, measuring body temperature, ear swelling and observing physiological phenomena, such as diarrhoea and skin responses by SPT. *Ex vivo* tests correspond to the collection of blood, tissue or organs and their further analysis. These tests include the evaluation of immunoglobulins in serum by ELISA, followed by immunoblotting and mediator release assay: rat basophilic leukaemia (RBL) assay or basophil activation

test (BAT); phenotyping of T-cell populations by the isolation of immune cells from organs (mesenteric lymph nodes, spleen, lung, skin, or intestine) analysed by flow cytometry; and the evaluation of cytokine secretion by the *ex vivo* restimulation of serum or lymphatic tissue cells with corresponding allergenic proteins or peptides [30].

Ultimately, clinical studies are the last step for the assessment and diagnosis of food allergies. Current testing methods include SPT, serum specific IgE (sIgE) and DBPCFC already mentioned in “Food Allergy” section. Component-resolved diagnosis (CRD) is an additional clinical study, which identifies allergen components at the molecular level by means of extracting natural allergen components and recombinant techniques [33].

1.5. How to detect food allergens

Presently, the European Union rules the mandatory labelling of fourteen groups of foods and substances causing allergies or intolerances, whose presence must be stated and highlighted in the list of labelled ingredients of pre-packaged foods, regardless of their amount (Regulation (EU) No 1169/2011). These groups are represented in Figure 3 and include cereals containing gluten, peanuts, tree nuts, celery, mustard, eggs, milk, sesame, fish, crustaceans, molluscs, soybean, lupine and sulphites [34].

Allergic individuals must rely on the labelled information to strictly avoid all products that contain food allergens, as the only way to protect their health. Therefore, the assessment of labelling compliance is essential to control the presence of hidden allergenic ingredients in processed foods and prevent any accidental exposure. It is, thus, important the development of sensitive and specific methods able to detect the eventual presence of food allergens at trace levels [35]. These methods need to meet high analytical demands, including high sensitivity to detect allergens at trace amounts and high specificity to avoid false positive results. They also should be robust with regard to food matrix, food processing and range of biological variation of allergenic ingredients and components, allowing reliable quantitative analysis [36]. The determination of allergens in foods is a challenging analytical issue because, in most cases, food allergens are within complex mixtures of proteins with different physicochemical properties and relative abundance, varying in origin, variety, growing and harvesting conditions, transportation, storage and processing.

In particular, the chemical and structural changes of proteins due to food processing make the development of reliable analytical methods for allergen detection and quantification a challenging task [37]. Besides, there are only few reference materials commercially available for allergen analysis, which are still lacking certification, thus hampering method harmonisation, with no general agreement on the expression units of results. In this context, the preparation of incurred materials, deriving from the addition of

allergenic ingredients to blank raw materials, is extremely important, allowing a rigorous quantitative performance. The use of these materials allows simulating food formulations with the realistic incorporation of allergenic ingredients and their modifications upon the application of processing, providing calibrants to enable accurate quantitative analysis [37].



Figure 3. List of the 14 groups of substances and products that should be emphasised in the list of ingredients of pre-packaged foods as stated on the European Regulation (Regulation (EU) No 1169/2011) [34].

The success of a food allergen detection method relies on the selection of the target marker, which can be allergenic proteins or other proteins and species-specific DNA sequences or allergen-encoding genes [35, 36]. The protein-based methods are the most commonly applied for allergen detection due to their rapid and easy performance, good specificity, low-cost of analysis and commercial availability. They are based on the direct recognition of an antigen by the use of specific antibodies, being available in different formats that include lateral flow devices (LFD), dipstick tests, ELISA and immunoblotting. Their major drawbacks are the high potential for cross-reactivity with similar proteins from different sources and their liability to be affected by harsh food processing conditions [35].

Recently, the development of cutting-edge technologies like biosensors and MS platforms provide alternative analytical tools for allergen analysis. Biosensors can be based on the biological interaction antibody-antigen (immunosensors), but also on the hybridisation between two complementary strands, a probe and a DNA sequence (genosensors). Alternative biosensors based on the molecular imprinting process rely on the polymerisation of functional monomers in the presence of a target molecule as a template agent (MIP-sensor) [38]. Their simple, fast, reproducible, potential for low-cost and

multitarget detection, combined with their automation feasibility make them attractive tools in allergen analysis.

The advances on MS technology allow the protein/allergen analysis and characterisation without requiring biorecognition, thus eliminating problems associated with cross-reactivity phenomena occurring with immunoassays. MS-based methods are capable of quantifying several allergenic ingredients in complex food matrices with high levels of confidence and within a single chromatographic run (multitarget approach) [39]. However, the high costs of the MS platforms, their maintenance and the need for specialised personnel are main drawbacks for their high-throughput application in food allergen analysis [35]. Additionally, as for any other method, the analytical performance of MS approaches can be affected by the food matrix and the applied food processing.

Finally, polymerase chain reaction (PCR)-based methods have been recognised as excellent tools for allergen detection, being currently implemented as standard methods by some governmental food control laboratories in Germany and Japan [36]. The indirect detection of allergens by PCR assumes that a positive result for a DNA marker is directly correlated with the presence of the allergenic food [40]. One of the major advantages of PCR-based methods is related with their exceptional specificity that may even surpass the level of single amino acid specificity, allowing the differentiation of closely related phylogenetic species. Additionally, real-time PCR technique provides quantitative results and high sensitivities in the same range of ELISA and MS methods. The high thermal stability of DNA molecules as target markers makes PCR particularly useful in the analysis of highly processed foods [35, 36].

The lack of harmonisation regarding the most suitable methodology for allergen detection, as well as the absence of reference materials, contribute to a general controversy on the analytical methodologies and the management of food allergens. None of the referred techniques assembles all the advantages for the unequivocal identification and quantification of food allergens. Therefore, the choice of a technique must be carefully assessed based on the type of food to analyse and the results needed. In some cases, the use of more than one technique might be necessary. In this context, more research is still needed to support the allergen management by the food industry and to safeguard the health and the quality of life of sensitised/allergic individuals.

1.6. Objectives and organisation of the thesis

Food allergy has become a subject of great concern in developed countries with a rising prevalence trend of sensitised/allergic individuals. Unfortunately, even after the implementation of an elimination diet to avoid severe allergic reactions, patients are still at

risk of unintended ingestion of the offending food. In order to protect their health, several regulations have been implemented by the EU concerning the labelling of processed foods containing allergenic proteins. However, the common excessive precautionary labelling restricts the choice of products that sensitised/allergic consumers can acquire, while the lack of information on the presence of hidden allergens due to mislabelling or cross-contamination during the production can pose health risks. This fact demands the enforcement of highly sensitive and specific techniques to detect food allergens at trace amounts, contributing to improve food allergen management. In the last years, many efforts have been made to produce hypoallergenic formulas based on the development of technological strategies to mitigate the allergenic potential of foods. Some food processing technologies have shown to have a high impact on the physicochemical properties of allergens, leading, in some cases, to a reduction of their allergenicity. However, more studies must be conducted to better understand the impact of processing on allergen structure, in parallel with the resultant alterations from gastrointestinal digestion in the final allergenicity. In this context, the main objectives of this thesis were: i) the development of highly sensitive and specific methods for the detection of allergenic foods; and (ii) to increase the knowledge on the effects of processing, food matrix and gastro-duodenal digestion on the structural features of food allergens. For this purpose, several specific goals were proposed:

- The development of new molecular technologies for the detection and quantification of milk and other milk protein substitutes, namely lupine, in food products by real-time PCR strategies;
- The evaluation of the effects of processing and food matrix on the immunoreactivity of lupine and soybean allergens by immunochemical assays (immunoblotting);
- The assessment of the effect of autoclaving and simulated gastrointestinal digestion on the IgE-binding capacity of milk protein concentrates (MPC) used as technological aids in meat products by immunoblotting and mass spectrometry (LC-MS/MS).

Other specific goals were:

- Selecting and preparing appropriate model mixtures (cooked-hams, sausages, breads) to be applied in the development of the analytical methods;
- Exploiting the best molecular markers (mitochondrial vs allergen-encoding genes) for the specific detection of bovine milk in meat products;
- Evaluating the use of a normalised calibration curve in the improvement of lupine detection and quantification by real-time PCR;
- Assessing the effect of processing (autoclaving, oven-cooking and baking) and food matrix on the performance of real-time PCR for the detection of milk and lupine in food products.

The developed work was carried out mostly on milk and lupine as case studies, with an additional and brief reference to soybean, mainly because of its common use as technological aids in a wide variety of processed foods and, as a consequence, frequently present as a “hidden allergen”.

This thesis was divided in four chapters: a general introduction in chapter 1; the research studies on milk allergens in chapter 2; the research studies on lupine allergens in chapter 3; and a final global discussion and conclusions in chapter 4. An overall definition of food allergy was presented in the introduction (chapter 1), followed by the use of some technological aids in food products, considered as “hidden allergens” and how conventional and novel food processing technologies can affect the allergenicity of these ingredients. Several strategies used in the evaluation of the allergenicity of foods and the analytical tools applied to food allergen detection were also focused. Chapter 2 was dedicated to the study of bovine milk allergens, including a state-of-the-art section, followed by an experimental part encompassing three research papers. The state-of-art section consists of an extensive review paper, addressing the most relevant aspects regarding cow’s milk allergy and respective allergens. The experimental part contains two research papers about the selection of the most appropriate molecular markers (mitochondrial vs allergen-encoding genes) and subsequent development of a highly sensitive and specific real-time PCR method for the detection of MPC in meat products, followed by a third research paper studying the effect of autoclaving and simulated gastro-duodenal digestion on the IgE-binding capacity of these ingredients added to meat products. Chapter 3, concerning lupine allergens, contains a review paper about lupine as an allergenic food and an experimental part composed of two research papers. Both research papers address the effects of food matrix and thermal processing, with the first being focused on the development of a quantitative real-time PCR method to detect lupine allergens in bakery products and the second one on the immunoreactivity of lupine and soybean allergens in bakery and meat products, as affected by moderate and harsh thermal processing conditions. The last section (chapter 4) presents the overall discussion of the results, referring the final remarks on the work, the main achievements and conclusions.

The research work was mostly performed at the Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, with a small part being carried out at the Institute of Sciences of Food Production, National Research Council (ISPA-CNR), Bari, Italy.

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CHAPTER 2. Milk allergens

State-of-the-art

Bovine Milk Allergens: A Comprehensive Review
Comprehensive Reviews in Food Science and Food Safety, **2018**, 17, 137-164

Experimental part

Cow's milk allergens: Screening gene markers for the detection
of milk ingredient in complex meat products
Food Control, **2020**, 108, 106823

Detection and quantification of milk ingredients as hidden allergens in
meat products by a novel specific Real-time PCR method
Biomolecules, **2019**, 9, 204

Effect of autoclaving and *in vitro* gastro-duodenal digestion on the modulation of
IgE binding capacity of milk proteins incurred in sausage model food
Nutrients (submitted)

2.1. State-of-the-art

Bovine Milk Allergens: A Comprehensive Review
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2.1.1. Bovine Milk Allergens: A Comprehensive Review

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ABSTRACT

Cow milk allergy is one of the most common food allergies in early childhood and often persists through adult life, forcing an individual to a complete elimination diet. Milk proteins are present in uncounted food products, such as cheese, yogurt or bakery item, exposing allergic persons to a constant threat. Many efforts have been made to overcome this global problem and to improve the life quality of allergic individuals. First, proper and reliable food labeling is fundamental for consumers, but the verification of its compliance is also needed, which should rely on accurate and sensitive analytical methods to detect milk allergens in processed foods. At the same time, strategies to reduce milk allergenicity, such as immunotherapy or the use of food processing techniques to modify allergen structure have to be extensively studied. Recent research findings on the applicability of food processing, such as heat treatment, fermentation, or high pressure, have revealed great potential in reducing milk allergenicity. In this review, significant research advances on cow milk allergy are explored, focusing on prevalence, diagnosis, and therapy. Molecular characterization of cow milk allergens and cross-reactivity with other non-bovine milk species are described, as well as the effects of processing, food matrix, and digestibility on milk allergenicity. Additionally, analytical methods for the detection of milk allergens in food are described, from immunoassays and mass spectrometry methods for protein analysis to real-time polymerase chain reaction for DNA analysis.

Keywords: Allergen, milk proteins, food processing, cross-reactivity, detection.

INTRODUCTION

The use of milk from domesticated mammalian animals in the human diet has a very long tradition. Today, cattle, buffaloes, sheep, goats, and camels are used in various parts of the world for the production of milk and milk products for human consumption (Goff 2016). Accordingly, about 82.4% and 13.6% of the world's fresh milk comes from cows and buffaloes, respectively, while the remaining 4.0% is produced by goats, sheep, and camels. In the last 3 decades, the world's milk production has grown by more than 63%, increasing from 500 million tons in 1983 to 819.3 million tons in 2016 (FAO 2017). Asia contributed with almost 41% to global milk production in 2016, with India as its leading producer (19.6%). Both American and European continents assure almost 50% of the world's milk production, with USA (11.8%) and Germany (4.1%) occupying the top positions, respectively. In terms of total trade of milk (share of production), only 8.7% is destined for import/export (FAO 2017). Per capita milk consumption is rather high (>150 kg/capita/year) in most developed countries (like USA, Finland, Netherlands, United Kingdom, Sweden), while in developing ones (such as Angola, Haiti, Guinea, Mozambique, Malawi, Liberia), the intake of milk and milk products is often less than 30 kg/capita/year. The average consumption of milk and dairy products in Europe is more than 218 kg/capita/year, corresponding to 8-9% of dietary energy, 19% of dietary protein and 11-14% of dietary fat intake (FAOSTAT 2017).

As a source of vitamins and minerals (calcium, vitamin A, and vitamin B6), which are needed for the growth and development of young children, and beneficial for human bones, hair, skin, and teeth, milk is among the first foods to be introduced into an infant's diet (Do and others 2016). However, it is also one of the first and most common causes of food allergy in early childhood (Hochwallner and others 2014). Food allergy is defined as an adverse reaction mediated by the immune system and caused by the intake of some kind of food that occurs reproducibly in allergic individuals (Boyce and others 2010). Almost 90% of worldwide reported food allergies are caused by 8 groups of food products, in which milk is included (CODEX STAN 1-1985). Milk allergy is typically mediated by immunoglobulin E (IgE), inducing adverse reactions to proteins present in milk that might occur following their ingestion by sensitized/allergic individuals. Different food products might be responsible for this type of allergy since milk proteins are used as processing aids, with the possibility of being present at trace amounts in a large number of food products (Monaci and others 2006). Moreover, infant formulas, milk powders, cheeses, and yogurts are among the food products that use milk as an ingredient for their production (Fox 2001). Another important fact is that milk of all ruminant species (such as, cow, sheep and goat) contains homologous proteins, which share the same structural, functional, and biological properties and, thus

contribute to the cross-reactivity phenomenon in allergic individuals (Monaci and others 2006).

Sometimes milk allergy is confused with milk intolerance, which is much more common and produces clinical symptoms very similar to those of milk allergy, but not so dangerous. Contrarily to milk allergy, milk intolerance is a non-immunological reaction to a certain milk component, causing disorders in digestion, absorption, or metabolism. A common example is the malabsorption of lactose due to an intestinal lactase deficiency, thus being classified as a metabolic disease.

In true milk allergy, typical IgE-associated symptoms appear immediately or within 2 hours after the intake of milk. It can affect the skin (atopic dermatitis or eczema, angioedema or urticaria), the respiratory system (rhinitis, asthma exacerbation, wheezing, pulmonary infiltrates, or acute rhinoconjunctivitis), and the gastrointestinal tract (vomiting, recurrent diarrhea, abdominal pain, excessive colic, or esophageal reflux). In some cases, the allergic reaction may also involve one or more target organs/systems, leading to a complex and systemic anaphylactic response, and often resulting in death (El-Agamy 2007; Hochwallner and others 2014; Martorell-Aragonés and others 2015). However, delayed adverse immunological manifestations may also occur, normally after 2 hours of milk ingestion. In this case, non-IgE mediated mechanisms are typically involved, including a wide range of clinical presentations, such as mild rectal bleeding in milk protein induced proctocolitis or severe vomiting in food protein induced enterocolitis syndrome (Venter and others 2017). Accordingly, mild to moderate clinical symptoms in milk allergic individuals are commonly attributed to non-IgE mediated mechanism, while the severe adverse immunological responses are often IgE-mediated (Venter and others 2017). Milk allergy is the third most common food allergy that triggers anaphylactic reactions, just after peanut and tree nuts, accounting for 10-19% of all food-induced anaphylactic cases (Kattan and others 2011).

Currently, there is no treatment for milk allergy. Once diagnosed, the prevention of an allergic reaction relies mostly on the total avoidance of the offending food. Therefore, to guarantee consumer protection and ensure life quality to sensitized individuals, a correct and truthful food labelling system has become imperative (Costa and others 2012; Rencova and others 2013; Gomaa and Boye 2015b). Legal measures have been established and adopted by the majority of countries in the world to protect the life of those individuals (Gendel 2012; Taylor and Baumert 2015). In 1985, the *Codex Alimentarius* Commission issued, for the first time, a recommendation for the mandatory labelling of pre-packaged food susceptible of containing potentially allergenic ingredients. Following this recommendation, 8 allergenic foods (milk, tree nuts, peanuts, gluten-containing cereals, soybean, fish, eggs, and crustaceans) and sulfites were proposed as priority for labelling systems (CODEX STAN 1-1985). Within the European Union (EU), Directive 2003/89 /EC

added sesame, celery, and mustard to the previous items (CODEX STAN 1-1985), therefore totaling 12 product groups. Since then, the EU has established legislation extending the priority list to 14 groups (with the addition of mollusks and lupine) that are required to be emphasized over the rest of the ingredients enumerated in processed foods, regardless of their quantity (Directive 2007/68/EC; Regulation (EU) No 1169/2011).

Nevertheless, the total avoidance of milk consumption can cause a nutritional deficiency and may influence the growth of infants and children. As an attempt to overcome this problem, the development and optimization of new strategies of milk processing in order to destroy or modify the structure of these allergens and, therefore, reduce or eliminate their allergenicity have been widely investigated (Bu and others 2013).

This review intends to provide an overview on the prevalence of milk allergy, its diagnosis, and therapy, with focus on the molecular characterization of milk allergens and cross-reactivity phenomena between milks of different species in allergic patients. Additionally, it describes the available methods for the detection of milk allergens in foods containing milk and milk proteins, and the effect of processing in the reduction of their allergenicity.

PREVALENCE, DIAGNOSIS AND THERAPY

It has been reported that nowadays 0.6-3% of children below the age of 6 years, 0.3% of older children and teens, and less than 0.5% of adults suffer from cow milk allergy, the most common type of milk allergy. Interestingly, the majority of milk allergic infants outgrow their allergy becoming able to consume milk and its products, although 15% of the affected children remain allergic throughout adulthood. One study reports that 45-50% of children outgrow milk allergy at 1 year of age, 60-75% at the age of 2 years, and 85-90% at 3 years, but the mechanisms underlying the development of this natural tolerance are not yet fully understood (Fiocchi and others 2010; Bu and others 2013). The development of natural tolerance seems to be attributed to the decline of IgE due to avoidance of milk ingestion at early stages of life or to the presence of IgE against mainly conformational epitopes (enabling the consumption of milk and milk products), rather than against sequential epitopes (Hochwallner and others 2014).

The diagnosis of IgE-mediated milk allergy is described in detail in a recent article from the World Allergy Organization (WAO) (Fiocchi and others 2010). The diagnosis begins with observation of clinical manifestations and medical history, followed by *in vitro* and *in vivo* diagnostic tests and oral provocation tests (oral food challenge - OFC and double-blind placebo-controlled food challenge - DBPCFC). The *in vitro* diagnostic tests include the measurement of milk allergen-specific IgE in blood serum (ImmunoCAP, Phadia AB, Uppsala, Sweden). The skin prick test (SPT) is applied *in vivo* using a commercial milk

fraction or milk protein fractions that is pricked into the epidermis of a patient, resulting in the appearance of a wheal greater than the control if the patient has IgE against milk allergens. The oral provocation tests, such as DBPCFC or OFC are considered the “gold standard” methods for the correct diagnosis of food allergies. They consist in the oral administration, on different days, of placebo and progressively increasing quantities of milk until the appearance of observable (positive result) or subjective clinical symptoms upon second administration of the same amount of the offending food. If the oral provocation test is considered negative (absence of clinical symptoms), the patient is advised to gradually reintroduce milk in the daily diet following a specific scheme.

The current effective treatment for milk allergy is the adoption of an elimination diet (Mousallem and Burks 2012). However, accidental exposure to milk proteins is recurrent, principally because these allergens are present in a great number of processed foods, such as meat products, fish products, desserts, bakery products, among others. In this case, medical treatment includes oral antihistamine for mild cutaneous or digestive reactions and an epinephrine auto-injector for systemic or respiratory reactions (Hochwallner and others 2014; Martorell-Aragonés and others 2015). More recently, some strategies have been developed and applied to induce desensitization or even tolerance, to different allergens. Immunotherapy has been advanced as a promising treatment approach, aiming at achieving a permanent state of tolerance in sensitized individuals (Mousallem and Burks 2012). Based on different routes of administration (subcutaneous, epicutaneous, sublingual, and oral), the patient is exposed daily to increasing doses of the offending food, to induce immunomodulation and a desensitization state. Recent studies using immunotherapy have been proposed for milk allergy with promising results. Oral immunotherapy (OIT) shows a success rate that varied from 37% to 70% (Longo and others 2008; Skripak and others 2008; Narisety and others 2009; Brożek and others 2012).

MOLECULAR CHARACTERIZATION OF MILK ALLERGENS

In recent years, the great increase of allergen identifications and the knowledge about their sequences have permitted the establishment of databases providing molecular, biochemical, and clinical data of allergens. The official list of allergens issued by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee and the ALLERGOME databases are two of the numerous accessible sources (ALLERGEN 2017; ALLERGOME 2017). The milk allergens included in the official list (WHO/IUIS) were all identified as belonging to bovine milk (*Bos domesticus*).

Cow milk contains about 3 g of protein per 100 mL and includes at least 25 different proteins, all of which may act as antigens (Martorell-Aragonés and others 2015). Cow milk proteins are classified into two main categories that can be separated based on their

solubility at pH 4.6 and 20 °C (Fox 2001). The group of proteins that precipitate are caseins (α_{S1} -casein, α_{S2} -casein, β -casein, and κ -casein) and the group that remain soluble are known as serum or whey proteins (β -lactoglobulin, α -lactalbumin, bovine lactoferrin, bovine serum albumin, and bovine immunoglobulins), corresponding to 80% and 20%, respectively. Caseins, β -lactoglobulin (β -LG), and α -lactalbumin (ALA) are considered the major allergens; however, lactoferrin (LF), bovine serum albumin (BSA), and immunoglobulins (Ig), which are present at lower quantities, have been shown to be of great importance in inducing milk allergies (Fox 2001; Hochwallner and others 2014). A summary on the known cow milk allergens, their biological functions, and accession numbers is provided in Table 1.

Caseins (Bos d 8)

Caseins are the major protein fraction of cow milk, amounting to about 80% of the total milk proteins, with sizes ranging from 19 to 25.2 kDa (Restani and others 2009; Hochwallner and others 2014). According to the WHO/IUIS official list of allergens, caseins are classified with the general designation of Bos d 8. However, in spite of this common name (Bos d 8), individual components of caseins have received different identifying names (ALLERGEN 2017). Caseins are encoded by different genes located in the same chromosome, being subdivided in distinct families. The most important are: α_{S1} - (Bos d 9), α_{S2} - (Bos d 10), β - (Bos d 11), and κ - (Bos d 12) caseins, representing 40%, 12.5%, 35%, and 12.5% of the casein fraction in milk, respectively (Wal 2002; Demeulemester and others 2006). They belong to a large family of secretory calcium-binding phosphoproteins (Smyth and others 2004), having a loose tertiary, highly hydrated structure with a phosphate group that binds strongly to polyvalent cations such as calcium, causing charge neutralization and precipitation of α_{S1} -, α_{S2} -, and β -caseins at >6 mM of Ca^{2+} and 30 °C. However, in the case of κ -casein, as it contains a small concentration of phosphate group, calcium binds weakly and is not precipitated by them (Fox 2001). In milk, where calcium is present at high concentrations, this fact leads to the formation of quaternary structures, named casein micelles, in suspension in the aqueous phase of lactoserum whey. These structures are characterized by a central hydrophobic core (calcium-sensitive α_{S1} -, α_{S2} -, and β -caseins) and a peripheral hydrophilic layer (κ -casein) (Figure 1) (Fox 2001; Wal 2001). The biological functions of casein micelles include the transportation and secretion of calcium and phosphate, and the digestion and absorption of nutrients by their retention in the stomach (Holt and others 2013). Caseins are considered poorly immunogenic, since their structures are non-compact and flexible.

Table 1. Identification of cow's milk allergens according to their biochemical classification, biological function and respective accession numbers.

Fraction	Protein Superfamily	Biochemical classification	Allergen	MW (kDa)	Biological function	Nucleotide (NCBI)	Protein (NCBI)	Protein (UniProt)
Caseins	Caseins	α S ₁ -casein	Bos d 8	20-30	Caseins (individual components Bos d 9-Bos d 12)	-	-	-
			Bos d 9	23.6 (199 aa)	Calcium binding protein. Major allergen.	NM_181029	NP_851372	P02662
			Bos d 10	25.2 (207 aa)	Calcium binding protein. Major allergen	NM_174528	NP_776953	P02663
			Bos d 11	24 (209 aa)	Calcium binding protein. Major allergen.	XM_005902037	XP_005902099	P02666
Whey proteins	Lysozyme	κ -casein	Bos d 12	19 (169 aa)	Stabilization and coagulation of milk. Major allergen	NM_174294	NP_776719	P02668
			Bos d 4	14.2 (123 aa)	Participates in synthesis of lactose, metal, lipid, and calcium binding. Major allergen.	M18780	AAA30615	P00711
	Lipocalin	β -lactoglobulin	Bos d 5	18.3 (162 aa)	Lipid binding, antioxidant activity. Major allergen.	X14712	CAA32835	P02754
			Bos d 6	66.3 (582 aa)	Transport, metabolism, and distribution of ligands. Defense. Major allergen.	M73993	AAA51411	P02769
	Serum albumin	Bovine serum albumin	Bos d 7	160	Defense. Minor allergen.	-	-	-
			-	80 (703 aa)	Iron binding protein, defense. Minor allergen.	-	-	-

Without a tertiary structure, caseins have high surface hydrophobicity. Due to the different nature of the residues (hydrophobic, polar, and charged), they do not present a uniform distribution throughout the molecular structure, being organized in hydrophobic or hydrophilic patches. For that reason, caseins are strongly amphipathic structures, making them highly surface-active and insoluble in water (Fox 2001; Wal 2001; Hochwallner and others 2014; Goff 2016). All the caseins present genetic polymorphisms that lead to several protein variants and contribute to their high heterogeneity. These variants are characterized by point substitution of amino acids (aa), by deletions of peptide fragments of varying size or by post-translational modifications, such as glycosylation, phosphorylation, or partial hydrolysis, which may affect their allergenic potential (Fox 2001; Wal 2001).

Bos d 9 (α_{S1} -Casein)

α_{S1} -Casein is a single-chain phosphoprotein of 199 aa, with a molecular size of 23.6 kDa and characterized by a high content of proline residues. It has around 70% unordered structure, with a small fraction of secondary structure, such as α -helix and β -sheets, and a reduction in tertiary structure due to the lack of disulfide bonds. It has 2 hydrophobic regions, containing all 17 proline residues, separated by a polar region containing phosphate groups. α_{S1} -Casein possesses 7 genetic variants, characterizing different cattle breeds (Wal 1998; Chatchatee and others 2001a; Goff 2016).

According to Natale and others (2004), approximately 50% of serum samples from patients with cow milk allergy react with α_{S1} -casein. However, the prevalence of sensitization to each casein fraction is not consensual, since IgE-binding capacity can be easily reduced even by a single aa substitution or increased by the unmask of hidden highly reactive epitopes (Bernard and others 1998). The identification of IgE recognition sites (IgE-binding epitopes) in the antigen is an important way to the development of new diagnostic strategies (Matsuo and others 2015). Thus, several studies have been made to identify the IgE-binding regions of α_{S1} -casein in humans. The lack of a clear tertiary structure on caseins suggests the presence of preferentially linear epitopes. Nakajima-Adachi and others (1998) identified a single immunodominant IgE-binding region at the C-terminal (residues 181-199), while Spuergin and others (1996) identified 3 immunodominant IgE-binding epitopes located in hydrophobic regions, where they are not accessible to antibodies, unless the casein is denatured or degraded during digestion. Chatchatee and others (2001a) identified 6 major IgE-binding regions, and they suggested that there is a difference in epitope recognition between patients with persistent and transient cow milk allergy. The region located at residues 28-50 recognized by Cerecedo and others (2008) was also identified by Spuergin and others (1996), Chatchatee and others (2001a), and Elsayed and others

(2004). Cong and others (2012) identified 4 different regions with the recognition of the critical residue for IgE-binding.

Bos d 10 (α_{S2} -Casein)

α_{S2} -Casein is comprised of 207 aa, with one disulfide bond per molecule and a molecular mass of 25.2 kDa. It presents 4 genetic variants (A, B, C, and D) with different amounts of phosphoryl groups (10 to 13), one of them (variant A) containing 11 residues of phosphoserine with a rather unstable structure to pH changes (Micinski and others 2013). The variant D has different structural characteristics, corresponding to the deletion of a cluster with 3 phosphoserine residues, which might affect the allergenic potential of this protein (Bernard and others 2000a; Wal 2001; Busse and others 2002).

According to Natale and others (2004) the prevalence of sensitization to α_{S2} -casein in patients with cow milk allergy is 90%. Six minor and 4 major (detected in 77% of patients) sequential IgE-binding epitopes were recognized in α_{S2} -casein by Busse and others (2002), while Cerecedo and others (2008) identified only 7 regions, one of them in common with the previous study.

Bos d 11 (β -Casein)

β -Casein is comprised of 209 aa and 5 phosphate groups, with a molecular mass of 24 kDa. It possesses 12 main genetic variants with different levels of phosphorylation. Its molecular structure is very similar to α_{S1} -casein with a globular hydrophobic domain at the C-terminal, a highly solvated and charged domain at the N-terminal, an even distribution of proline content, and without disulfide bonds. The acidic peptide sequence containing a cluster of phosphoserine residues is homologous among caseins, namely in β -casein (13VESLSSSEE21) and α_{S1} -casein (62AESISSSEE70). In α_{S2} -casein variant A, the homolog cluster of phosphoserine residues is partially repeated twice at residue positions 7-12 (VSSSEE) and 55-60 (GSSSEE). Additionally, β -casein possesses a reduced secondary structure and no rigid tertiary interactions, as in α_{S1} -casein, suggesting that their important allergenic epitopes are linear rather than conformational.

β -Casein can be cleaved by the milk plasmin (native protease) originating another family of caseins (γ_1 -, γ_2 - and γ_3), although they are considered to be non-allergenic (Kumosinski and others 1991; Wal 1998; Chatchatee and others 2001b; Micinski and others 2013; Goff 2016). Regarding the identification of IgE-binding epitopes on β -casein, Otani and others (1987) concluded that there are at least 6 antigenic sites on the molecule and that the epitopes are sequential (Wal 1998). Chatchatee and others (2001b) have also identified 6 major and 3 minor IgE-binding epitopes, but the data about this protein are still scarce.

Bos d 12 (κ -Casein)

κ -Casein has a molecular mass of 19 kDa, and a primary sequence of 169 aa, with a N-terminal strongly hydrophobic and a C-terminal highly hydrophilic. This protein promotes the steric and electrostatic repulsion between micelles, thus preventing aggregation. It is also the only glycosylated casein, containing galactose, galactosamine, and sialic acid. This protein occurs as tri- or tetrasaccharides attached to threonine residues in the C-terminal region, a fact that increases its hydrophilicity. Depending on the degree of glycosylation, multiple isoforms of κ -casein can coexist in milk. There are 11 variants of κ -casein due to the differences in the number of the attached oligosaccharides (Fox 2001; Farrell and others 2004). Since it is very resistant to calcium precipitation, it contributes to the stabilization of other caseins. However, this ability is eliminated by rennet cleavage at the Phe105-Met106, leaving a hydrophobic portion, para- κ -casein, and a hydrophilic one, called κ -casein glycomacropeptide or caseinomacropeptide (CMP). The CMP has 64 aa and it is responsible for the reduction of gastric acid and serum gastrin secretion, increasing the efficiency of digestion. It also exhibits anticoagulant properties, prevents platelet agglomeration, and serotonin secretion (Micíński and others 2013; Goff 2016). The number of O-glycosylation sites in CMP can vary from 0 to 7, so both non-glycosylated and glycosylated isoforms exist in digested milk. According to Boutrou and others (2008), the glycosylated forms of CMP are less digested than the non-glycosylated ones, suggesting that the former might be responsible for the potential immunoreactivity of CMP.

Eight major IgE-binding epitopes were detected in κ -casein by Chatchatee and others (2001b): 3 of them being recognized by 93% of patients' serum samples with cow milk allergy (9IRCEKDERFFSDKIAKYI26, 21KIAKYIPIQYLLSRYPYGLNYY44, and 47KPVALINNQLPYPYAKPAAVR68), and 6 epitopes by the majority of older patients. Cerecedo and others (2008) identified 2 regions (16RFFSDKIAKYIPIQYVLSRY35 and 34RYPYGLNYYQQKPVALINN53) as dominant epitopes. Thus, the region between residues at positions 9-68 (at the N-terminal) may play an important role in the allergenicity of this protein. Han and others (2008) reported a total of 13 aa (at positions 17, 18, 29, 32, 35, 58, 61, 72, 97, 105, 118, 146, and 160) as critical residues for IgE-binding to linear epitopes of κ -casein. The substitution of the native residues by others resulted in overall loss/decrease of IgE-binding by pooled sera and each individual patient's serum for each epitope (Han and others 2008).

Whey Proteins

The whey proteins represent 20% of cow milk protein. The main allergenic components are the globular proteins Bos d 5 (β -LG) and Bos d 4 (ALA), representing 50% and 25% of

the whey protein fraction, respectively, followed by minor constituents, such as Bos d 6 (BSA), Bos d 7 (Ig), and lactoferrin (LF) (Monaci and others 2006). Contrarily to the caseins, the whey proteins possess high levels of secondary, tertiary, and, in the case of β -LG, quaternary structures. They are not phosphorylated and contain intramolecular disulfide bonds, which stabilize their structure (Fox 2001). The three-dimensional (3D) structure seems to play an important role in maintaining the conformational epitopes, therefore contributing to the allergenic potential of the protein (Monaci and others 2006).

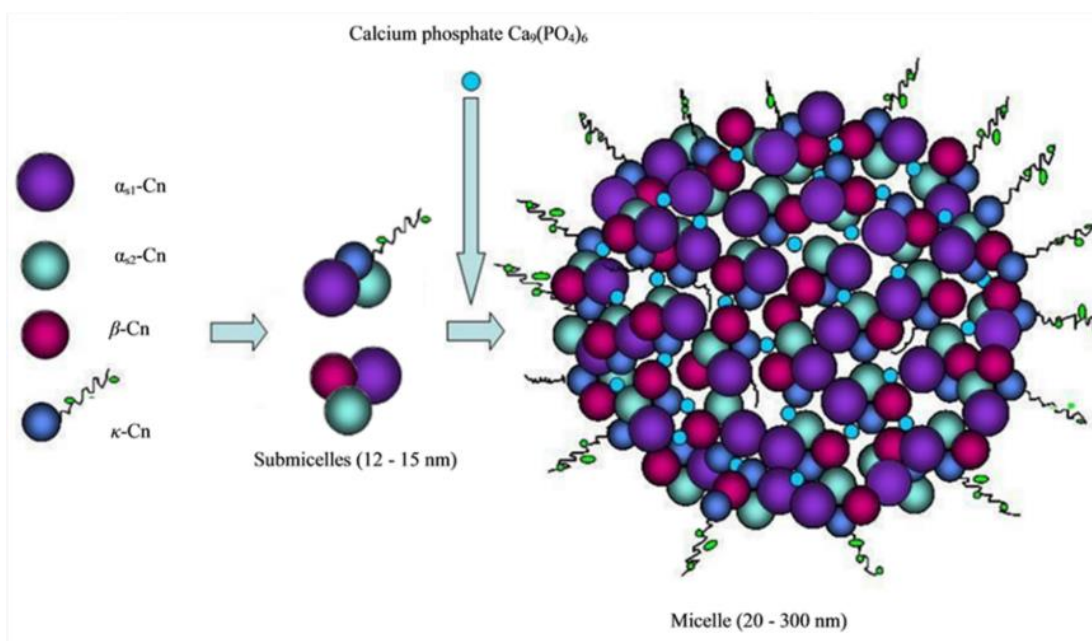


Figure 1. Structure of casein submicelles and casein micelles composed of submicelles held together by calcium phosphate. Retrieved from Rebouillat and Ortega-Requena (2015).

Bos d 4 (ALA)

Bos d 4 is a monomeric globular calcium-binding metalloprotein that belongs to the family of glycosyl hydrolase (lysozyme c superfamily) and it has been identified as a major allergen in cow milk. Its primary structure is built of 123 aa, with a molecular mass of 142 kDa, and reported as having 3 genetic variants. This protein is a regulatory component of the enzymatic system of β -galactosyl transferase, responsible for the synthesis of lactose by the formation of the lactose synthetase complex. It is also known to interact with lipid membranes (stearic and palmitic acids), and it binds metals such as cobalt, magnesium, and zinc. It possesses 4 disulfide bridges and a high-affinity binding site for calcium, which stabilizes its secondary structure. Furthermore, ALA shows high thermal stability and refolding capacity. It has a highly ordered secondary structure and a compact, spherical tertiary structure; it has an “elbow- Ca^{2+} -binding loop” with 2 structural domains: a large α -helical domain at the N-terminal and a short β -sheet domain at the C-terminal, flanking the

calcium-binding loop (Demeulemester and others 2006; Micíński and others 2013; Hochwallner and others 2014; Goff 2016). Depending on the study population, the prevalence of ALA-specific IgE in milk allergic patients ranges from 27.6% to 62.8% (Matsuo and others 2015). The peptide 5KCEVFRELKDLKGY18, which corresponds to a homologous sequence in β -LG (Bos d 5) at positions 124-135, is considered the major antigenic site, with a large capacity for binding with specific IgE from human sera (Adams and others 1991). Maynard and others (1997) showed IgE-binding to native ALA and to large peptides, suggesting the importance of conformational epitopes in the development of milk allergy. However, they also showed that protein denaturation might expose some linear epitopes. Hochwallner and others (2010) identified 6 IgE-reactive peptides, 3 located at the surface of the protein and one of them corresponding to the major antigenic site mentioned above. Jarvinen and others (2001) identified the same region and 3 additional conformational IgE epitopes. Hopp and Woods (1982) recognized another highly antigenic region accounting for 20-25% of whole ALA antigenicity at the peptide that includes the loop 60WCKNDQDPHSSNICNISCDKF80. More recently, Li and others (2016) identified 6 linear IgE-binding epitopes, corresponding the ones of the previous studies of Maynard and others (1997) and Jarvinen and others (2001).

Bos d 5 (β -LG)

Bos d 5 is a retinol-binding protein that belongs to the lipocalin superfamily, and it is classified as a major allergen in milk. It binds a wide variety of molecules like cholesterol, vitamin D2, saturated and unsaturated fatty acids, Cu^{2+} and Fe^{2+} ions, hydrophobic ligands such as retinol, and it possesses antioxidant activity. It is a 36-kDa dimer with 2 main isoforms that differ by only 2 point mutations on residues 64 and 118. Each subunit consists of 162 aa that possesses one free cysteine and 2 disulfide bonds responsible for the dimerization of the molecule. This protein has a well-characterized tertiary structure common in the lipocalin superfamily, with a globular shape built up by an 8-stranded antiparallel β -barrel with a 3-turn α -helix on the outer surface and a ninth β -strand flanking the first strand. This structure is responsible for its main physicochemical properties and for its sulfhydryl/disulfide interactions with κ -casein during heat treatments above 75 °C. β -LG has a relative resistance to acid hydrolysis, as well as to protease activities. These features enable preserving some structural integrity after digestion, allowing its absorption through the intestinal mucosa and further presentation to the immunocompetent cells, having high allergenic potential (Wal 1998; Fox 2001; Jarvinen and others 2001; Wal 2001; Demeulemester and others 2006; Micíński and others 2013; Hochwallner and others 2014).

Food allergies associated with this protein are estimated in 80% of the population (Micíński and others 2013). For this reason, several studies focusing on the identification of

IgE-binding regions have been performed. Many authors described the peptide 121CLVRTPEVDDEAL134, located at the protein surface, as a major allergenic site (Adams and others 1991; Williams and others 1998). Jarvinen and others (2001) identified 7 IgE-binding epitopes, but only 3 were considered immunodominant, one of them corresponding to the same region (residues 121-134). Moreover, they stated that the presence of IgE to multiple linear allergenic epitopes might be an indicator of persistent allergy. This fact was also evidenced by Picariello and others (2010, 2013), since they suggested the same region (residues 125-135) with the most important allergenic potential among the β -LG determinants due to its high resistance to proteolysis. Cerecedo and others (2008) identified 3 epitopes recognized by more than 75% of sera from allergic patients with the region 58LQKWENDECAQKKIIAEKTK77, being significantly associated with milk protein-reactive patients. Cong and others (2012) determined 4 IgE-binding epitopes, namely 17LIVTQTMKGLDIQKV31, 72ILLQKWENGECAQKK86, 92TKIPAVFKIDAL-NEN106, and 152FDKALKALPMHIRLS166, and 2 IgG-binding epitopes (22TMKGLDIQKVAGTWY36 and 127AEPEQSLACQCLVRT141). Accordingly, the authors identified different critical residues for IgE- and IgG-binding, located at epitopes 17LIVTQTMKGLDIQKV31 (Thr20, Met23, and Asp27) and 22TMKGLDIQKVAGTWY36 (Leu26 and Val31), respectively. Ball and others (1994), Heinzmann and others (1999), and Selo and others (1999) also identified some major and minor linear epitopes, some of them not exposed at the surface of the molecule and, therefore, well protected against enzymatic attacks, which suggests their relative minor importance in terms of immunoreactivity.

The recognition of specific proteins/peptides that behave as potent allergens can be considered a step forward in component-resolved diagnosis as new and highly efficient diagnostic tools (microarrays). These microarrays can determine different epitope-binding patterns, thus allowing differentiating the clinical phenotypes of milk allergy. Ultimately, Bos d 5 is considered a major allergen with multiple IgE-binding linear epitopes, highlighting the importance of its specific peptides as molecular markers for the diagnosis of persistent milk allergy (Jarvinen and Sicherer 2012; Hochwallner and others 2014; Sicherer and Sampson 2014).

Bos d 6 (BSA)

Bos d 6, although present in milk at low quantities, reacted with IgE from the sera of 50% of milk allergic patients, which rendered its classification as a major allergen. It has 582 aa and a molecular weight of 66.3 kDa, with a stable tertiary structure. Its main biological role is related to the transport, metabolism, distribution of several substances (fatty acids, ions, hormones, drugs), and protection from free radicals. It contributes to regulate the colloidal osmotic pressure of blood and it imparts free radical protection. This protein is organized in

3 homologous domains and consists of 9 loops connected by 17 disulfide bonds, many of which are protected in the core of the protein, therefore not easily accessible. The disulfide bonds play an important role in maintaining the native antigenic determinants of this molecule, mainly because of the great stability of its tertiary structure, even under denaturing conditions (Farrell and others 2004; Restani and others 2004; Hochwallner and others 2014). It has been shown that the fragment, comprising residues at positions 524-598, is an epitopic area for human species, from which the region of 524AFDEKLFTFHADICTLPDT542 corresponds to the most critical sequence (Beretta and others 2001). Tanabe and others (2002) also identified a few epitopes from BSA to be involved in beef allergy. However, the epitopes reported by various studies were not always the same (Atassi and others 1976; Peters and others 1977; Beretta and others 2001).

Bos d 7 (Ig)

Bos d 7 accounts for about 3% of total milk protein and 6% of whey proteins. It possesses a conformational structure very similar to those of human origin, occurring as polymers or protomers of a basic “Y shaped” unit composed of 4 polypeptide chains linked by inter- and intramolecular disulfide bonds. The monomers are composed of heavy (H) and light (L) chains, each of them with variable (V-) and constant (C-) domains. The V-domains of H- and L-chains converge to form the antigen-binding site, while the C-domains characterize the isotype of the Ig in cow milk: IgG, IgA, or IgM. The potential allergenicity of bovine Ig is still under study and their IgE-binding epitopes have not yet been identified. However, IgG was proposed as milk allergen due to the observation that IgE from milk allergic patients specifically binds to bovine IgG (Lefranc-Millot and others 1996; Farrell and others 2004; Natale and others 2004; D'Urbano and others 2010). Approximately 10% of patients with cow milk allergy are IgE-positive to cow IgG; therefore, this protein is considered as a minor allergen in milk (Matsuo and others 2015).

Lactoferrin (LF)

LF is an iron-binding glycoprotein that belongs to the transferrin family and it is found at levels <1% in the milk of most species. It consists of a single polypeptide chain folded into two globular lobes, each of them having high-affinity iron binding sites, connected by a 3-turn helix. The molecular weight of this protein varies, depending on the extent of its glycosylation. Besides its function as a scavenger of free radicals and as an antioxidant, its main role is to defend the organism against infections and inflammations owing to its ability to sequester iron from the environment and, thereby, removing this essential nutrient for bacterial growth (Ward and Conneely 2004). In addition, it is involved in detoxification processes and it has an antineoplastic effect by inhibiting the attachment of tumor growth

factors (Micíński and others 2013). Some studies declare that some milk-allergic individuals possess LF-specific IgE, although the relevance of the allergenicity of this protein is still under discussion because these patients also present IgE against one of the major milk allergens. Until today, no data about the identification of IgE-binding epitopes has been reported (Adel-Patient and others 2005; D'Urbano and others 2010).

CROSS-REACTIVITY OF MILK ALLERGENS

Although the milk proteins officially recognized as food allergens are of bovine origin, there are many other dairy animals whose milk is used for human consumption and, therefore, liable to initiate an allergic reaction in susceptible individuals by the ingestion of homologous proteins. Milk and milk proteins from buffalo, sheep, goat, pig, camel, mare, donkey, reindeer, and yak can be used to produce dairy products or be added to cow milk. Therefore, homologous milk proteins of different species can lead to cross-reactivity phenomena in sensitized/allergic individuals (Restani and others 2009).

Different studies reveal that the vast majority of patients with cow milk allergy have high cross-reactivity to milk from sheep, buffalo, and goat, which might be explained by their great similarity in protein composition, although presenting a different distribution. Contrarily, very few cow milk allergic individuals present cross-reactivity to donkey, mare, and camel milk, whose structures are more similar to human milk. Mare milk presents a reduction in the casein fraction, while camel milk shows a high proportion of β -casein and the lack of β -LG, as in human milk (Jarvinen and Chatchatee 2009; Restani and others 2009; Hinz and others 2012). Restani and others (1999) tested sera from cow milk allergic patients with milk proteins from mammalian species. Accordingly, the authors showed a strong IgE-reactivity of sera with the majority of milk proteins from sheep, goat, and buffalo, while no IgE-binding was observed when testing camel milk, which might be explained by the phylogenetic differences between cow and camel. Using animal monoclonal antibodies specific for cow milk proteins, Restani and others (2002) confirmed the previous results, but also observed weak immunoreactivity with mare and donkey milk. These results were also obtained by Katz and others (2008) when performing skin prick tests in patients with a clinical history consistent with IgE-mediated cow milk protein allergy. The authors added deer as a cross-reactive species and pig as a less reactive one, suggesting the existence of a “kosher epitope” responsible for this common allergenicity (Katz and others 2008). Suutari and others (2006) demonstrated that β -LG of reindeer milk has weak cross-reactivity with bovine β -LG, in spite of being a ruminant species, probably due to the lack of homolog bovine epitopes in the protein or a weak bond with those that are recognized.

Since goat milk contains a lower quantity of α -caseins, it has been suggested as a substitute of cow milk for allergic patients, though the results about its cross-reactivity are

still controversial. Some reports suggest that cow milk allergic children can tolerate goat and sheep milk because of the weak IgE-binding to caseins (Restani and others 2009). However, the majority of studies demonstrates high cross-reactivity between cow and goat proteins (Bellioni-Businco and others 1999; Besler and others 2002; Pina and others 2003). On the other hand, there are individuals, normally older children that present relevant, or even severe, allergic reactions to goat and sheep milk, without clinical manifestation towards cow milk. In those cases, the IgE binds the caseins (α_{S1} -, α_{S2} -, and β -caseins) with high specificity and efficiency, but not to the whey proteins, despite their pronounced sequence homology (Ah-Leung and others 2006). Several studies report the fact that patients with cow milk allergy have cross-reactivity with sheep and goat milk, but not the reverse (Wüthrich and Johansson 1995; Calvani Jr and others 1998; Umpierrez and others 1999; Muñoz-Martín and others 2004; Viñas and others 2014). Nonetheless, the reason for this phenomenon is still unclear.

Donkey and mare milk have been revealed to be less allergenic, with a very weak IgE cross-reactivity. Some authors suggest the utilization of donkey milk in children with severe cow milk allergy, confirming that 80% of children tolerated donkey milk better than goat milk, and that it is more effective in ameliorating atopic dermatitis (Businco and others 2000; Alessandri and Mari 2007; Monti and others 2007; Vita and others 2007).

The casein proteins are present in milk of different ruminant species with high sequence homologies, varying from 80% to more than 90%, sharing the same structural, functional, and biologic properties. For example, α_{S1} -, α_{S2} -, and β -caseins from cow, goat, and sheep share 87–98% of sequence identity, with an IgE-sensitization to sheep and goat casein ranging from 93% to 98% in children with cow milk allergy. Moreover, it was demonstrated that human and bovine β -caseins also share approximately 50% of sequence homology. These regions correspond to clusters of phosphorylated seryl residues conserved in bovine caseins, as well as in the caseins of other species, probably playing an important role in cross-reactivity among the milk of different species (Spuergin and others 1997; Restani and others 1999; Bernard and others 2000b). Bernard and others (1998) demonstrated that 99% of the patients' sera ($n=58$) reacted to more than one casein, and 88% presented IgE against each of the 4 bovine caseins. This finding suggests the presence of common or closely related IgE epitopes, possibly associated with phosphorylation sites, described as being immunoreactive and resistant to digestive degradation, or with the polysensitization to different casein components after disruption of the casein micelles during the digestive process (Bernard and others 1998; Wal 2004). In another study, Bernard and others (2000b) showed cross-reactivity between human and bovine β -caseins, though with a lower affinity of IgE to human β -caseins. The similarity of human and bovine caseins was also demonstrated in the study of Han and others (2008), which shows 2 potential cross-reactive

sites of IgE-binding epitopes between human and bovine κ -casein. Additionally, bovine and human ALA sequences share 74% of sequence homology (Wal 2001). The distribution of proteins in human milk is rather different from that of bovine milk, but more similar to donkey and mare milk because they have a minor content in caseins. Besides, β -LG is absent from human milk, in opposition to other mammalian milks. Still, sequence homology between human and bovine milk is rather high, leading to cross-allergic reactions in some patients (Tsabouri and others 2014).

Until now, camel milk seems to be the most appropriate substitute for cow milk, mainly because of the high proportion of β -casein, low proportion of α -casein, deficiency in ALA, and similarity of the Ig (Kumar and others 2016). Camel milk shows the lowest level of similarity (about 60%) with cow milk proteins (Jarvinen and Chatchatee 2009; Restani and others 2009; Tsabouri and others 2014). Many efforts have been made to study the reliability of using camel milk in allergic patients with interesting and promising results (Shabo and others 2005; Ehlayel and others 2011; Boughellout and others 2016).

Cross-reactivity in sensitized patients can occur, not only between milk proteins of different species, but also with proteins present in other tissues, as in meat or epithelia of different mammals. BSA is very similar to human serum albumin and owing to its widespread availability, it has numerous applications in medical formulations, namely as a component of several vaccines. Most of the individuals with persistent milk allergy are known to be reactive to serum albumins of different mammalian meats, which increases their risk of developing clinical symptoms, such as rhinoconjunctivitis or asthma, due to animal epithelia (Chruszcz and others 2013). Therefore, BSA is a common example of cross-reactivity phenomenon and it is involved in the co-sensitization to milk and beef with a prevalence of 13-20% among cow milk allergic patients (Martelli and others 2002). Vicente-Serrano and others (2007) showed that the sera from patients allergic to cow milk with IgE-binding to BSA also recognized the native serum albumin in different meats (beef, lamb, deer, and pork) and epithelia (dog, cat, and cow). However, none of them reacted with heated meats, suggesting the implication of heat denaturation in the reduction of serum albumin allergenicity. The authors also stated that albumins are involved as a panallergen (allergens responsible for wide IgE cross-reactivity between related and unrelated allergenic sources) in mammals.

Table 2. Milk proteins of other species associated with cross-reactivity phenomena and respective allergen names.

Allergens	Buffalo (<i>Bubalus bubalis</i>)	Goat (<i>Capra aegagrus hircus</i>)	Sheep (<i>Ovis aries</i>)	Reindeer (<i>Rangifer tarandus</i>)	Mare (<i>Equus caballus</i>)	Donkey (<i>Equus asinus</i>)	Mule (<i>Equus mulus</i>)	Camel (<i>Camelus dromedarius</i>)	Pig (<i>Sus scrofa domestica</i>)
α S ₁ -casein	Bub b 9	Cap h 9	Ovi a 9	Not found	Equ c 9	Not found	Not found	Cam d 9	Sus s 9
α S ₂ -casein	Bub b 10	Cap h 10	Ovi a 10	Not found	Equ c 10	Not found	Not found	Cam d 10	Sus s 10
β -casein	Bub b 11	Cap h 11	Ovi a 11	Not found	Equ c 11	Not found	Not found	Cam d 11	Sus s 11
κ -casein	Bub b 12	Cap h 12	Ovi a 12	Not found	Equ c 12	Not found	Not found	Cam d 12	Sus s 12
α -lactalbumin	Bub a 4	Cap h 4	Ovi a 4	Not found	Equ c ALA	Not found	Not found	Cam d 4	Sus s 4
β -lactoglobulin	Bub a 5	Cap h 5	Ovi a 5	Ran t 5	Equ c BLG	Equ as BLG	Equ mu BLG	Absent	Sus s 5
Serum albumin	Not found	Cap h 6	Ovi a 6 (also present in meat, and urine)	Not found	Equ c 3* (also present in meat, and skin)	Equ as 6	Not found	Not found	Sus s 1* (also present in meat, and urine)

*Present in WHO/IUIS Official List of Allergens.

Soy formulas are common substitutes to cow milk allergic individuals. Nonetheless, it has been demonstrated that some patients are intolerant to such products, suggesting the cross-reactivity between soy and cow milk proteins. It was reported that the α -subunit of beta-conglycinin (Gly m 5.0101) from the vicilin-like protein family, the G4 subunit of glycinin (Gly m 6.0401) from legumin-like proteins, and, more recently, the cysteine protease P34 (Gly m Bd30K) and the globulin P28 (Gly m Bd28K) are involved in cross-reactivity phenomena with bovine caseins (Rozenfeld and others 2002; Katz and others 2008; Smaldini and others 2012; Curciarello and others 2014; Candreva and others 2015; Candreva and others 2016).

Due to the great allergenicity of milk proteins from other species, databases such as ALLERGOME include some of these proteins in their allergen list (non-official). Table 2 summarizes the allergens involved in cross-reactivity to milk proteins (official and non-official), with the respective names from the databases ALLERGOME and WHO/IUIS.

EFFECT OF PROCESSING, FOOD MATRIX, AND DIGESTIBILITY ON MILK ALLERGENICITY

A wide range of food products can be manufactured from milk as raw material. On a global scale, 36% of cow milk is used for cheese production, 30% for butter and yellow products, 13% for the fabrication of cream products, 11% is consumed as drinking milk, and 3% is used for powders products. Condensed, evaporated, and fermented milks are also consumed, but at smaller amounts; casein and whey proteins are used as ingredients in several products, including cheeses, bakery products, and glues. Milk from other species, such as sheep and goat, is also used predominantly for the manufacture of fermented milks and cheeses (Fox 2001; Goff 2016; FAOSTAT 2017; Eurostat 2017). Thus, milk and milk proteins can be present in several food matrices, being submitted to different types of processing, until they become available to consumers as final products. Food processes applied to milk and milk products can include pasteurization or ultra-high temperature (UHT) treatment to eliminate pathogens from liquid milk, fermentation to produce yogurts, and evaporation and spray-drying to obtain concentrates and milk powders for infant formulas, respectively (Verhoeckx and others 2015).

Food processing can induce different modifications in the structure of proteins, including aggregation, unfolding, and glycation, and also the occurrence of Maillard reaction products. All these alterations may affect the IgE-binding capacity and, consequently, increase or reduce the allergenicity of proteins (Rahaman and others 2016). The latter is normally attributed to the destruction of conformational epitopes or to the occurrence of chemical reactions in the food matrix between proteins, fat, and sugars, limiting the availability of the protein to the immune system. On the other hand, the formation of neoepitopes and the

effect of food matrix that decrease protein digestibility (and consequently the preservation of the existing epitopes) might potentially increase protein allergenicity (Nowak-Węgrzyn and Fiocchi 2009). The high content of proteins in a food matrix seems to enhance the stability against simulated gastrointestinal degradation and create a competitive environment for enzyme cleavage, thereby delaying gastrointestinal proteolysis of food allergens (Schulten and others 2011).

Many efforts have been made to study the influence of milk processing technologies on the reduction of allergenicity, to find new and effective processes to be applied to milk products and, therefore, control milk allergy. Tables 3 and 4 summarize several recent studies about the modification of milk allergens and allergenicity upon conventional and novel food processing technologies.

Conventional food processing

Heat treatment

Heat treatment is an important step in the manufacturing of most dairy products with the use of techniques such as pasteurization, sterilization, and UHT processing. By nature, caseins are considered as intrinsically disordered proteins, possessing very little secondary and tertiary structures (such as the case of β - and κ -caseins), but still able to perform their function. Consequently, they are very stable to heat treatments, showing only a partial reduction or no change in their allergenicity (Bhat and others 2016). Bloom and others (2014) demonstrated the presence of caseins after 60 min at 95 °C, not affecting substantially their immunoreactivity. Although casein allergenicity can be influenced by the period, temperature, and presence of other foods (for example, wheat) during the heat process, all serum sample taken from milk-allergic subjects remained IgE-reactive to caseins, even after extensive thermal treatment. Similarly, Morisawa and others (2009) showed that α -caseins submitted to thermal treatment did not affect the amount of histamine released from basophils, but a combination of heat treatment with enzymatic digestion led to a decrease of histamine release, confirming the relation of α -casein specific-IgE with linear epitopes.

In opposition, whey proteins are thermolabile, with changes on their allergenicity (Verhoeckx and others 2015). β -LG shows an increased antigenicity and allergenicity, when subjected to temperatures ranging from 50-90 °C due to the exposure of hidden allergenic epitopes after an unfolding of the native structure of the protein. Above 90 °C, the allergenicity of β -LG seemed to decrease because of sulfhydryl/disulfide exchange, which enhances conformational changes with the subsequent destruction or mask of conformational epitopes on the surface of the molecule (Bu and others 2009b). In addition

to disulfide-mediated aggregation at those temperatures (90-120 °C), Maillard reactions might cause the loss of linear epitopes that lead to a general reduction in the allergenicity of β -LG (Kleber and Hinrichs 2007; Bu and others 2009b; Bloom and others 2014; Xu and others 2016). Figure 2 presents a schematic representation of the effect of heat treatment at different temperatures in the β -LG structure. The combination of heat treatment with pepsin digestion also showed a reduction in the allergenicity of β -LG (Sletten and others 2008; Morisawa and others 2009).

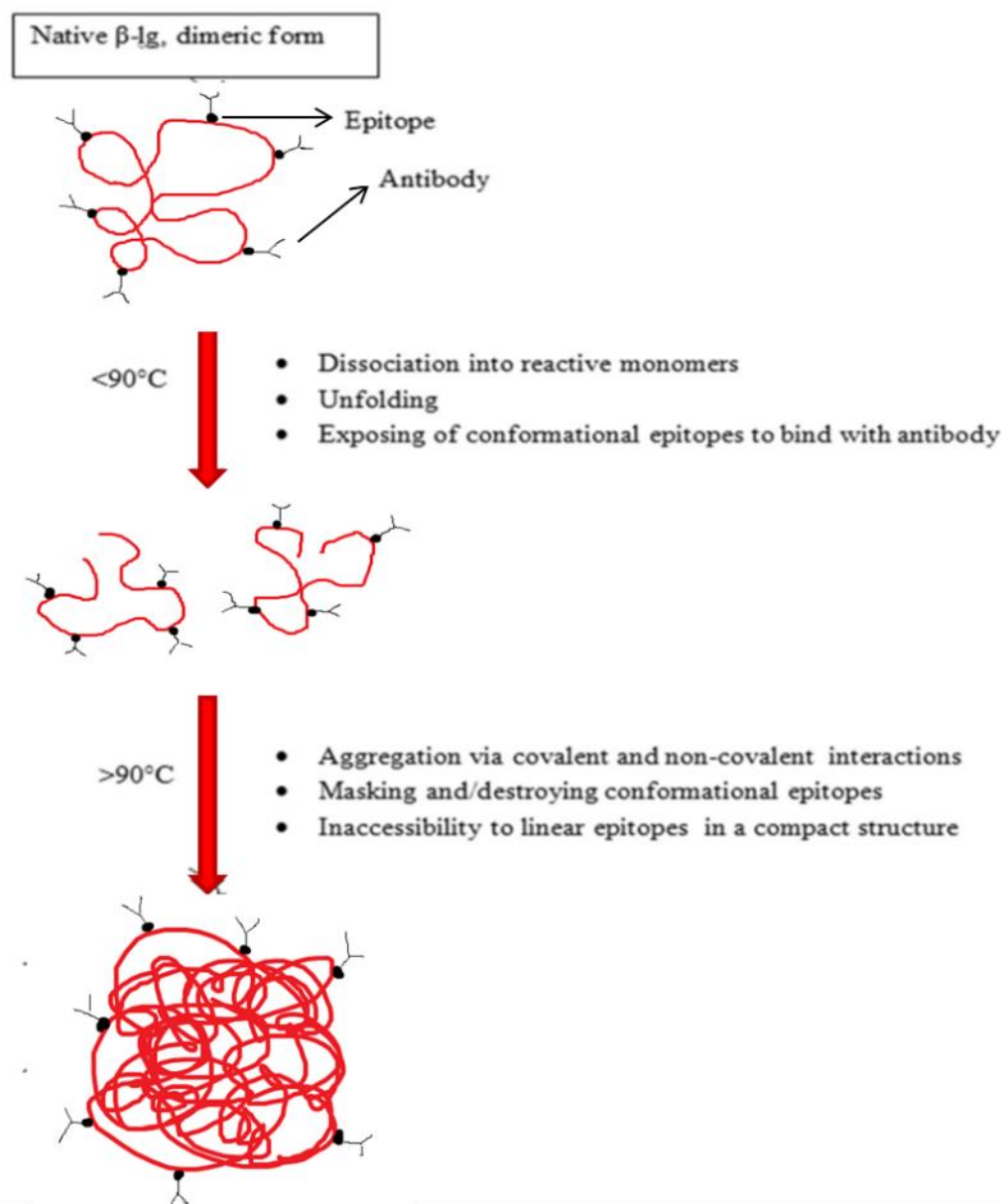


Figure 2. Schematic representation of the alterations in β -LG epitopes to bind with antibody at different level of heat treatment. Reprinted from Rahaman and others (2016) with permission from Elsevier Ltd.

ALA is more heat-stable than β -LG, but at high temperatures it presents a greater decrease in antigenicity because its conformational epitopes are thought to be more IgE-reactive, while the most relevant epitopes in β -LG are linear (Jarvinen and others 2001; Bu

and others 2009b). In addition, heating of β -LG or ALA results in the formation of inter-molecular disulfide bonds and subsequent binding to other food proteins. Therefore, these mechanisms of aggregation make IgE-epitopes less accessible and, consequently, less allergenic (Nowak-Węgrzyn and Fiocchi 2009; Bloom and others 2014). This matrix effect has led some authors to suggest a diet with baked milk, since in their study about 70% of tested children were able to ingest a muffin containing baked milk without any immediate clinical symptoms (Nowak-Węgrzyn and others 2008). After sequential food challenges with baked cheese and unheated milk in a test population of children that previously tolerated extensively heated (baked) milk products, Kim and others (2011) revealed that 28% and 60% of them were able to tolerate baked milk/baked cheese and unheated milk, respectively. Sopo and others (2016) evaluated the effect of wheat matrix on baked milk tolerance in children with IgE-mediated cow milk allergy. They demonstrated that 81% of children tolerated baked cow milk in a wheat matrix (ciambellone), 56% tolerated liquid baked cow milk, 78% Parmigiano Reggiano (a typical Italian cheese), and 82% partially hydrolyzed formula, revealing that matrix effect was relevant only in half of the cases. The tolerance to Parmigiano Reggiano was also studied by Alessandri and others (2012) in patients with suspected cow milk allergy, reporting that 56% of children tolerated the Italian cheese after 36 months of its maturation. These data were correlated with the extent of cheese maturation, in which the milk proteins, especially caseins, are gradually and constantly broken by the proteolytic enzymes of lactic acid bacteria and milk rennet, resulting in a decrease of allergenicity during gut digestion.

Techniques such as sterilization cause the denaturation of 75% of whey proteins and promote Maillard reactions, which occur between free amino acids and aldehyde/ketone groups of sugars present in milk or in other food matrices, and are known to change conformational structures and to affect protein allergenicity (Thomas and others 2007; Verhoeckx and others 2015). The effect of conjugating allergenic proteins with reducing sugars through Maillard reactions has been widely studied as a possible solution to reduce milk allergenicity. Even in proteins highly resistant to proteolysis, it has been reported that there is an increase in *in vitro* digestibility and a reduction in their immunoreactivity (Kobayashi and others 2001; Corzo-Martínez and others 2010; Wu and others 2013). Glucose (Bu and others 2009a; 2010a), chitosan (Aoki and others 2006), nystose, fructofuranosyl nystose, and fructo-oligosaccharides (Zhong and others 2013, 2015), oligoisomaltose and maltose (Aalberse 2007; Li and others 2011, 2013), and carboxymethyl dextran (Kobayashi and others 2001) are some sugars with a reported effect on the reduction of β -LG and ALA antigenicity and allergenicity. Maillard reactions between lactose (disaccharide) and the accessible amino groups of lysine residue in whey proteins might also occur, leading to the formation of Amadori products (Liu and others 2016). As reported

for other sugars, heating at 130 °C for 20 min lead to the formation of Maillard products from lactose and whey proteins (mainly ALA and β -LG), with a decrease in the IgG-binding capacity of at least 67% compared to unheated samples. Since both ALA and β -LG present several residues of lysine located at the IgG-binding regions, the blockage of lysine residues during the Maillard product formation probably induced conformation alterations in their epitopes, thus affecting the IgG-binding capacity of whey proteins (Liu and others 2016). On the other hand, proteins that undergo lactosylation process are more resistant to proteolysis, which might contribute to the formation of immunoreactive species and, thus increase the allergenicity of whey proteins (ALA and β -LG) (Milkovska-Stamenova and Hoffmann 2016). Although the heat treatment seems to reduce their immunoreactivity (Taheri-Kafrani and others 2009; Liu and others 2016).

Masking native/conformational epitopes is a possible explanation (Taheri-Kafrani and others 2009), but new epitopes can also emerge after conjugation with some substances due to the exposure of hydrophobic regions (Bu and others 2013). In addition, conditions such as pH, temperature, duration of exposure, weight ratio of sugar/protein, and previous digestion assays need to be well established to induce the maximum effect on antigenicity and allergenicity (Corzo-Martínez and others 2010; Li and others 2011, 2013).

Unfortunately, there are no reports about the effects of other heat treatments, such as UHT, vacuum evaporation, or spray-drying on allergenicity, despite the fact that some recent studies describe their effect on functional properties of milk proteins (Schuck and others 2013; Verhoeckx and others 2015).

Fermentation and enzymatic hydrolysis

Fermentation by lactic acid bacteria is a process commonly used to produce different types of milk products, such as yogurt and ripened-cream butters. These bacteria possess a complex proteolytic system that includes peptidases, proteinases, and transport systems, all essential for their growth in milk and dairy products. During fermentation, these enzymes hydrolyze milk proteins into peptides and amino acids, which greatly increase the possibility of cleaving relevant epitopes and, consequently, decrease their antigenicity and allergenicity (Shi and others 2014). Wróblewska and others (2016) reported a significant reduction in the immunoreactivity of ALA, β -LG, α -casein, β -casein, κ -casein, BSA, and LF after buttermilk fermentation by *Lactobacillus casei*, which was even higher after simulated digestion. Despite the 21% reduction of α -casein immunoreactivity, this protein was still the most reactive. This was due to the higher concentration of anti-casein-specific serum IgE compared to anti-ALA and anti- β -LG, because the patients were mostly sensitized to caseins (98%) and less to β -LG (69%) or ALA (51%). The change on allergenicity was also explained by the lactic acid bacteria species, the fermentation, and the storage conditions.

Fotschki and others (2015) tested 3 different strains of bacteria and verified that *L. casei* LCY caused the highest decrease in the immunoreactivity of mare milk after fermentation, while *Streptococcus thermophilus* MK10 caused the lowest effect. Bu and others (2010b) also concluded that the combination of *L. helveticus* and *S. thermophilus* induced a decrease in β -LG and ALA antigenicity. Fermentation with a proper cold storage also seems to have an interesting effect since the activity of microorganisms is then higher, producing more proteases that contribute to a reduction of protein antigenicity (Bu and others 2010b; Yao and others 2014). Several authors have studied the effect of simulated gastric digestion with saliva, pepsin, and pancreatin/bile salts after fermentation with lactic acid bacteria. The results showed a synergistic effect on the reduction of immunoreactivity with different rates at each stage of digestion for each tested allergen (Fotschki and others 2015; Wróblewska and others 2016). Wróblewska and others (2016) showed the fragmentation of ALA dimeric structure, the hydrolysis of BSA and β -LG after the pepsin step, and the complete degradation of caseins by porcine pancreatin/bile extract.

A matrix effect seems to be involved in the reduction of allergenicity. In the study of Pescuma and others (2011), fermentation with *L. delbrueckii* subsp. *bulgaricus* CRL 656 showed a greater hydrolysis percentage of β -LG in whey protein concentrates than in free protein, possibly due to the co-denaturation of ALA with β -LG, increasing their aggregation, which led to complete exposure of peptic cleavage sites. An interesting approach carried out by Phromraksa and others (2008) was the identification of different proteolytic bacteria from a Thai traditional fermented food with reducing allergenic potentials. The concentrated crude enzyme of *Bacillus subtilis* reduced β -LG allergenicity, making it suitable for use in the production of hypoallergenic milk food products. The use of proteolytic bacteria has received much attention for their application in the design of new hypoallergenic dairy products. Biscola and others (2016) isolated a new proteolytic strain of *Enterococcus faecalis* from raw bovine milk, whose proteases demonstrated strong activity against α - and β -caseins at optimal conditions of 42 °C and pH 6.5, in both skim milk and sodium caseinate.

Enzymatic hydrolysis has been used in the development of a variety of protein hydrolysate-based infant formulas to feed infants with cow milk allergy, proving to be an effective method to change the immunoreactivity of allergens (Duan and others 2014). Prioult and others (2004, 2005) studied the effect of hydrolysis with *Lactobacillus paracasei* and *Bifidobacterium lactis* enzymes on the allergenicity of acidic peptides from bovine β -LG. Their results indicated that the IgE binding capacity was reduced by the hydrolysis of β -LG peptides, by repressing the lymphocyte stimulation. Moreover, these peptide fragments significantly up-regulated interferon (IFN)- γ and interleukin (IL)-10 production and down-regulated IL-4 secretion by murine splenocytes.

Table 3. Recent studies about the effect of traditional processing techniques on milk allergens.

Allergen	Treatment	Allergenicity/antigenicity	Digestibility	Matrix	Reference
β-LG	Heat treatment	80-90 °C – increased allergenicity; Above 100 °C – decreased allergenicity	-	Purified protein, skim milk and sweet whey	Ehn and others (2004); Kleber and Hinrichs (2007)
Caseins, β-LG	Heat treatment Enzymatic hydrolysis	Caseins – decreased allergenicity after hydrolysis; β-LG – little effect on allergenicity	k-caseins – unaffected without heat treatment; caseins – proteolytic degradation with heat treatment; β-LG – increased digestibility by pepsin after heat denaturation, matrix fat content did not affect digestibility	Whole, semi-skimmed, skimmed and UHT; Caseins – IgE epitopes more stable to digestion with UHT process; β-LG – high reactivity to IgE in both UHT and semi-skimmed milks;	Sletten and others (2008)
ALA, β-LG	Heat treatment	50-90 °C – increased antigenicity (higher in β-LG); 90-120 °C – decreased antigenicity (higher in ALA)	-	Whey protein isolates	Bu and others (2009b)
α-caseins, β-LG	Heat treatment Enzymatic hydrolysis	Decreased allergenicity with the combination of heat treatment and pepsin digestion	Pepsin digested all untreated and heat treated α-caseins in 30 min; pepsin digested β-LG only after heat treatment	Purified proteins	Morisawa and others (2009)
β-LG	Heat treatment	-	Native β-LG resistant to simulated gastric digestion; 90°C – increased digestibility	Purified protein	Peram and others (2013)
Caseins, β-LG, ALA	Heat treatment	Caseins – unaffected; β-LG/ALA – decreased allergenicity at 90-100°C after 20 min	-	Fresh skim milk. Caseins – unaffected by matrix; β-LG/ALA – decreased allergenicity with a wheat matrix	Bloom and others (2014)
α-caseins, β-caseins, β-LG, ALA	Heat treatment	Caseins- unaltered β-LG – increased allergenicity at 65-85 °C during 25 min; decreased allergenicity at 85-90 °C during 25 min; ALA - decreased allergenicity	-	Milk protein concentrates	Xu and others (2016)
β-LG	Glycation reaction, (acidic oligosaccharides, carboxymethyl dextran, chitosan)	Decreased allergenicity	-	Purified protein (genotype AA)	Kobayashi and others (2001); Hattori and others (2004); Aoki and others (2006)
β-LG	Heat treatment, glycation reaction	Decreased allergenicity	-	Purified protein (variant A)	Taheri-Kafrani and others (2009)
β-LG, ALA	Glycation reaction (glucose)	Decreased antigenicity: β-LG - 51.88 °C, 75.7 h and 2.59 WR*; ALA - 52.8 °C, 78 h and 5.96 WR*	-	Whey protein isolates	Bu and others (2009a, 2010a)

Table 3. Recent studies about the effect of traditional processing techniques on milk allergens (continued).

Allergen	Treatment	Allergenicity/antigenicity	Digestibility	Matrix	Reference
β -LG	Glycation (galactose, tagatose, dextran)	Glycation (gal/tag) at 40 °C, 24 h – unaffected allergenicity; Glycation (gal/tag) at 50 °C, 48 h – decreased allergenicity; Glycation with dextran – unaffected allergenicity	Glycation (gal/tag) at 40 °C, 24 h – unaffected digestibility; Glycation (gal/tag) at 50 °C, 48 h – increased digestibility with an aggregation inhibitor (Pyridoxamine); Glycation with dextran – unaffected digestibility	Mixture of variants A and B	Corzo-Martínez and others (2010)
β -LG, ALA	Glycation reaction (oligoisomaltose, maltose)	Decreased antigenicity: β -LG (oligoisomaltose) – 68.48 °C, 29 h and 4.7 weight ratio (WR)*; β -LG (maltose) – 60.8 °C, 54.3 h and 1.04 WR*; ALA (maltose) – 61.6 °C, 57.6 h and 1.1 WR*	β -LG/ALA – More susceptible to simulated gastric digestion after glycation with oligoisomaltose; Reduced antigenicity after glycation with oligoisomaltose and digestion	Purified proteins	Li and others (2011, 2013)
β -LG	Glycation (fructooligosaccharides, galactooligosaccharides and isomaltooligosaccharides)	Decreased allergenicity	-	Purified protein	Wu and others (2013)
β -LG	Glycation reaction (mPEG; nystose and fructofuranosyl nystose; fructooligosaccharides)	Decreased antigenicity	-	Purified protein	Zhong and others (2013, 2015, 2016)
ALA β -LG	Fermentation (<i>L. helveticus</i> and <i>S. thermophilus</i>)	Lower antigenicity at 6 h of fermentation with combined strains and 0.5 d of cold storage	-	Skim milk	Bu and others (2010b)
β -LG	Fermentation (<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> CRL 656)	Higher decrease of allergenicity induced by proteases of <i>L. delbrueckii</i> than with heat treatment	-	Purified protein and whey protein isolates. Greater hydrolysis of β -LG in WPI	Pescuma and others (2011)
α _{S1} -caseins, β -caseins	Fermentation (<i>L. helveticus</i> A75)	Decreased allergenicity induced by proteases of <i>L. helveticus</i>	-	Reconstituted skim milk	Ahmadova and others (2013)
ALA, β -LG, α -caseins, β -caseins	Fermentation (<i>L. casei</i>)	Decreased allergenicity of all proteins; Cold storage after fermentation did not affect allergenicity significantly. Heat treatment before fermentation: reduced allergenicity α -LA and β -LG	-	Reconstituted skim milk	Shi and others (2014)
ALA, β -LG, α -caseins, β -caseins	Fermentation (<i>L. rhamnosus</i> GG)	Decreased allergenicity of all proteins; Lower antigenicity at 12 h of fermentation and 0.5 d of cold storage; Heat treatment before fermentation: reduced allergenicity α -LA and β -LG	-	Reconstituted skim milk	Yao and others (2014)

Table 3. Recent studies about the effect of traditional processing techniques on milk allergens (continued).

Allergen	Treatment	Allergenicity/antigenicity	Digestibility	Matrix	Reference
ALA, β -LG, α -caseins, β -caseins, k-caseins, BSA, LF	Fermentation (<i>L. casei</i> LCY, <i>S. thermophilus</i> MK10, <i>B. animalis</i> B30)	Decreased allergenicity: different rates for each allergen, bacteria or stage of simulated gastric digestion	Simulated gastric digestion with fermentation: decreased allergenicity	Mare's milk	Fotschki and others (2015)
ALA, β -LG, α -caseins, β -caseins, k-caseins, BSA, LF	Fermentation (<i>L. casei</i> LCY)	Fermentation with or without simulated digestion: decreased allergenicity (higher with digestion)	Simulated gastric digestion with and without fermentation: decreased allergenicity	Buttermilk	Wróblewska and others (2016)
Whey proteins	Enzymatic hydrolysis	Decreased allergenicity (proteins degradation, inhibition of mast cell degranulation, reduction of ear swelling, reduction of T-cell proliferation)	Hydrolysis has a time-dependent effect on allergenicity	Whey protein concentrates	Knipping and others (2012)
Whey proteins	Enzymatic hydrolysis (trypsin)	Decreased allergenicity (reduced spleen lymphocyte proliferation, low levels of specific IgE and plasma histamine, increased secretion of IFN- γ)	-	Whey protein concentrates	Duan and others (2014)
Caseins, β -LG, ALA	Enzymatic hydrolysis	Decreased allergenicity (decrease IgE recognition, basophil activation and T cell response)	In most patients, increasing time of hydrolysis decrease allergenicity and immunogenicity	Whey protein hydrolysates	Meulenbroek and others (2014)
β -LG, ALA, α -caseins, β -caseins	Enzymatic hydrolysis (proteases of <i>E. Faecalis</i>)	-	α -caseins and β -caseins: complete hydrolysis; β -LG and ALA: partial hydrolysis	Skim milk and sodium caseinate	Biscola and others (2016)
β -caseins	<i>In vitro</i> protein digestion	Increased immunoreactivity after gastric digestion, higher with commercial porcine pepsin; Decreased immunoreactivity after duodenal digestion particularly with human enzymes	Faster degradation of β -caseins with human digestive enzymes than with commercial enzymes; Similar hydrolysates after gastric digestion; Less numerous and shorter peptides after gastroduodenal digestion with human fluids	Purified protein	Benedé and others (2014)
Caseins, β -LG	<i>In vitro</i> protein digestion	Simulated gastric digestion (0-60 min): decreased allergenicity; Simulated intestinal digestion (0-60 min): unaltered immunoreactivity	Different rates of hydrolysis in simulated digestions for each allergen, caseins and β -LG	Pasteurized non-fat dry milk	Do and others (2016)

*WR, weight ratio of sugar:protein isolates

A decreased allergenicity, confirmed by protein degradation, inhibition of mast cell degranulation, reduction of ear swelling, and reduction of T-cell proliferation was also observed in whey protein concentrates after hydrolysis with a time-dependent effect (Knipping and others 2012). Meulenbroek and others (2014) stated that in some patients this time-effect is not evident, indicating that the degree of hydrolysis is not decisive, but the presence and stability of IgE and T-cell epitopes in the hydrolysates are recognized by individual patients. As stated previously, heat treatment seems to increase the effect of enzymatic hydrolysis due to the possible exposure of cleavage sites as a result of thermal denaturation and, subsequently, enhancing the susceptibility of protein to undergo proteolysis. The molecular weight of peptides obtained after hydrolysis has also different effects on allergenicity, though there is a disagreement about the optimal molecular weight to be used depending on the chosen hydrolysis process. The specificity of enzyme, the sensitivity of the patients against the antigen, and the optimization of hydrolysis conditions may alter the final effect on allergenicity (Bu and others 2013). For example, the enzymatic digestion of β -LG may generate new antigenic substances, suggesting the existence of numerous epitopes scattered in hydrophobic regions of the molecules that became bio-available after enzymatic digestion (Selo and others 1999). Thus, the development of hypoallergenic formulas for cow milk patients requires a careful evaluation of all these parameters.

Digestibility

During the gastrointestinal digestion, the majority of proteins is extensively cleaved throughout the digestive tract by gastrointestinal enzymes and the peptidases of the intestinal brush border to small peptides and amino acids (Sanchón and others 2018). However, some larger peptides are known to survive to the harsh conditions of the digestion process, being absorbed by the intestinal mucosa and further presented to the immune system. When a significant portion of the protein (large peptide) resists to digestion, it is more likely to be presented to the inductive mucosal immune system, thus increasing its potential for sensitization (Bøgh and Madsen 2016). Upon a re-exposure to the allergenic peptide, which retained the proper size and conformation to be recognized by the immunocompetent cells, it increases the probability for eliciting an allergic response. The most common route of exposure to food allergens is via the gastrointestinal tract or the skin, which may occur at different pre- and postnatal stages. In the specific case of cow milk allergy, the gastrointestinal tract is the principal route of sensitization in children, normally during their first year of age, although the exposure by inhalation (especially in patients with asthma) to milk proteins might also be relevant as primary sensitizer (Leonardi and others 2014; Tran and others 2017).

As already stated, some authors combined the evaluation of the effect processing with the simulation of *in vitro* human gastrointestinal digestion to identify large stable fragments that come massively in contact with the mucosa and the immunocompetent cells. Some allergens are resistant to gastric and intestinal luminal digestion, easily reaching the intestinal mucosa where absorption can occur and trigger an immune response. However, alterations in allergen conformation caused by processing may affect the ability of food allergens to reach the jejunal mucosa and reduce their allergenicity. In addition, the intestinal digestion may potentiate the effect of processing on allergens and reduce even more their allergenicity (Do and others 2016). Heat treatment (Peram and others 2013), glycation (Li and others 2011, 2013), and fermentation (Fotschki and others 2015; Wróblewska and others 2016), when combined with simulated gastric digestion, presented an increased digestibility of milk proteins and, consequently, a reduction in their allergenicity. Studies focusing on the evaluation of different digestibility models are also very relevant for improved results (Mandalari and others 2009; Benedé and others 2014; Do and others 2016; Sanchón and others 2018). The study of Benedé and others (2014) evaluated the effect of commercial enzymes in comparison with human fluids during gastric and duodenal digestion. They found a faster degradation of β -caseins with the production of less numerous and shorter peptides and a decreased immunoreactivity after duodenal digestion, particularly with human digestive enzymes. An increased immunoreactivity of β -caseins after gastric digestion, higher with commercial porcine pepsin, was also observed, suggesting the unmasking of some IgE epitopes following hydrolysis. Sanchón and others (2018) compared the peptides obtained from *in vitro* digestion process with the ones collected *in vivo* from human jejunum. The authors verified that the common resistant regions of milk proteins were similar *in vitro* and *in vivo* digestion processes, revealing that the *in vitro* process might present a good approximation to the physiological gastrointestinal digestion of milk proteins. Damodaran and Li (2017) evaluated a two-step enzymatic approach to reduce the immunoreactivity of whey protein isolate and casein. The method consisted of a partial hydrolysis using different proteases (chymotrypsin, trypsin, or thermolysin) followed by repolymerization with microbial transglutaminase. After partial hydrolysis with chymotrypsin, trypsin, and thermolysin, whey protein hydrolysates preserved about 80%, 30%, and 20% of the original immunoreactivity, which decreased to 45%, 35%, and 5% upon repolymerization, respectively. Accordingly, the results suggested the possibility of producing hypoallergenic milk products. In another study performed by Quintieri and others (2017), whey protein concentrate digested with pepsin followed by ultrafiltration markedly reduced the antigenicity of whey hydrolysates, suggesting this method as a potential tool for the production of hypoallergenic infant food formulas.

Novel food processing technologies

High-pressure processing

High-pressure processing (HPP) is a novel technology able to inactivate microorganisms and enzymes in food, maintaining its original flavor and nutritional value, with the use of ultra-high pressures above 100 mPa at room temperature (Huang and others 2014). It is known that high pressures alter the conformational state of milk proteins, leading to enhanced flexibilities, unfolding, and aggregation. This causes the exposure of epitopes buried in the native molecule and increases allergenicity of whey proteins, but it also enhances susceptibility to the action of key digestive proteases with an eventual decrease of allergenicity. Moreover, aggregation of casein monomers reveals new determinants absent in monomeric forms, shown by the high IgE-reactivity of some patients only against these aggregates, but not against their individual components (Kleber and others 2007; Zhong and others 2011; López-Expósito and others 2012). The effect of high pressure in whey proteins is time-dependent, being influenced by different milk matrix and temperature levels. Kleber and others (2007) observed that skim milk and sweet whey (by-product of rennet-coagulated cheese) presented an augmented antigenicity with increasing pressure and time, but decreased antigenicity with increasing temperature, while in whey protein isolates the antigenicity was enhanced in all tested conditions of pressure and temperature. The diversity among the allergenicity rates detected in distinct types of milk and other products highlights the complexity of food ingredients and the importance of conducting studies on the effect of HPP in different food matrices (Huang and others 2014). High-pressure treatment is still unable to completely eliminate the allergenicity directly, but the combination with other strategies may result in possible solutions, such as HPP with enzymatic hydrolysis. López-Expósito and others (2012) demonstrated that β -LG hydrolysates (obtained with chymotrypsin and pepsin digestion) lost their allergenicity as revealed by the absence of anaphylactic reactions, mast cell activation, and a decrease in body temperature. A novel strategy named instant controlled pressure drop (DIC) that combines the effect of pressure and high temperature in a short time, followed by an instant pressure drop to vacuum was also tested. An augmented allergenicity of caseins and a reduction in whey protein immunoreactivity was caused by the dissociation of the casein micelles or aggregation of casein monomers and by changes in tertiary and secondary molecular structures of whey proteins, respectively (Boughellout and others 2015). The development of hypoallergenic milk formulas after HPP, combined with heat treatment or enzymatic hydrolysis, can reduce milk allergenicity and maintain sensory quality and nutritional value.

Food irradiation

Food irradiation uses ionizing radiation such as X-rays, high-energy electron beams (β -particles), or γ -rays for food sterilization, thereby improving the safety and shelf-stability without compromising nutritional or sensory quality, when applying the appropriate dose. Irradiation creates changes in the ability of IgE-allergen binding by the induction of structural denaturation, fragmentation, and/or aggregation of proteins, and, at least, the destruction of IgE epitopes (Ham and others 2009; Odueke and others 2016). Using γ -irradiation, Lee and others (2001) successfully reduced the allergenicity of β -LG by 7-fold. Meng and others (2016a) also proved the low potential *in vivo* allergenicity of irradiated ALA by the decrease in ALA-specific IgE levels, the inhibition of mast cells, and basophil activation, a significant decrease of histamine levels and a reduction of anaphylactic reactions in mice.

Ultraviolet and infrared radiation

Similar effects on the changes in conformational epitope structures of milk allergens were observed after the applications of ultraviolet (UV) and infrared (IR) radiations (Anugu 2009; Tammineedi and others 2013; Hu and others 2016). UV radiation has been used as a bactericidal agent since the year 1928. More recently, it has been used by the food industry as a sanitizing and disinfecting agent. Similarly, IR radiation can inactivate microorganisms by damaging intracellular components, such as DNA, RNA, and ribosomes in the cell, and modify the protein structure in food. According to Hu and others (2016), the allergenicity of α -caseins decreased after 15 min of the UV-C treatment and 5 min of far-IR treatment, with the first treatment being the most efficient as confirmed by the simulated digestion tests. Tammineedi and others (2013) also showed a reduction in the allergenicity of α -caseins and whey proteins in 25% and 27.7%, respectively, after 15 min of the UV-C treatment. However, it seems that these alterations are not enough for the production of hypoallergenic formulas. Microwave radiation combined with enzymatic hydrolysis could be an alternative to reduce the antigenicity of milk proteins, based on few studies that reported interesting results (Izquierdo and others 2008; El Mecherfi and others 2015).

Other technologies

Ultra-sound has gained much attention because it seems to have an effect on the allergenicity of shrimp and soy allergens (Li and others 2006). This new technology alters the conformation and reactivity of allergens by the implosion of sonication bubbles formed during the process, causing localized high pressure and temperature (Tammineedi and Choudhary 2014). However, ultra-sound seems to have no effect in reducing the allergenicity of milk proteins, as stated by Stanic-Vucinic and others (2012), and Tammineedi and Choudhary (2014).

Table 4. Recent studies about the effect of novel food processing techniques on milk allergens.

Allergen	Treatment	Allergenicity/antigenicity	Digestibility	Matrix	Reference
ALA β-LG	High pressure (HP)	HP treatment alone – unaffected allergenicity; HP in combination with pepsin and trypsin – decreased antigenicity of ALA and β-LG	β-LG – hydrolyzed by chymotrypsin and trypsin with and without HP; hydrolyzed by pepsin only with HP; ALA – hydrolyzed by pepsin and trypsin with and without HP; not hydrolyzed by chymotrypsin	Skimmed bovine milk	Peñas and others (2006)
β-LG	High pressure Heat treatment	Skim milk and sweet whey – increased antigenicity with increasing pressure and time but decreased with increasing temperature; Whey protein isolate – increased antigenicity with increasing temperature and pressure	-	Skim milk and sweet whey – antigenicity more susceptible to heat treatment	Kleber and others (2007)
β-LG	High pressure	-	400 mPa, 10 min – slight increased digestibility; 600-800 mPa – complete proteolysis by pepsin in 1 min	Spray-dried powder (85% pure)	Zeece and others (2008)
β-LG	High pressure	>200 mPa – decreased antigenicity; proteolysis at atmospheric pressure – unaffected allergenicity; 400 mPa and proteolysis – decreased allergenicity	Atmospheric pressure- unaffected digestibility; Above 400 mPa – increased digestibility by pepsin and chymotrypsin	Purified protein	Chicon and others (2009)
β-LG	High hydrostatic (HHP) pressure Proteolysis	Chymotrypsin and pepsin in combination with HHP - decreased allergenicity (absence of anaphylaxis, mast cell activation and basophils)	-	Purified protein	López-Expósito and others (2012)
β-LG	Dynamic high-pressure microfluidization (DHPM)	DHPM in combination with heat treatment – increased antigenicity; DHPM in combination with tryptic hydrolysis – decreased antigenicity	Improved digestibility by trypsin at increased pressure	Purified protein	Zhong and others (2011, 2014)
Caseins, ALA, β-LG	Controlled pressure drop (DIC)	Caseins – increased allergenicity; Whey proteins – decreased allergenicity at 0.4 and 0.6 mPa	-	Whey protein isolates Skimmed milk	Boughellout and others (2015)
α-caseins, β-LG	Irradiation (γ-radiation)	Decreased allergenicity (doses up to 10-kGy)	-	Purified proteins	Lee and others (2001)
β-LG	Irradiation (γ-radiation)	Decreased allergenicity	-	Purified protein	Byun and others (2002)
α-caseins, β-caseins	Irradiation (γ-radiation)	Decreased total ratio of proteins (10 kGy)	-	Cow's milk and Queso Blanco: similar results in both matrix	Ham and others (2009)
ALA	Irradiation (γ-radiation)	Decreased allergenicity (low levels of ALA-specific IgE; inhibition of mast cells and basophils activity, reduced levels of plasma histamine and reduced anaphylaxis in mice)	-	Purified protein	Meng and others (2016a, 2016b)
α-caseins	High pressure, UV-C, far-IR radiation	HPP – decreased allergenicity, high after simulated gastric and intestinal digestion; UV-C and FIR – decreased allergenicity	Increased digestibility after HPP treatment	Purified protein	Hu and others (2016)

Table 4. Recent studies about the effect of novel food processing techniques on milk allergens (continued).

Allergen	Treatment	Allergenicity/antigenicity	Digestibility	Matrix	Reference
α -caseins	UV-C, high intensity	UV-C – decreased allergenicity; high intensity	-	Purified proteins	Tammineedi and others (2013)
β -LG, ALA	ultrasound, NAPT	ultrasound and NAPT – unaffected allergenicity			
Whey proteins	Microwave irradiation Enzymatic treatments	Decreased allergenicity with the combination of microwave (200W) and hydrolysis with pronase, papain and alcalase	Increased hydrolysis by pronase, papain, alcalase and chymotrypsin after microwave treatment	Whey protein concentrates	Izquierdo and others (2008)
β -LG, whey proteins	Microwave irradiation Enzymatic treatments	Decreased allergenicity with the combination of microwave (200W) and peptic hydrolysis	Increased β -LG and whey proteins digestion with microwave treatment	Whey protein isolates and purified β -LG	El Mecherfi and others (2015)
β -LG	Genetic modification (Ala86Gln)	Decreased IgE-binding (mutated β -LG 9 times less recognized than native and recombinant wild type)	-	Purified protein (variant A)	Kazem-Farzandi and others (2015)
β -LG	Genetic modification (Lys69Asn)	Decreased IgE-binding (mutated β -LG 9 times less recognized than wild type)	-	Purified protein (variant A)	Taheri-Kafrani and others (2015)

The application of non-thermal atmospheric plasma (NTAP) also revealed having no effect on α -casein and whey protein allergenicity (Tammineedi and others 2013), despite the recent developments on the reduction of shrimp and wheat allergenicity (Nooji 2001; Shriver 2001). As demonstrated, it destroys mainly the 3D structure and the conformational epitopes of proteins, without the selective damage of allergenic IgE epitopes.

Genetic modification of key residues in binding sites of the allergens, without interrupting the global structure would be a possible solution for allergen-specific immunotherapy. Until now, 2 mutations on major epitopes of β -LG, namely Ala86Gln (Kazem-Farzandi and others 2015) and Lys69Asn (Taheri-Kafrani and others 2015), have been studied. The results indicated that both mutated proteins are recognized with 9-fold less potency by IgE in cow milk allergic patients than the native or recombinant β -LG. Mutations were responsible for the disappearance of important epitopes and, consequently, for the reduced IgE-binding to mutated β -LG.

Many efforts have been made to study the effect of processing on the allergenicity of milk proteins, with promising results showing the applicability of these technologies on the production of hypoallergenic formulas. Moreover, it seems that all the nutritional and sensory characteristics are maintained, in opposition to some conventional processes, such as heat treatment and enzymatic hydrolysis. The applicability of other novel processing technologies, such as pulsed ultraviolet light, pulsed electric field, and ohmic treatment, not yet evaluated in milk allergenicity, could be interesting approaches in the near future (Johnson and others 2010; Tammineedi and Choudhary 2014; Verhoeckx and others 2015; Cappato and others 2017).

ANALYTICAL METHODS FOR THE DETECTION OF MILK ALLERGENS IN PROCESSED FOODS

The increased awareness about the public health implications of food allergies has resulted in the need of developing analytical methodologies to control the presence of hidden allergenic ingredients in processed foods, allowing the enforcement of labeling regulations (Taylor and others 2014). Allergic consumers are fully dependent and supposedly protected by the label information of processed foods. However, accidental exposure to hidden allergens in foods owing to mislabeling or cross-contaminations during food processing constitutes a real risk for these individuals (Costa and others 2014, 2015). Milk proteins are often applied as technological aids, and, thus, they are present in several types of foods as an ingredient. However, owing to the common practice of using shared production lines to manufacture different food formulations, accidental cross-contaminations are very likely to occur. To increase the well-being and safety of sensitized individuals, food products for human consumption must declare all potentially allergenic

ingredients irrespective of their amount. Therefore, proper and highly sensitive analytical methodologies represent essential assets to aid the industrial management of allergenic foods and, subsequently, to facilitate allergen control/monitoring by regulatory authorities.

The choice of the best method for allergen analysis depends on specific criteria, such as target analyte (proteins or DNA), basis of detection (chemical or biological), cost per run/analysis, setup, cross-reactivity phenomena, need for expertise knowledge, and possibility for multi-target detection (Johnson and others 2011). Moreover, appropriate sensitivity and specificity to trace minute amounts in complex food matrices are important requirements (Costa and others 2012). The ideal limit of detection (LOD) for allergens in food products has been considered in the range of 1-100 mg/kg (Poms and others 2004), although these values of reference are currently being revised. Morisset and others (2003) established a threshold of clinical reactivity to milk of 30 mg/kg for milk proteins, to guarantee 95% safety for patients who are allergic to milk, based on the consumption of 100 g of product. More recently, using appropriate statistical dose-distribution models, the reference dose for milk was defined as 0.1 mg of protein, considering the eliciting dose that protects 99% of the milk-allergic population (ED01). According to the conversion factors available at USDA (Department of Agricultural National Nutrient Database), this reference dose represents 3.03 mg of liquid milk per kg of food and 0.28 mg of non-dry fat milk per kg of food (Taylor and others 2014). Presently, there are several technical possibilities for the detection of milk allergens in foodstuffs, and recent developments are summarized in Table 5.

Protein-based methods

The classical protein-based methods are still the most commonly used for the detection of allergens in foods. They are based on allergen-antibody interactions and available in different formats, such as lateral flow devices (LFD), dipstick tests, enzyme-linked immunosorbent assay (ELISA), and immunoblotting. Currently, leading-edge technologies have reached particular attention for allergen analysis, namely immunosensors and mass spectrometry (MS) platforms (Costa and others 2017).

Immunoassays

Antibodies play an important role in most of the allergen detection methods due to their specific binding to respective antigens, which create very sensitive and specific systems. Most immunoassays for the detection of milk allergens are based on ELISA, which can provide quantitative results through the comparison of optical or fluorescent signals of the unknown samples with standard curves (Costa and others 2017). Different formats are available, namely direct ELISA, indirect ELISA, competitive ELISA, although the sandwich

ELISA is the most commonly used. Presently, owing to the increased demand for rapid and reliable tests for the detection of specific allergens in food, various commercial ELISA kits have entered the market (Table 6). They are able to detect specific proteins, such as caseins and β -LG, or total milk proteins with reported LOD values ranging from 0.015 mg/L to 2 mg/L, though the type of food matrix and the effect of processing can affect these values. Heating can result in the formation of insoluble protein aggregates, which may be undetectable by ELISA. In addition, the interaction with compounds of the food matrix and differences in antibody recognition of heat-denatured proteins can affect the detection and, particularly, the quantitative determination of allergenic proteins in food products. Therefore, a careful choice of the proper kit is always needed (Downs and Taylor 2010). Table 6 presents a set of commercially available ELISA kits for the detection of milk allergens.

Table 5 gathers not only the information regarding the application of commercial kits to assess the detection/quantification of milk in foods, but also the in-house developed ELISA. Deckwart and others (2014) have developed 2 systems (indirect and indirect sandwich ELISA) for the detection of caseins in white and red wines, achieving a LOD between 10 μ g/L and 200 μ g/L. The reported sensitivities were in accordance with the threshold of 0.25 mg/L requested by the OIV (Organisation Internationale de la Vigne et du Vin, Paris, France), regarding the presence of fining agents (milk caseins, ovalbumin) in wines. Their work also demonstrated the influence of a complex food matrix on the performance of this type of assay. The development of two ELISA formats (indirect competitive and sandwich) was performed by de Luis and others (2009) for the detection of β -LG in processed foods. The competitive and sandwich systems, with LOD of 0.5 mg/kg and 0.05 mg/kg, respectively, were able to detect undeclared milk ingredients in 14% of the tested commercial samples. The sandwich format proved to be more specific and sensitive because of being less affected by the matrix than the indirect competitive one.

LFD are other rapid and specific immunochemical tests for allergen detection. The principle of the method is the same as that of ELISA, but it allows a simpler and faster performance with qualitative or semi-quantitative results that can be interpreted visually. The lack of quantitative information and the susceptibility of these devices in providing false-negative results are the major drawbacks associated with their use. However, they are largely applied in the food industry to monitor the cleaning of food processing equipment and food product contamination (Courtney and others 2016). There are several LFD kits in the market that detect milk allergens in food products in a few minutes and on-site, with LOD down to 0.5 mg/L, as demonstrated in Table 6.

Table 5. Methods for the detection of cow's milk allergens in different food products.

Method	Matrix	Target allergen	Limit of detection	Analytical range	References
IC-ELISA and S-ELISA	Model processed foods (sausage, bread and pâté); commercial samples labelled with milk ingredients	β -LG	0.05 mg/kg (S-ELISA) 0.5 mg/kg (IC-ELISA)	5 – 100 μ g/L (S-ELISA) 15 – 500 μ g/L (IC-ELISA)	de Luis and others (2009)
ELISA, commercial kits	Flours and bread	Casein	Not reported	Not reported	Heick and others (2011a)
S-ELISA, Commercial kit	Meat-based products	β -LG	0.2 mg/kg	0.5 – 13.5 mg/kg	Decastelli and others (2012)
ELISA, commercial kits	Cookie dough and cookies	Caseins, β -LG	Not reported	Not reported	Khuda and others (2012b)
ELISA, commercial kits	Chocolate	Caseins, β -LG	Not reported	Not reported	Khuda and others (2012a)
I-ELISA and IS-ELISA	Red and white wines	Caseins	0.1 and 0.2 mg/L (White and Red Wine, I-ELISA), 0.01 mg/L and 0.1 mg/L (White and Red Wines, S-ELISA)	10-1000 and 10-3000 μ g/L, (white and red wines, indirect ELISA) 10-1000 and 10-10000 μ g/L (white and red wines, sandwich ELISA)	Deckwart and others (2014)
ELISA, commercial kits	Desserts	Caseins, β -LG, Milk proteins	3 mg/kg	0 – 30 mg/kg	Johnson and others (2014)
ELISA, commercial kits	Cookie dough, cookies	Caseins	0.04 mg/kg	0.2 – 6 mg/kg	Török and others (2014)
ELISA, Commercial kits	Dry mixes and processed cookies	Caseins	10 mg/kg	2.5 – 15 mg/kg	Gomaa and Boye (2015b)
ELISA, commercial kit	Working surfaces	β -LG	0.2 μ g	Not reported	Galan-Malo and others (2017)
Optical Immuno-biosensor (SPR)	Bovine milk, colostrum, and infant formulas	LF	19.9 μ g/mL	0 – 1000 ng/mL	Indyk and Filonzi (2005)
Optical immunosensor (resonance enhanced absorption)	Processed milk matrices	β -LG	Not reported	100 μ g/ml – 100 ng/ml	Hohensinner and others (2007)
SPR immunosensor	Bovine milk	Caseins	0.01 mg/L	0.1 – 10 mg/L	Hiep and others (2007)
SPR immunosensor	Milk	ALA, β -LG, BSA, LF	Not reported	0 – 1000 ng/mL	Billakanti and others (2010)
Optical Immuno-biosensor (SPR)	Milk-based products	ALA	0.12 mg/mL	10 – 1000 ng/mL	Indyk (2009)

Table 5. Methods for the detection of cow's milk allergens in different food products (continued).

Method	Matrix	Target allergen	Limit of detection	Analytical range	References
Optical immunochip biosensor (resonance enhanced absorption)	Milk- and whey protein-based food samples	β -LG	1 ng/mL	Not reported	Maier and others (2009)
Antibody-microarrayed chip using iSPR	Cookies, chocolates	k-casein	0.2 mg/kg (cookies)	0.1 – 10 mg/L	Raz and others (2010)
Electrochemical immunosensor	Cheese	Caseins	0.05 ng/mL	0.1 – 10 ng/mL	Cao and others (2011)
Electrochemical immunosensor	Cake, cheese snacks, and biscuits	β -LG	0.85 pg/mL	0.001 – 100 ng/mL	Eissa and others (2012)
Amperometric Magnetoelectrode	Cow and human milk (raw, UHT and pasteurized)	β -LG	0.8 ng/mL	2.8 – 100 ng/mL	Ruiz-Valdepeñas and others (2015)
Electrochemical immunosensor	Cow and human milk (raw, UHT and pasteurized)	ALA	11.0 pg/ml	37.0 – 5000 pg/ml	Ruiz-Valdepeñas and others (2016)
LC-MS	Fruit juices	ALA (2 peptides), β -LG (4 peptides)	1 μ g/mL	Not reported	Monaci and van Hengel (2008)
LC-ESI-Q-TOF-MS	Cookies	α_{S1} -casein, α_{S2} -casein, BSA, β -casein	100 mg/kg	10 – 1000 mg/kg	Monaci and others (2010b)
LC-ESI-MS/MS	Wine	α_{S1} -casein (3 peptides) α_{S2} -casein (2 peptides) β -casein (3 peptides) k-casein (1 peptide)	50 μ g/mL	10 – 100 g/hL	Monaci and others (2010a)
LC-MS/MS	Bread	α_{S1} -casein (2 peptides) α_{S2} -casein (2 peptides)	5 mg/kg	10 – 500 mg/kg	Heick and others (2011b)
LC-MS/MS	Bread, flours	α_{S1} -casein (2 peptides)	5 mg/kg	10 – 500 mg/kg	Heick and others (2011a)
LC/HCD-MS	Wine, cookies	β -casein (1 peptide) α_{S1} -casein (3 peptide)	1.6 mg/kg	1.6 – 22 mg/kg (Cookies) 1.6 – 6 mg/kg (Wines)	Monaci and others (2011)
MALDI-TOF/MS and MS/MS analysis	Wheat and glucose, matrix, chocolate, cookies	ALA (3 peptides), β -LG (6 peptides)	Not reported	Not reported	Cucu and others (2012)
LC-MS/MS	Chocolates, milk based products	ALA (1 peptide) β -LG (2 peptides) α_{S1} -casein (2 peptides) β -casein (2 peptides)	1 ng/mL		Ansari and others (2011)
LC-ESI-3D-IT-MS	White wine	β -casein (1 peptide)	0.09 – 0.23 mg/kg	10 – 500 mg/L	Losito and others (2013)

Table 5. Methods for the detection of cow's milk allergens in different food products (continued).

Method	Matrix	Target allergen	Limit of detection	Analytical range	References
Protein standard absolute Quantification (PSAQ) using UPLC-ESI-MS	Cookies, biscuits	Labelled α_{S1} -casein	0.6 fmol	0 – 100 mg/kg	Newsome and Scholl (2012)
UPLC-TQ-MS/MS	Pasta, dough	β -casein	0.5 mg/kg	1 – 100 μ g/mL	Chen and others (2015)
LC-ESI-MS/MS	Red wine	α_{S1} -casein (1 peptide) β -casein (1 peptide)	0.5 mg/L (α_{S1} -casein) 0.01 mg/L (β -casein)	1 – 100 mg/L 0.03 – 50 mg/L	Mattarozzi and others (2014)
microHPLC-ESI-MS/MS	Cookies	α_{S1} -casein (2 peptides)	0.1 mg/kg	0.5 – 20 mg/L	Monaci and others (2014)
Orbitrap monostage MS vs hybrid linear ion trap MS	Wine	α_{S1} -casein (6 peptides) α_{S2} -casein (1 peptide) β -casein (2 peptide) k-casein (1 peptide)	0.2–2 μ g/mL	0.2–2 μ g/mL	Pilolli and others (2014)
LC-MS	Dry mixes and processed cookies	Caseins (5 peptides)	10 mg/kg	10 – 1000 mg/kg	Gomaa and Boye (2015b)
HPLC-MS/MS	Meat products	Caseins (2 peptides) Whey proteins (2 peptides)	1 mg/kg	0 – 139 mg/kg 0 – 4000 mg/kg	Jira and Schwägle (2015)
LC-ESI-MS/MS	Bakery products	α_{S1} -casein (1 peptide)	0.11 mg/kg	1 – 150 mg/kg	Lamberti and others (2016)
UHPLC-MS/MS	Tomato sauce, cookies, chocolate, ice-cream	Caseins (4 peptides), whey proteins (3 peptides)	0.5 mg/kg (caseins), 5 mg/kg (whey)	0 – 5 mg/kg	Planque and others (2016)
LC-MRM/MS	Cookies, biscuits, peanut jam, waffles, patisseries, yolk pie, wheat meal	β -LG (2 peptides), ALA (2 peptides), α_{S1} -casein (1 peptide)	0.2 mg/L (β -LG), 0.39 mg/L (ALA), 0.2 mg/L (α_{S1} -casein)	0.48 – 31.25 mg/L (β -LG) 0.97 – 31.25 mg/L (ALA) 0.48 – 31.25 mg/L (α_{S1} -casein)	Ji and others (2017)
Tetraplex real-time PCR	Bakery products	Mitochondrial DNA (tRNA-Lys)	0.64 μ g DNA/L	0.64–20 μ g DNA/L	Köppel and others (2010)
Hexaplex Real-time PCR	Sausages, cookies, chocolates, sandwiches, parfais	Mitochondrial DNA (tRNA-Lys)	2 μ g DNA/L	2–20 μ g DNA/L	Köppel and others (2012)
Real-time PCR with TaqMan probe	Candies, biscuits, chocolate, drinks	ALA	0.025 mg/L	0.00025 – 25 mg/L	Xiao and others (2016)

C-ELISA, competitive ELISA; I-ELISA, indirect-ELISA; S-ELISA, Sandwich ELISA; LC, liquid chromatography; MS, mass spectrometry; ESI, electrospray ionization; UHP, ultra-high pressure; MRM, multiple reaction monitoring; IT, ion trap.

Biosensors are considered emerging tools for allergen detection, since they are fast, repeatable, and highly sensitive approaches with great potential for full automation. In brief, biosensors are based on the direct recognition of a biological interaction between a receptor (antibody or probes) and a target molecule (protein or DNA) by means of a transducer that produces a measurable signal (Schubert-Ullrich and others 2009; Prado and others 2016; Costa and others 2017). Several studies have been performed using SPR immunosensors to detect milk allergens in different food matrices, reaching sensitivities of 1 ng/L to 0.12 mg/mL (Indyk and Filonzi 2005; Hiep and others 2007; Indyk 2009; Billakanti and others 2010; Raz and others 2010). Electrochemical immunosensors are also used for milk protein detection (Cao and others 2011; Eissa and others 2012; Ruiz-Valdepeñas and others 2015; Ruiz-Valdepeñas and others 2016). Eissa and others (2012) developed an immunosensor able to detect down to 0.85 pg/mL of β -LG in food products, which is the lowest LOD reported for this protein by electrochemical immunosensors (Table 5). Nonetheless, owing to the fact that these systems are based on the biological interaction between an antibody and the respective allergen/marker protein, a careful interpretation is always needed to avoid false positive or false negative results (Johnson and others 2011; Costa and others 2012; Khuda and others 2012b; Gomaa and Boye 2015b; Gomaa and Boye 2015a).

In general, the immunoassays are able to detect major allergenic milk proteins, such as ALA and β -LG, in a wide diversity of food matrices. However, information on their development is still lacking to determine the presence of hydrolyzed milk proteins in foods, as they often retain their immunoreactivity. So far, few studies have been carried out to evaluate the immunoreactivity of pure whey and casein hydrolysates (Pessato and others 2016; Damodaran and Li 2017), but with no application to the detection of peptides from hydrolyzed milk proteins in different food matrices.

MS platforms

Mass spectrometry has played an important role in proteomic research and has proved to be a powerful analytical technique for both protein and peptide analysis, encompassing the identification, characterization, and determination of food allergens (Monaci and Visconti 2009). MS platforms offer several advantages, such as high rapidity, accuracy, sensitivity, specificity, and reproducibility (Picariello and others 2011). Its sensitivity is comparable to ELISA and quantitative polymerase chain reaction (PCR), allowing a multi-target detection in a single run with high specificity. Additionally, the problems related to cross-reactivity often linked to immunoassays are eliminated because the detection of the target peptide/protein does not require interaction with a biological receptor (antibody), enabling a direct and unequivocal identification of the target analytes. The proteomic analysis of a sample commonly consists of one/several separation steps at protein and/or peptide level

(gel electrophoresis, liquid chromatography - LC), followed by MS analysis. There are 2 main approaches for allergen detection, quantification, and characterization: the bottom-up approach, where proteins are digested with enzymes, such as trypsin, prior to MS analysis; and the top-down approach, where the whole proteins are fragmented directly inside the mass spectrometer, avoiding the variable step of protein digestion. The bottom-up approach is the most commonly used due to the current limited performance of top-down-based instruments (Prado and others 2016).

There is an increasing number of reports regarding the detection of milk allergens in foodstuffs by MS technologies. The majority of these works use multi-target approaches, enabling the discrimination of different milk allergens (caseins, ALA, and β -LG) in different food matrices, such as wines, cookies, infant formulas, and bakery products, with sensitivities ranging from 0.01 mg/kg to 5 mg/kg (Table 5). Very recently, Ji and others (2017) developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the confirmation and quantification of 3 milk allergens (ALA, β -LG, and α_{S1} -casein) in different food products, namely cookies, biscuits, waffles, patisseries, yolk pie, among others, with a reported LOD of 0.2 mg/kg for β -LG and α_{S1} -casein, and 0.39 mg/kg for ALA. Similar sensitivities were obtained by Losito and others (2013) whose method, based on liquid chromatography-electrospray ionization-ion trap-mass spectrometry (LC-ESI-IT-MS), was able to detect caseinate at trace levels in different Italian white wines, with LOD ranging from 0.09 mg/L to 0.29 mg/L, depending on the wine.

Like in immunochemical methods, food matrix also affects the sensitivity of MS methods. Accordingly, the reported LOD values are higher for the analysis of allergens in complex food matrices such as chocolates. The effect of food processing on the target allergens should also be accounted for because their structure is known to be differently affected by distinct types of processing, requiring the identification of marker peptides in both raw and processed matrices. Food processing alters the extractability and solubility of allergenic or other marker proteins, which can compromise the good performance of the MS-based method. However, the advantages of high accuracy, specificity, and multi-target analysis, make the MS-platforms more widely used for allergen analysis than the classical immunochemical assays (Prado and others 2016).

DNA-based methods

Recently, DNA-based methods for allergen detection have been received with increasing interest due to their high specificity, sensitivity, independence from possible biological effects associated with antibody production, and high thermal stability of DNA molecules, particularly relevant to analyze processed foodstuffs. Therefore, DNA-based methods have proved to be excellent alternatives to protein-based methods, especially when analyzing

highly processed foods (Costa and others 2017). DNA targets might be genes that encode allergenic proteins or other specific sequences, therefore they are considered as indirect markers of the presence of an allergenic ingredient. Most of the published works using DNA-based methods consist of the amplification of the initial target DNA sequences by PCR with the use of specific primers, responsible for conferring a high specificity level to the assays (Mafra and others 2008; Prado and others 2016). The main approaches used for allergen detection are end-point PCR, multiplex PCR, real-time PCR, and PCR-ELISA, but recently new promising advances have gained much interest, such as real-time PCR coupled to high-resolution melting (HRM) analysis, single-tube nested real-time PCR, DNA arrays, and genosensors. Most reports apply DNA-based methods for the authentication of milk products, such as cheeses (Dalmasso and others 2011), and for the identification of different species in milk products (Bottero and others 2003; Mafra and others 2004; Lopez-Calleja and others 2007; Zhang and others 2007; De and others 2011). In contrast, few studies describe the detection of milk allergens in foodstuffs, with real-time PCR being the main technique used for this purpose. Real-time PCR methods have the advantages of providing quantitative results with adequate setup cost, reasonable running time, and moderate requirements for specialized equipment and personnel. Köppel and others (2010, 2012) developed 2 tetraplex and 2 hexaplex real-time PCR systems with TaqMan probes for the simultaneous detection of several allergens in food, including milk allergens (Table 5).

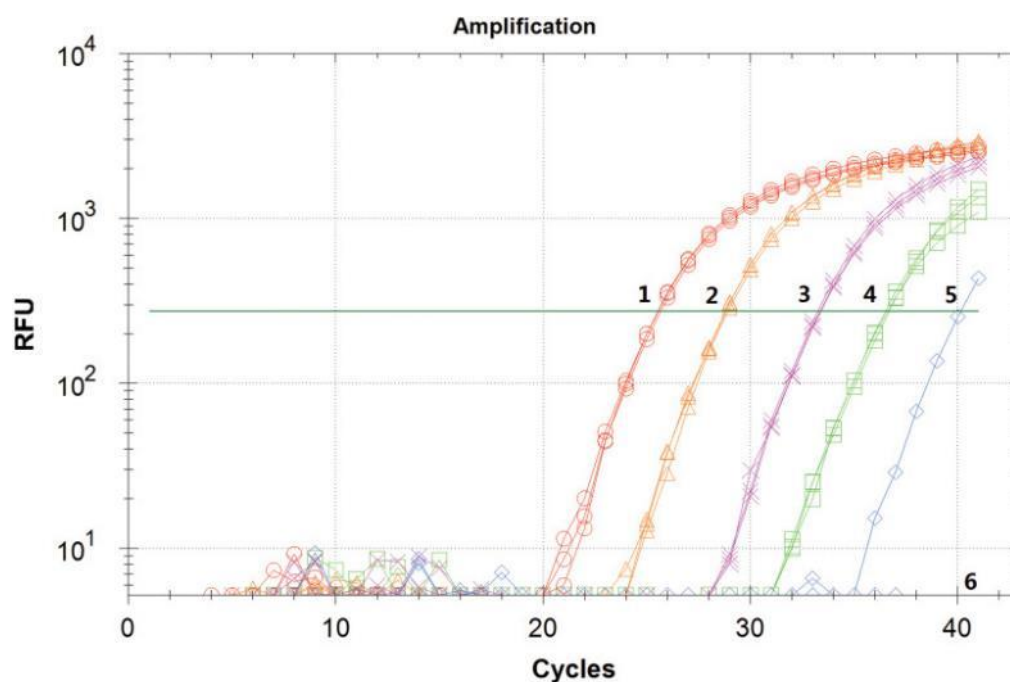


Figure 3. Amplification of an ALA gene fragment from 10-fold serial dilutions of cow's milk DNA. Legend: 1-6: 50 ng (circle), 5 ng (triangle), 0.5 ng (cross), 0.05 ng (square), 0.005 ng (diamond), and 0 (straight line) ng of DNA, respectively. Reprinted from Xiao and others (2016) with permission from Elsevier Ltd.

The methods exhibited good specificity with a sensitivity down to 0.64 µg/mL of bovine DNA. A real-time PCR method with a TaqMan minor groove binder probe for the specific detection of ALA gene in food was developed by Xiao and others (2016). The method showed a sensitivity of 0.05 ng of bovine DNA (Figure 3) and it was applied to 42 commercial samples in order to verify the compliance with the label for the presence of milk as an ingredient.

FINAL REMARKS

The concern with milk allergy has increased over the last few years, mainly because most of the affected individuals are infants below the age of 3. Currently, there is no treatment for food allergies and, consequently, the sensitized individuals have to avoid milk products and all foodstuffs containing milk derivatives. In the case of accidental exposure, different pharmaceuticals (H1- and H2-antihistamines, beta-2 agonists, or glucocorticosteroids) can be used to relieve the clinical symptoms associated with adverse immunological responses, although epinephrine is commonly used to treat very severe and life-threatening allergic reactions (anaphylaxis).

Recent advances have been made in the development of effective strategies to treat milk allergy and induce tolerance in allergic patients. Oral immunotherapy seems to be a promising approach, with a success rate varying from 37 to 70%. Another approach is focused on the reduction of milk allergenicity by the use of new food processing technologies. Processing induces changes to milk proteins that can largely affect their susceptibility to gastrointestinal digestion, absorption kinetics, and, consequently, their immunoreactivity. Therefore, the allergenic potential of milk proteins may be diminished by selecting appropriate parameters during processing. In spite of reported reductions in allergenicity with some types of processing, no method is completely effective. Due to differences in the degree of allergenic reactions and in the tolerance among different patients, it is important to conduct more *in vitro* and *in vivo* studies to test different conditions and combinations of milk processing methods.

To improve consumer protection and to ensure life quality of sensitized individuals, several regulations and directives have been established, which state the obligation of labeling the potentially allergenic ingredients/foods, including milk. Thus, the development of methodologies to detect and quantify allergens is imperative to allow the enforcement of the labeling regulations and control the presence of hidden allergenic ingredients. ELISA have provided sensitive and specific methods for the detection of milk proteins in food products, although with the limitation of being seriously affected by food processing that could lead to false negative results. Immunosensors have also been applied, resulting in fast, repeatable, and potentially fully automated analysis. MS platforms, such as LC-

MS/MS, have shown their efficacy in the detection of allergenic milk proteins and peptides, which can be used as confirmatory tools for the identification of multiple allergens. DNA-based methods, despite consisting of indirect detection approaches, are considered efficient alternatives, being less prone to be affected by food processing. The capability of methods to detect allergens in food products at trace levels depends on many factors, including the food matrix, the extraction method, the food processing operation, and the form in which the allergen is present. Despite these limitations, the currently available methods for the detection of milk allergens are playing a crucial role in the provision of information to allergic consumers, which is essential for an elimination diet required to protect their health.

Acknowledgements

This work was supported by FCT (Fundação para a Ciência e Tecnologia) through project UID/QUI/50006/2013 – POCI/01/0145/FEDER/007265 with financial support from FCT/MEC through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 and by the project NORTE-01-0145-FEDER-000011. C. Villa and J. Costa are grateful to PhD (PD/BD/114576/2016) and post-doctoral (SFRH/BPD/102404/2014) grants from FCT financed by POPH-QREN (subsidized by Fundo Social Europeu [FSE] and Ministério da Ciência, Tecnologia e Ensino Superior [MCTES]).

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2.2. Experimental part

Cow's milk allergens: Screening gene markers for the detection
of milk ingredient in complex meat products

Food Control, **2020**, 108, 106823

Detection and quantification of milk ingredients as hidden allergens in
meat products by a novel specific Real-time PCR method

Biomolecules, **2019**, 9, 204

Effect of autoclaving and *in vitro* gastro-duodenal digestion on the modulation of
IgE binding capacity of milk proteins incurred in sausage model food

Nutrients (submitted)



2.2.1. Cow's milk allergens: Screening gene markers for the detection of milk ingredients in complex meat products

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ABSTRACT

Cow's milk proteins are food allergens present in uncounted foodstuffs. By exploiting and comparing different regions of bovine genome, this work intended to develop a real-time PCR method to detect/quantify traces of milk in complex foods. Two mitochondrial (cytb and 12S rRNA) and two nuclear (β -lactoglobulin and β -casein) genes showed the most promising results by qualitative PCR. However, real-time PCR assays targeting nuclear genes revealed cross-reactivity with some meat species, while the 12S rRNA assay was the most specific (only minor reactivity with two species above 36 cycles of amplification), providing a linear dynamic range of 10–0.05% of cow's milk protein concentrate (MPC) in raw meat and cooked ham, with acceptable performance parameters. The results highlight the potentiality of the assay for the development of a novel method to quantify milk ingredients in meat products.

Keywords: Food allergen, *Bos taurus*, gene markers, real-time PCR, meat products, cooked ham.

INTRODUCTION

Milk is one of the eight groups of foods accountable for about 90% of food-allergic reactions. Cow's milk allergy is a common food allergy in early childhood, with an incidence estimated from 2.0% to 7.5% in the first year of life (Mousan & Kamat, 2016). It is typically mediated by immunoglobulin E (IgE), inducing adverse immunological reactions to milk proteins in sensitised/allergic individuals, even when ingested at trace amounts (Monaci, Tregoat, van Hengel, & Anklam, 2006; Villa, Costa, Oliveira, & Mafra, 2018). β -Lactoglobulin, caseins and α -lactalbumin are the most abundant proteins in cow's milk, being also classified as major allergens (Monaci et al., 2006; Villa, Costa, Oliveira, & Mafra, 2018).

Besides the well-known dairy products, such as milk beverages, cheeses and yogurts, milk proteins are present in a great number of processed foods, namely meat and fish products, desserts and bakery products, among others. Enriched milk powders, such as milk protein concentrates (MPC, protein content <90%) or milk protein isolates (MPI, protein content >90%), are typical dairy ingredients used by the food industry due to their functional properties (Fox, 2001; Meena, Singh, Panjagari, & Arora, 2017). MPC are described as concentrated forms of milk proteins that contain both caseins and whey proteins in the same proportions as the whole milk, providing an increased heat stability, solubility and gel-forming capacity to food products (Uluko, Liu, Lv, & Zhang, 2016).

The accidental exposure to these proteins is recurrent, posing allergic individuals to a constant threat. Therefore, the EU has established specific legislation requiring the mandatory labelling of fourteen groups of foods (thirteen potentially allergenic), including milk and dairy products, that must be emphasised over the rest of the ingredients enumerated in processed food labels, regardless of their quantity (Directive 2007/68/EC; Regulation (EU) No 1169/2011). However, mislabelling or unintentional cross-contaminations during food processing can occur, making the development of analytical methodologies with high specificity and sensitivity a demanding task.

Presently, there several methods for the detection of milk allergens in processed foodstuffs, targeting allergenic proteins by enzyme-linked immunosorbent assay (ELISA) (de Luis, Lavilla, Sánchez, Calvo, & Pérez, 2009; Decastelli, Gallina, Manila Bianchi, Fragassi, & Restani, 2012; Deckwart et al., 2014) and mass spectrometry (MS) platforms (Boo, Parker, & Jackson, 2018; Jira & Schwägele, 2015; Pilolli, De Angelis, & Monaci, 2017; Pilolli, De Angelis, & Monaci, 2018; Planque et al., 2019). More recently, DNA-based methods have revealed as promising techniques in allergen detection due to their high specificity and sensitivity, taking advantage of their applicability to processed food analysis because of the high thermal stability of DNA molecules (Costa, Fernandes, Villa, Oliveira,

& Mafra, 2017a). Some DNA-based methods have been developed targeting milk allergen-encoding genes, such as α -lactalbumin (Xiao, Qin, Wenju, & Qin, 2016) or mitochondrial genes (Köppel et al., 2010; Köppel, Velsen-Zimmerli, & Bucher, 2012), as molecular markers for milk detection in foods. However, DNA-based methods focusing on the detection of cow's milk ingredients in meat products, such as milk protein concentrates, which are frequently used in the preparation of hams and sausages (Spychaj, Pospiech, Iwańska, & Montowska, 2018), are still lacking.

In this context, the present work intended to investigate different mitochondrial and nuclear regions as potential DNA markers for the development of a highly specific and sensitive PCR method. Different regions of bovine genome (mitochondrial vs allergen-encoding genes) were extensively exploited and compared in order to develop a method able to detect trace quantities of cow's milk in complex/processed foods. The selected regions were used in the development of qualitative PCR and real-time PCR assays, evaluating their sensitivity and specificity with the final goal of selecting the best molecular marker for the detection/quantification of cow's milk as a potential allergenic food in processed meat products.

MATERIALS AND METHODS

Sampling

Two sets of binary model mixtures containing 10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% (w/w) of MPC in turkey meat were prepared. To simulate ham preparation, turkey meat was previously minced using a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany), with the addition of 8 g of salt and 4 g of powder sugar in a total of 1 kg. To facilitate homogenisation, a sterile phosphate-buffered saline solution (0.2 M) was added to this mixture. MPC was provided by FORMULAB (Maia, Portugal) and the turkey meat (muscle) was acquired at a local retail market. The exact milk protein content of MPC was determined by the Kjeldahl method, corresponding to 83.4%. Taking into consideration this value, the first mixtures containing 10% of MPC were prepared by adding the required amount (24.0 g) to minced turkey meat (mixture for ham) (in a total of 200 g). The other model mixtures were prepared by successive additions of the mixture for ham. A set of binary mixtures was cooked in the oven at 67 °C for 5 h simulating ham production, while the other set remained raw.

For assay specificity testing, 15 different meat species were tested: boar, duck, partridge, hare, quail, pheasant, deer, rabbit, chicken, turkey, lamb, goat, ostrich, horse and pork.

To avoid contaminations, all mixtures and meat samples were homogenised separately, and all materials and different containers were previously treated with a DNA

decontamination solution. All mixtures and meat samples were immediately stored at -20°C until further analysis.

DNA extraction

The NucleoSpin food kit (Macherey-Nagel, Düren, Germany), with some minor modifications, was the selected protocol to extract the DNA from the binary model mixtures and from MPC. DNA from meat species was extracted using the Wizard method as described by Mafra, Silva, Moreira, da Silva, and Oliveira (2008). The extractions were performed at least in duplicate assays using 200 mg of each sample. The extracts were kept at -20°C until further analysis.

To evaluate the yield and purity of DNA extracts, UV spectrophotometric DNA quantification was performed on a SynergyHT multimode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), using a Take 3 micro-volume plate accessory. The DNA content was assessed by the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA).

Oligonucleotide primers and probes

The primers used in this work were designed on different sequences of mitochondrial and nuclear genes of *Bos taurus* retrieved from NCBI database or previously reported in the literature (Table 1). Both hydrolysis probes, targeting the mitochondrial 12S rRNA and *cytb* genes sequences of cow (916-P – FAM-TCTAGAAGGATATAAAGCACCGCCAAGT-BHQ1 and Bos-P – FAM-CCGATACATACACGCAAACGGAGCTTCAA-BHQ1, respectively), were specifically designed for this work and labelled with FAM as fluorescent reporter and BHQ-1 as quencher. All the primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

For specificity purposes, *in silico* analysis of the nucleotide and primer sequences was performed using the basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), respectively. OligoCalc software (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) was also used to check primer properties and to ensure the absence of primer hairpins and self-hybridisation.

To evaluate the amplification capacity of extracts, all the samples were amplified by qualitative PCR using universal eukaryotic primers (18SRG-F – CTGCCCTATCAACTTTCGATGGTA and 18SRG-F – TTGGATGTGGTAGCCGTTTCTCA) targeting a sequence of 113 bp of a highly conserved 18S rRNA nuclear region (NCBI accession no. HQ873432.1) (Costa, Oliveira, & Mafra, 2013).

Table 1. Key data of primers designed to specifically target DNA sequences from mitochondrial and nuclear genome of cow.

Target region	Primers	Sequence (5' → 3')	Amplicon (pb)	Concentration (nM)	Annealing temperature (°C)	NCBI (Accession no.)	Reference
cyt b	Bos-F	CTGCCGAGACGTGAAC TACG	99	160	65	D34635.1	Amaral, Santos, Melo, Oliveira, and Mafra (2014)
	Bos-R	AAGCCTCGTCTCTACGTGCATA					
12S rRNA	Bcyt-F	ACGGGTCTTACACATTTTCTAGAAAC	139	240	65	AY526085.1	This work
	Bcyt-R	AAGAGGTTGGTGATGACTGTTGCT					
	Beef-F	CCTTCTCTATCCTAATCTTGCTC	115	240	60	KF926377.1	This work
	Beef-R	AGTAGGCTCTGCTACTAGGGC					
	B-F1	CCTAGCCCTTCTCTATCCTAATTC	124	240	60	KF926377.1	This work
	B-R1	GTGAGTGTCTAGTAGGCTCTGCTA					
	B-F2	TTCCTGCTCTAATCCCTTACT	112	240	60	KF926377.1	This work
	B-R2	CATGTGAGTGTCTAGTAGGCTCTG					
	916	GTACTACTAGCAACAGCTTA	256	200	55	AY526085.1	Bottero et al. 2003
	1171	GCTTGATCTCTTGGTAGAG					
	916	GTACTACTAGCAACAGCTTA	121	200	56	AY526085.1	This work
	916-R	AGACTGTATTAGCAAGAATTGGTG					
	916-F	AGAGTACTACTAGCAACAGCTTA	123	160	56	AY526085.1	This work
	916-R	AGACTGTATTAGCAAGAATTGGTG					
COI	916-F1	CTACTAGCAACAGCTTAAACTC	117	160	56	AY526085.1	This work
	916-R	AGACTGTATTAGCAAGAATTGGTG					
	B12S-F	CTACACCAAGAGAATCAAGCACG	170	240	60	AY526085.1	This work
	B12S-R	GTGCGTTTAAATAGGTTAGATGC					
	mit-F	TCTACACCAAGAGAATCAAGCA	163	240	60	AY526085.1	This work
	mit-R	CCAAGAGAATCAAGCACGAAAGT					
	mit-F2	CCAAGAGAATCAAGCACGAAAGT	157	240	60	AY526085.1	This work
	mit-R	CCAAGAGAATCAAGCACGAAAGT					
	BCOI-F	TACTCTCGCTCCCTGTATTAGCA	140	160	65	AY526085.1	This work
	BCOI-R	TCGGGGTGTCCAAAGAATCAGA					
	Bmt-F1	AGCCATATATCTCTTGGCTGACA	146	240	65	AY526085.1	This work
	Bmt-R	TGGTGTCAAGTTCTGGATTGTGATA					
	Bmt-F2	TATACTCTCCTTGGTGACATGCC	140	240	65	AY526085.1	This work
	Bmt-R	TGGTGTCAAGTTCTGGATTGTGATA					

Table 1. Key data of primers designed to specifically target DNA sequences from mitochondrial and nuclear genome of cow (continued).

Target region	Primers	Sequence (5' → 3')	Amplicon (pb)	Concentration (nM)	Annealing temperature (°C)	NCBI (Accession no.)	Reference
tRNA-Pro/D-loop	tRD-F tRD-R	AACTGCAGTCTCACCATCAACC GGGTGCAAAATTCGTGTGAGTTA	147	160	65	AY526085.1	This work
NADH subunit 2	ND2-F ND2-R	ACTCACGTATTCTACCCACACTAAC CTGATAGTATTGGCGTGAGTGGT	149	240	60	AY526085.1	This work
NADH subunit 4	ND4-F ND4-R	GTAATATAGTAATCACCCGCCCTA CCGTGTAAAGGAAAGCGAGATA	105	240	62	AY526085.1	This work
NADH subunit 4L	ND4L-F ND4L-R	CACAGTATCTCTTGAGGACTAC TGAGTTGAGGATTGTTAGGGCTG	118	240	62	AY526085.1	This work
β-casein	BCAS-F1 BCAS-R2	CACCAGCCTCTTCTCCTCCAACTG TCTGGGGATAGGGCACTGCTTT	106	200	64	XM_005902037.2	This work
β-lactoglobulin	BLG-F1 BLG-R1	GCTGGCCACATGCTCGCTCT GTGCCACTTCTGAGCCTC	120	200	65	Sequencing data	This work

Qualitative PCR

The PCR amplifications were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total reaction volume of 25 µL, containing 2 µL of template DNA (40 ng for binary mixtures and 100 ng for meat samples), 1× buffer (67 mM of Tris-HCl (pH 8.8), 16 mM of (NH₄)₂SO₄, 0.1% of Tween 20), 200 µM of each dNTP (Grisp, Porto, Portugal), 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of MgCl₂ (2.0 mM for primers BLG-F1/BLG-R1 and 1.5 mM for primers 18SRG-F/18SRG-R), and 160–240 nM of each primer (according to Table 1). The amplification programmes were defined as following: initial denaturation at 95 °C for 5 min; 40 cycles (except for primers 18SRG-F/18SRG-R with 33 cycles and 43 cycles for both sets of primers BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2) at 95 °C for 30 s, 55–65 °C (according to Table 1) for 30 s (except for primers 916–1171 for 60 s) and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

PCR products were verified by electrophoresis in a 1.5% agarose gel stained with GelRed 1×(Biotium, Inc., Hayward, CA, USA) and carried out in 1×SGTB (Grisp, Porto, Portugal) for 25–30 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was recorded using Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each extract was amplified at least in two independent assays.

Sequencing

β-Lactoglobulin region of cow (NCBI accession no. X14712.1) was selected to be sequenced. This region was amplified with primers BLG-F – GCCACATCTAGGTGAGCCCCT and BLG-R – AGGGGTGAATGTGGTCCCGTT producing fragments of 520 bp with the following amplification conditions: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 45 s, 65 °C for 45 s and 72 °C for 60 s; and a final extension at 72 °C for 10 min. In order to remove contaminants such as proteins, divalent cations, unincorporated nucleotides and enzyme inhibitors, PCR products were purified with GRS PCR & gel band purification kit (GRISP, Porto, Portugal) and sent to a specialised research facility (GATC Biotech AG, Constance, Germany) for sequencing. The target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality. Data were analysed using the available BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and FinchTV (Geospiza, Seattle, WA, USA) softwares.

Real-time PCR

For the real-time PCR amplifications, the reaction mixture of 20 μ L included 1 $\times$ of SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA) or 1 $\times$ of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in the case of primers BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2, 240 nM of each primer (Bos-F/Bos-R, 916/916-R, BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2), 150 nM of each probe (Bos-P or 916-P) for *cytb* or 12S rRNA genes, respectively, and 2 μ L of DNA extract (40 ng for binary mixtures and 100 ng for meat samples).

Target sequences were amplified using a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 65 °C for 45 s (for primers Bos-F/Bos-R, BLGF1/BLG-R1 and BCAS-F1/BCAS-R2) or 50 cycles at 95 °C for 10 s, 56 °C for 10 s and 72 °C during 30 s (for primers 916/916-R), with collection of fluorescence signal at the end of each cycle. For melting curve analysis, in real-time PCR assays using EvaGreen dye, the amplicons were denatured at 95 °C for 1 min and then annealed at 70 °C for 3 min in order to allow the correct annealing of the double strands. These two steps were further followed by melting curve ranging from 70.0 to 95.0 °C with temperature increments of 0.2 °C every 10 s. The fluorescence data were acquired by the end of each melting temperature. The software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) was used to evaluate the data from each real-time PCR run. Cycle of quantification (Cq), also known as cycle threshold (Ct) values were calculated using the software at automatic threshold settings. Real-time PCR trials were performed using n=4 replicates in each run.

RESULTS

In this study, several sequences targeting different genes from cow's mitochondrial genome and some nuclear genes encoding important milk allergenic proteins were evaluated for their capacity to detect trace amounts of cow's milk in meat products by PCR. Different DNA extraction methods with the optimisation of some steps were assayed, followed by the design of specific primers and their application in qualitative PCR to evaluate their sensitivity and specificity. The most promising primers were tested by real-time PCR using both TaqMan probes or binding dyes, to study their suitability for quantitative purposes by the analysis of the analytical performance parameters.

DNA extraction

DNA extracts from meat species were obtained with the Wizard method (Mafra et al., 2008), presenting a wide range of yields (195.0–817.7 ng/ μ L) and adequate purities (1.8–

2.1). MPC sample and binary mixtures were extracted using the Wizard method and NucleoSpin food kit (Macherey-Nagel, Düren, Germany) with and without the addition of 2 μL of RNase (2 mg/mL) after the cell lysis step, for comparative purposes. Regarding the first method, the obtained yields varied between 37.0 and 397.6 ng/ μL and purities were in the range of 1.8–2.1. NucleoSpin Food kit provided purities between 2.0 and 2.3 and yields of 109.4–888.3 ng/ μL without RNase or purities of 1.9–2.0 and yields between 34.2 and 301.0 ng/ μL with its use. It was considered that the use of RNase improves the quality of extracts by eliminating PCR inhibitors. All DNA extracts tested positively with universal primers targeting the eukaryotic nuclear 18S rRNA gene (section 2.3), thus confirming the presence of amplifiable DNA and eliminating any potential false negative results in the specific PCR assays.

Screening gene markers for cow's detection (sensitivity and specificity)

The primers used in this work and the respective optimised qualitative PCR conditions are summarised in Table 1. Firstly, for sensitivity testing, the primers were assayed using 10-fold serial diluted extracts of the model mixture of 10% of MPC in turkey meat (corresponding to 10 ng of cow's milk DNA). The PCR amplifications with all primer sets produced the expected fragments, except for the BLG region. In this case, a fragment of around 500 bp was obtained instead of the expected 129 pb, suggesting the presence of introns probably spliced during the transcription process and, therefore, not reported in the target sequence of bovine mRNA β -lactoglobulin available at NCBI database (accession no. X14712.1). For that reason, the PCR products obtained from the amplification of MPC extracts (10 ng) with primers BLG-F/BLG-R were sequenced. The results showed high-resolution electropherograms, enabling the complete and correct sequencing of 520 bp amplicons. Therefore, a new primer set was designed for this region (BLG-F1/BLGR1), to specifically amplify fragments of 120 bp (supplementary material, Fig. S1). The PCR assays with all the primer sets showed absolute sensitivities between 1 and 100 pg of bovine DNA, which can be observed in Fig. S2 (supplementary material) presenting some of the obtained results.

In addition to *in silico* analysis, the specificity of the proposed primers for cow's identification was determined experimentally using different meat species commonly used in food products (as described in 2.1 section). Table 2 shows the resumed PCR results of specificity tests, demonstrating a high level of cross-reactivity with some animal species, namely chicken, goat, pork and turkey, which were amplified by the majority of primers targeting mitochondrial genes.

Table 2. Results obtained by qualitative PCR showing the level of amplification of each meat species tested in cross-reactivity studies.

Primers	Cow	Boar	Duck	Partridge	Hare	Quail	Pheasant	Deer	Rabbit	Chicken	Turkey	Lamb	Goat	Ostrich	Horse	Pork
Bos-F/R	+++	+/-	-	-	+/-	-	-	-	-	+	+	-	+	-	-	+
Bcyt-F/R	+++	-	-	-	-	-	-	-	-	+/-	+/-	-	+++	-	-	++
Beef-F/R	+++	-	-	-	-	-	-	-	-	++	-	-	++	-	-	++
B-F1/R1	+++	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+++
B-F2/R2	+++	-	-	-	-	-	-	-	-	+/-	-	-	+	-	-	+++
916/1171	+++	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	+/-
916/916-R	+++	-	-	-	-	-	-	-	-	-	-	-	++	-	-	+/-
916-F/R	+++	-	-	-	-	-	-	-	-	-	+	-	+++	-	-	+/-
916-F1/R	+++	-	-	-	-	-	-	-	-	-	+/-	-	+++	-	-	+/-
B12S-F/R	+++	-	-	-	-	-	-	-	-	+/-	++	-	++	-	-	++
mit-F/R	+++	-	-	-	-	-	-	-	-	+/-	-	-	+++	-	-	++
mit-F2/R	+++	-	-	-	-	-	-	-	-	+/-	-	-	+	-	-	++
BCOI-F/R	+++	-	-	-	-	-	-	-	-	+/-	+	-	+	-	-	++
Bmt-F1/R	+++	-	-	-	+/-	-	-	-	-	+/-	+	-	++	-	-	++
Bmt-F2/R	+++	-	-	-	-	-	-	-	-	+	+	-	+++	-	-	+
tRD-F/R	+++	-	-	-	-	-	-	-	-	-	+	-	+++	-	-	++
ND2-F/R	+++	-	-	-	-	-	-	-	-	+/-	++	-	++	-	-	++
ND4-F/R	+++	-	-	-	-	-	-	-	-	++	+	-	++	-	-	++
ND4L-F/R	+++	-	-	-	-	-	-	-	-	-	+	-	++	-	-	++
BCAS-F1/R2	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BLG-F1/R1	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(+++) very strong positive amplification; (++) strong positive amplification; (+) weak amplification; (+/-) very weak amplification; (-) negative amplification.

The specificity results combined with the sensitivity data (supplementary material, Fig. S2), highlighted the primer sets targeting the *cytb* (Bos-F/Bos-R), 12S rRNA (916/916-R), β -casein (BCAS-F1/BCAS-R2) and β -lactoglobulin (BLGF1/BLG-R1) genes, with the production of fragments of 99 bp (Fig. S2A), 121 bp (Fig. S2C), 106 bp (Fig. S2I) and 120 bp (Fig. S2J) (supplementary material), respectively, as the most promising. Thereafter, the sensitivity of these primers was further evaluated using the binary mixtures of MPC in meat (supplementary material, Fig. S3). Both assays targeting mitochondrial genes presented sensitivities down to 0.01% (100 mg/kg) of MPC in cooked ham and 0.1% (1000 mg/kg) in raw meat (supplementary material, Figs. S3A–S3D), while the assay targeting β -lactoglobulin region was able to amplify 0.05% (500 mg/kg) and 0.01% (100 mg/kg) of MPC in raw turkey meat and cooked ham, respectively (supplementary material, Figs. S3E and S3F). Regarding Bos-F/Bos-R primers, there is a very weak amplification at the level of 0% (supplementary material, lane 9, Figs. S3A and S3B) probably due to the residual cross-reactivity with the matrix. The β -casein PCR assay presented the lowest sensitivity, down to 0.5% (5000 mg/kg) of MPC in cooked ham and 1% (10,000 mg/kg) of MPC in raw turkey meat mixture (supplementary material, Figs. S3G and S3H).

Real-time PCR

The primers targeting the mitochondrial (Bos-F/Bos-R and 916/916-R) regions and the milk allergen-encoding genes (BLG-F1/BLG-R1 and BCAS-F1/BCAS-R2) were tested by real-time PCR in order to verify their suitability for a quantitative method. Firstly, cross-reactivity with the critical species, namely chicken, goat, pork and turkey was verified for each system, followed by their application to binary mixtures. The Ct mean values obtained by each run are showed in Table 3. Contrarily to qualitative PCR results (Table 2), where no positive non-target amplification appeared, the real-time PCR assays with EvaGreen dye using primers targeting the allergen-encoding genes presented cross-reactivity with all the tested meat species. Ct mean values ranged from 29.49 ± 0.12 to 35.40 ± 1.75 and from 28.10 ± 0.13 to 32.48 ± 0.33 for β -lactoglobulin and β -casein genes, respectively (Table 3). In addition, the melting temperature obtained in the amplification of β -lactoglobulin gene in MPC sample (84.50°C) is very similar to the value obtained by the amplification of turkey species (84.40°C), suggesting the production of a very similar PCR product (data not shown). When both allergen-encoding gene approaches were applied to raw binary mixtures, it was observed a lack of reproducibility between qualitative PCR and real-time PCR data.

Table 3. Real-time PCR results for the cross-reactivity test of each system with the meat species, chicken, turkey, goat and pork and with the binary mixtures of raw turkey meat with MPC-85 ranging from 0% to 10%.

Target	Primers			
	916/916-R	Bos-F/Bos-R	BLG-F1/BLG-R1	BCAS-F1/BCAS-R2
Ct $\pm$ SD ^a				
	Raw	Cooked	Raw	Raw
Meat species				
Chicken	38.79 $\pm$ 0.37 (3/3) ^b		34.59 $\pm$ 1.18 (3/3)	32.30 $\pm$ 0.54 (3/3)
Turkey	NA		38.60 $\pm$ 0.33 (2/3)	32.48 $\pm$ 0.33 (3/3)
Goat	36.91 $\pm$ 0.59 (3/3)		NA	28.10 $\pm$ 0.13 (3/3)
Por	NA		35.45 $\pm$ 0.97 (2/3)	31.35 $\pm$ 0.20 (3/3)
Milk protein concentrate (MPC)				
0	22.63 $\pm$ 0.53 (3/3)		21.16 $\pm$ 0.07 (3/3)	23.00 $\pm$ 0.05 (3/3)
Spiked level of MPC in raw meat (%)				
0.005	NA	NA	37.44 $\pm$ 0.00 (1/4)	30.45 $\pm$ 0.23 (4/4)
0.01	39.85 $\pm$ 0.51 (4/4)	39.86 $\pm$ 1.00 (4/4)	38.71 $\pm$ 0.76 (4/4)	30.42 $\pm$ 0.34 (4/4)
0.05	39.35 $\pm$ 0.39 (4/4)	38.67 $\pm$ 0.32 (4/4)	36.55 $\pm$ 0.33 (4/4)	30.69 $\pm$ 0.07 (4/4)
0.1	37.51 $\pm$ 0.40 (4/4)	36.87 $\pm$ 0.75 (4/4)	35.25 $\pm$ 0.74 (4/4)	30.60 $\pm$ 0.07 (4/4)
0.5	36.54 $\pm$ 0.35 (4/4)	36.55 $\pm$ 0.12 (4/4)	34.47 $\pm$ 0.59 (4/4)	31.25 $\pm$ 0.13 (4/4)
1	34.61 $\pm$ 0.17 (4/4)	33.53 $\pm$ 0.14 (4/4)	32.63 $\pm$ 0.25 (4/4)	30.90 $\pm$ 0.05 (4/4)
5	33.26 $\pm$ 0.16 (4/4)	32.55 $\pm$ 0.28 (4/4)	31.28 $\pm$ 0.27 (4/4)	30.55 $\pm$ 0.40 (4/4)
10	30.26 $\pm$ 0.09 (4/4)	29.19 $\pm$ 0.12 (4/4)	28.58 $\pm$ 0.08 (4/4)	29.75 $\pm$ 0.01 (4/4)
	28.47 $\pm$ 0.17 (4/4)	27.82 $\pm$ 0.07 (4/4)	27.31 $\pm$ 0.19 (4/4)	28.74 $\pm$ 0.40 (4/4)

^aMean cycle threshold (Ct) values $\pm$ standard deviation (SD); ^bPositive amplifications/total replicates; NA - no amplification.

The clear gradient observed in qualitative PCR results (supplementary material, Figs. S3E and S3G) is not confirmed by the Ct values obtained in real-time PCR (Table 3), showing a difference of only 1.0 (Ct values from 30.82 ± 0.03 to 31.82 ± 0.35) and 1.7 (Ct values from 28.74 ± 0.40 to 30.42 ± 0.34) cycles between the first spiked level (10%) and the last one (0.005%), for β -lactoglobulin and β -casein gene systems, respectively. Moreover, both systems showed amplification for the blank mixture (0%), without containing MPC, at 31.47 ± 0.04 and 30.45 ± 0.23 cycles for the β -lactoglobulin and β -casein systems, respectively. This finding is in accordance with the referred cross-reactivity with turkey species that is present in mixture.

Regarding real-time PCR trials targeting the two mitochondrial genes (*cytb* and 12S rRNA), one TaqMan probe was designed for each (Bos-P and 916-P) in an attempt to eliminate the residual cross-reactivity showed in qualitative PCR results (Table 2) for some meat species. The results were only slightly improved in the case the *cytb* primers, which did not amplify goat, while chicken, turkey and pork showed a delayed amplification. In the case of 12S rRNA primers, turkey was not amplified, though chicken and goat were amplified, but with very high Ct values. Such high Ct values suggest that 12S rRNA system presented the weakest cross-reactivity with goat and chicken compared with the other tested systems (Table 3).

Comparing the application of the two mitochondrial gene systems to raw binary mixtures, the target species is amplified two cycles earlier with *cytb* primers than with 12S rRNA primers in almost all spiked levels, including the sample without the addition of MPC (Table 2), as also obtained by qualitative PCR (supplementary material, Figs. S3A and S3C). Although both mitochondrial systems present consistent Ct values in conformity with the gradient observed in qualitative PCR, only the real-time PCR assay targeting the 12S rRNA gene showed no amplification for the blank matrix (0% level) and amplifications above 38 cycles only for chicken species (Table 3). Thus, Ct values above the threshold of cross-species amplification (> 38) were considered negative. Regarding the amplification of goat (36.91 ± 2.59), it was considered not problematic since this species is not added to this type of meat products due to economic reasons.

As a result, the most promising system targeting the 12S rRNA gene was further assessed with both raw and cooked binary mixtures as a potential candidate for the development of a quantitative method for MPC detection in processed meat products. The assessment of assay performance was according to the general guidelines for method development described by Bustin et al. (2009) and ENGL (2015), whose criteria established for real-time PCR assays were carefully considered: the limit of detection (LOD) should be defined as the lowest concentration at which 95% of the replicates are detected, the PCR efficiency should range between 90 and 110%, the slope should be within -3.6 and -3.1

and the correlation coefficient (R^2) above 0.98. Fig. 1 presents the amplification curves and respective calibration curves for the real-time PCR 12S rRNA system applied to both raw and cooked model mixtures. The calibration curves presented slopes of -3.504 and -3.647 , R^2 of 0.976 and PCR efficiency of 92.9% and 88.0% for raw and cooked hams, respectively (Fig. 1), which were within or very close to the adequate performance criteria parameters. Both calibration curves enabled a dynamic range of 4 orders of magnitude, covering the amplification down to 0.005% (w/w) (50 mg/kg) of MPC in raw and cooked hams (Table 3). However, considering that amplifications above 38 cycles should not be accounted due to non-target amplification, the relative LOD should be defined as 0.05% (w/w) (500 mg/kg) for both raw and cooked binary mixtures.

DISCUSSION

Some food proteins are used as technological aids in the preparation of uncounted food products providing functional and nutritional properties to the final product. However, in most cases, these type of aids are considered important allergens, such as celery, soy and cow's milk proteins (Spychaj et al., 2018). They are often present only at trace amounts or masked by the food matrix, which usually makes their detection very difficult to achieve, representing a constant threat to allergic individuals. In the specific case of meat products, such as hams and sausages, milk protein concentrates and isolates are frequently added (Uluko et al., 2016). Presently, there are numerous technical possibilities for the detection of allergens in foodstuffs, targeting either the allergen itself (MS spectrometry and immunochemical methods) or an indirect molecular marker (DNA-based techniques) that indicates the presence of the offending food. The selection of the target/biomolecule becomes critical since it will greatly affect the sensitivity, the reliability and the quantification capacity of the technique (Costa et al., 2017a). Meat products are submitted to different thermal treatments during their preparation (autoclaving and cooking procedures, such as roasting, baking, grilling or boiling), which can induce alterations in the properties of the food components, such as hydrolysis and structural changes of proteins (Costa, Amaral, Grazina, Oliveira, & Mafra, 2017b). In these cases, techniques targeting DNA markers are advantageous alternatives for the analysis of highly processed foods. DNA targets might be genes that encode allergenic proteins or other specific sequences, thus being considered as indirect markers of the presence of an allergenic ingredient (Villa et al., 2018).

In order to develop a sensitive and accurate method to detect and quantify milk ingredients in meat products, a complete screening and evaluation of several mitochondrial and nuclear genes as candidate markers for cow detection were performed to be used as specific and indirect targets for milk allergen detection. For this purpose, binary mixtures of MPC in turkey meat, simulating the production of cooked ham were prepared and submitted

to a thermal treatment at 67 °C during 5 h in the oven, accounting with the interference of food matrix and processing to assess the most promising genes for method development. In order to achieve better sensitivity results, multi-copy genes (mitochondrial genes) were mostly used. However, it was observed a strong cross-reactivity with some meat species using almost of the designed primers due to the highly conserved nucleotide sequences of these genes in different species (Holzhauser, Stephan, & Vieths, 2006). Since single-copy genes are reported to be more specific (Holzhauser et al., 2006), they were also tested in the present work. Based on the best sensitivity and specificity results obtained by qualitative PCR, four primer pairs targeting two mitochondrial (*cytb* and 12S rRNA) and two nuclear (β -lactoglobulin and β -casein) genes were chosen from a total of 22 (Table 1), mostly designed in this work and further tested in binary mixtures. Generally, it was observed a better relative sensitivity in cooked binary mixtures than in raw, possibly due to a strong reduction in water content during thermal treatment, thus causing an improvement in DNA extraction and PCR efficiency. Additionally, raw meat has high enzymatic activity, which is greatly reduced during mild food processing (oven cooked at 67 °C for 5 h) by inactivation phenomena and water loss, thus contributing to stabilise total DNA, including the target one. Although thermal processing is known to contribute to shearing and degradation of DNA of high molecular mass (Fernandes, Oliveira, & Mafra, 2013), other factors such as enzymatic activity together with high water content and the presence of PCR inhibitors (Costa et al., 2013; 2017b) are also determinant for the success of PCR amplification. The relative sensitivity in qualitative PCR was down to 0.01% (100 mg/kg) of MPC in cooked ham with primers targeting both mitochondrial and β -lactoglobulin genes (supplementary material, Fig. S3).

To improve specificity of the two selected multi-copy regions, specific TaqMan probes were designed on the *cytb* and 12S rRNA sequences in real-time PCR assays using binary mixtures. On the other hand, allergen-encoding sequences were amplified with a third generation binding dye (EvaGreen), which offers enhanced fluorescence, increased sensitivity and excellent stability (Wang, Chen, & Xu, 2006). However, these trials were not consistent with qualitative PCR results probably due to the enhanced real-time PCR amplification conditions, highlighting reactivity with non-target species (chicken, turkey, goat and pork) (Table 3) and disabling their utility. The use of mitochondrial genes combined with specific TaqMan probes seemed to provide better specificity results, comparing with nuclear genes, suggesting only minor or no cross-reactivity for chicken, turkey, goat and pork. The 12S rRNA system was considered as the best choice for the development of a quantitative real-time PCR method since turkey meat was not amplified, while the other three cross-reactive meat species exhibited delayed amplification (above 38 cycles).

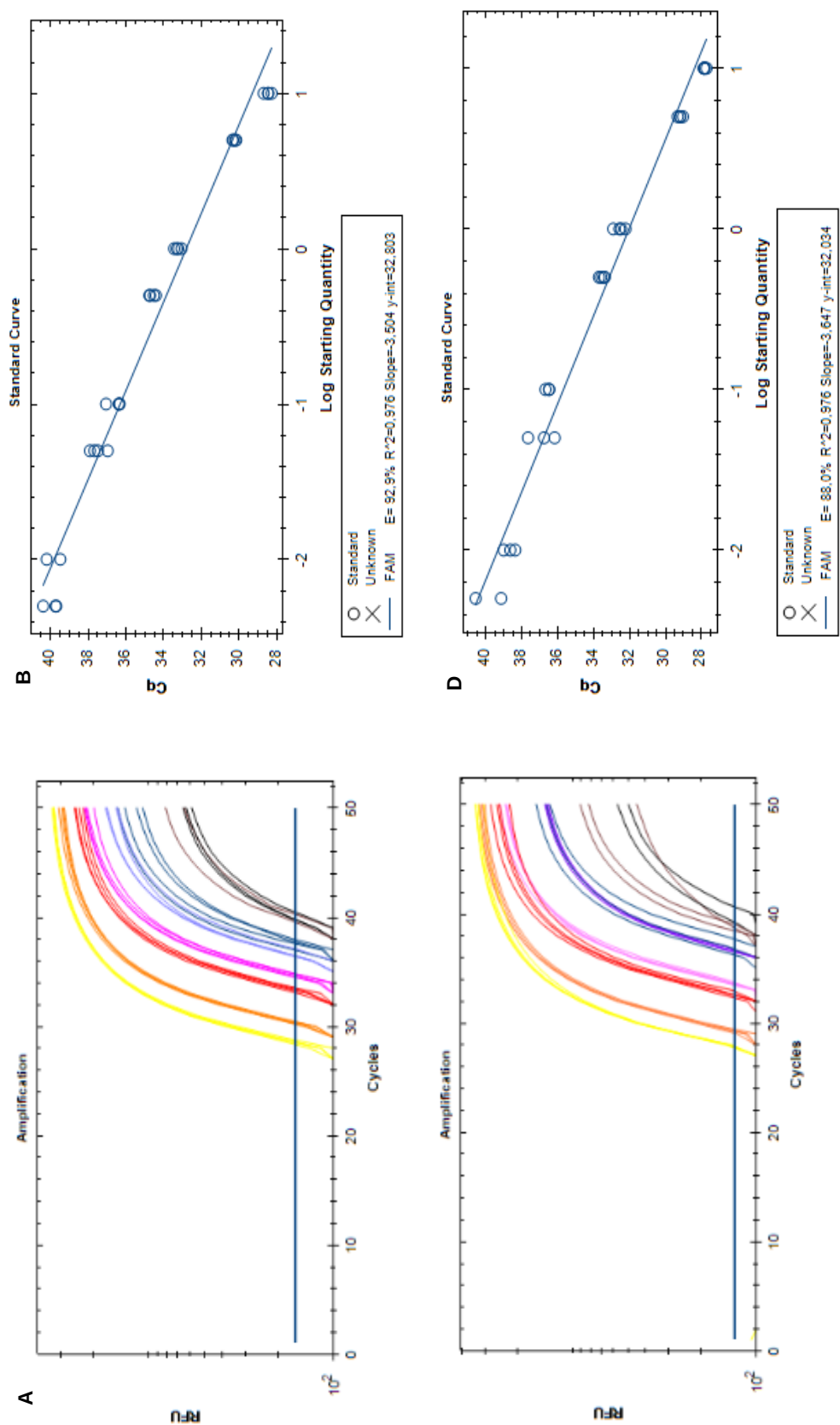


Fig. 1. Amplification curves (A, C) and respective calibration curves (B, D) obtained by real-time PCR targeting the bovine 12S rRNA mitochondrial gene using the binary mixtures of cow's milk protein concentrate (MPC) in turkey raw meat (A, B) and in cooked hams (C, D) (10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005% (w/w)) (n=4).

The obtained calibration curves using both raw and processed binary mixtures presented, generally acceptable PCR performance parameters, achieving a relative LOD of 0.05% (500 mg/kg). The calibration curve for the thermally treated binary mixtures displayed a PCR efficiency slightly lower (88%) than the acceptance criteria generally recommended (90–110%). However, since it is a complex processed food matrix, it is acceptable to be within the range of 75–110% (ENGL, 2015).

The majority of works using DNA-based methods detecting cow in food products focuses on dairy product authentication. Some authors using multi-copy genes reported the problem of cross-reactivity with other meat species. In those cases, Ct values above the threshold of cross-species DNA amplification should be considered negative (Drummond et al., 2013; Zhang, Fowler, Scott, Lawson, & Slater, 2007). With such a high level of unspecific amplifications, LOD are never below 0.1% of cow DNA (Kalogianni, 2018). Using the *cytb* region, Cottenet, Blancpain, and Golay (2011), and Drummond et al. (2013) were able to detect 1% of bovine DNA, while Lopparelli, Cardazzo, Balzan, Giaccone, and Novelli (2007) and Di Domenico, Di Giuseppe, Wicochea Rodríguez, and Cammà (2017) reached a sensitivity of 0.5% and 0.1%, respectively. Rentsch et al. (2013) detected 0.2% of cow DNA in cheeses using the bovine beta actin nuclear gene. A method developed by Liao, Liu, Ku, Liu, and Huang (2017) targeting the mitochondrial 12S rRNA gene was able to detect 0.1% of cow's milk in goat milk powders. However, in the field of food allergen detection, methods need to be more sensitive and able to detect trace amounts of the offending food. Xiao et al. (2016) performed a real-time PCR with a TaqMan minor groove binder probe to detect the α -lactalbumin-encoding gene, obtaining a sensitivity down to 0.05 ng of bovine DNA. However, they only used 10-fold serial dilutions of bovine DNA isolated from cow milk as template, without attempting any reference mixture to provide a realistic and practical level to estimate cow's milk content as an ingredient. Köppel et al. (2010, 2012) were able to detect milk as an allergen in processed foods, but without quantitative results, presenting a sensitivity of 0.64 μ g/mL of bovine DNA.

In the present work, we have realised that the development of a DNA-based method to detect milk as a potential allergenic ingredient, complying with high levels sensitivity and specificity, was a very challenging task, requiring concerted *in silico* and experimental efforts. Nevertheless, with the real-time PCR approach targeting the 12S rRNA gene, we could reach an acceptable compromise of sensitivity/specificity. The assay was the most specific since it provided only minor reactivity with two species at delayed amplification cycles (> 36), linearity in the range of 10–0.005% of cow's MPC in raw meat and cooked ham, but a LOD of 0.05%, considering cross-reactivity data. The preliminary real-time PCR assay provided acceptable performance parameters, being the starting point to propose a

novel method to quantify trace amounts of milk ingredients as potential allergens in complex meat products, which should be further validated and applied.

Acknowledgements

This work was supported by UID/QUI/50006/2019 with funding from FCT/MCTES through national funds and by the projects AlleRiskAssess – PTDC/BAA-AGR/31720/2017 and NORTE-01-0145-FEDER-00001. Caterina Villa is grateful to FCT grant (PD/BD/114576/2016) financed by POPH-QREN (subsidised by FSE and MCTES).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2019.106823>.

Declaration of interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

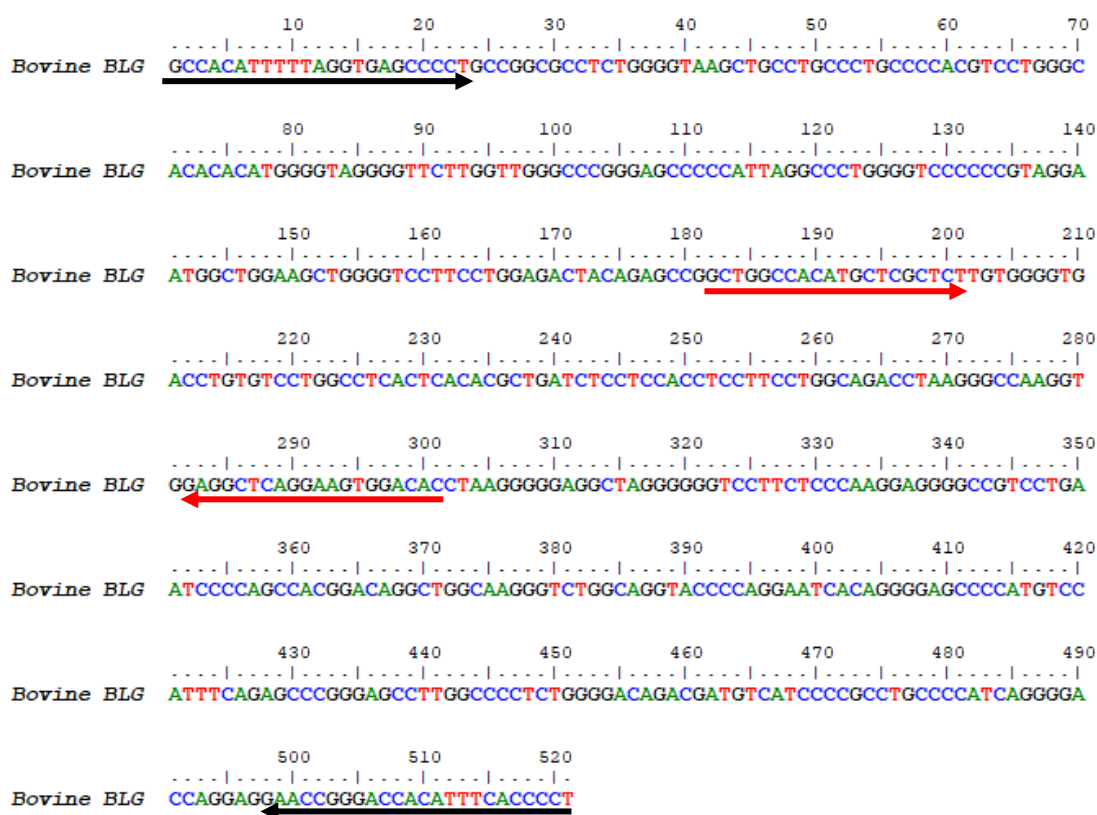


Fig. S1. Sequencing product of bovine BLG region (520 bp). Black arrows represent sequencing BLG-F/BLG-R primers and red arrows represent the new BLG-F1/BLG-R1 primers designed to amplify a fragment of 120 bp.

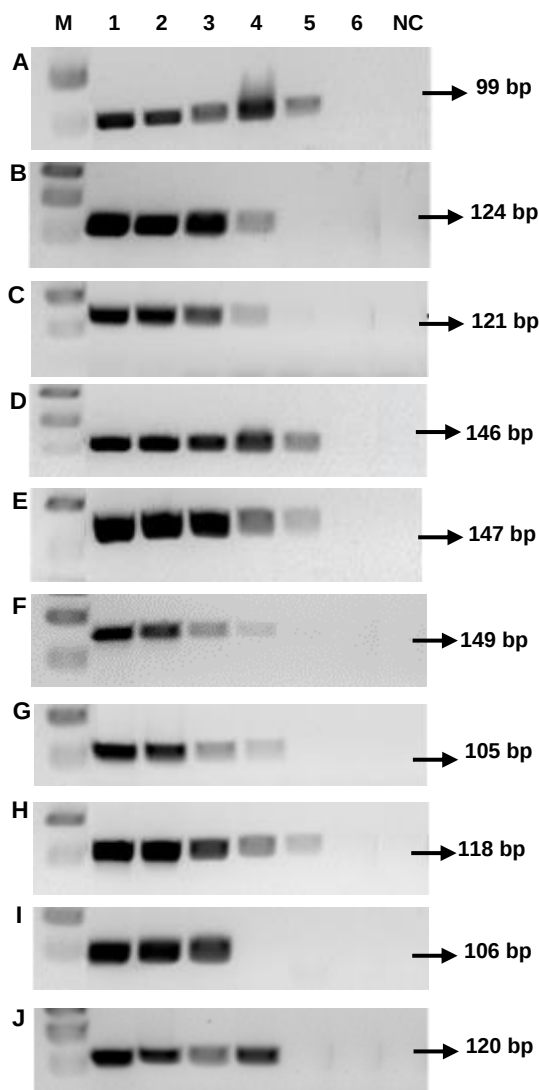


Fig. S2. Agarose gel electrophoresis of PCR products obtained using some of the primers designed in this work to specifically amplify cow DNA. (A) Bos-F/Bos-R and B-F1/B-R1 (B) primers targeting *cytb* gene; (C) 916/916-R primers targeting 12S rRNA gene; (D) Bmt-F1/Bmt-R1 primers targeting tRNA-Lys/ATP synthase F0 subunit 8 genes; (E) tRD-F/tRD-R primers targeting tRNA-Pro/D-loop genes; (F) ND2-F/ND2-R primers targeting NADH dehydrogenase subunit 2 gene; (G) ND4-F/ND4-R primers targeting NADH dehydrogenase subunit 4 gene; (H) ND4L-F/ND4L-R primers targeting NADH dehydrogenase subunit 4L gene; (I) BCAS-F1/BCAS-R2 primers targeting β -casein gene; (J) BLG-F1/BLG-R1 primers targeting β -lactoglobulin gene; M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany); lane 1, 10000 pg; lane 2, 1000 pg; lane 3, 100 pg; lane 4, 10 pg; lane 5, 1 pg; lane 6, 0.1 pg; NC, negative control.

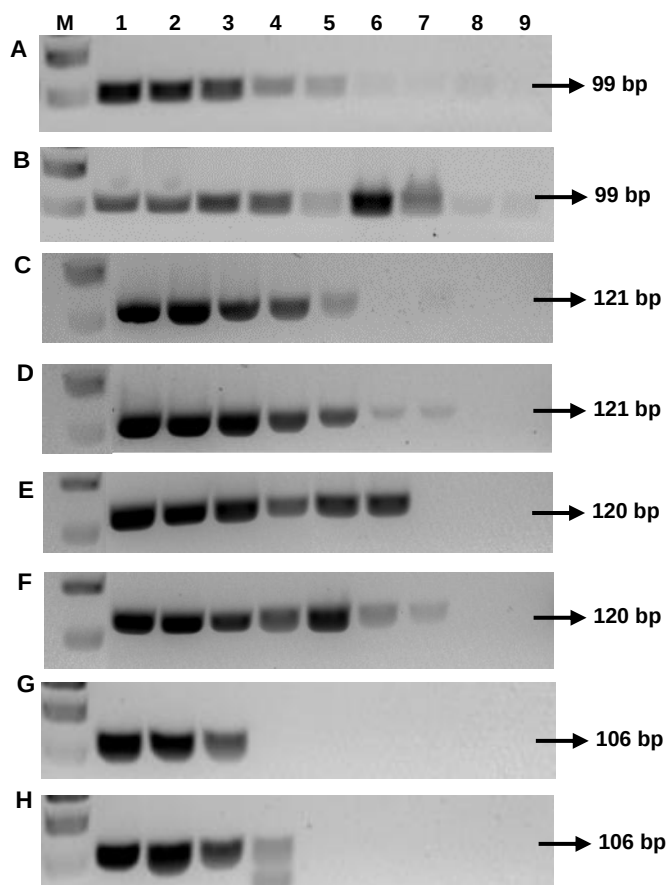


Fig. S3. Agarose gel electrophoresis of PCR products targeting *cytb* (A, B), 12S rRNA (C, D), β -lactoglobulin (E, F) and β -casein (G, H) genes using DNA extracts of binary mixtures of MPC in raw (A, C, E, G) meat and in cooked hams (B, D, F, H) from 10% to 0% (w/w). M, 100 bp DNA molecular marker (Bioron, Ludwigshafen, Germany); lane 1, 10%; lane 2, 5%; lane 3, 1%; lane 4, 0.5%; lane 5, 0.1%; lane 6, 0.05%; lane 7, 0.01%; lane 8, 0.005%; lane 9, 0%.

2.2.2. Detection and quantification of milk ingredients as hidden allergens in meat products by a novel specific real-time PCR method

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ABSTRACT

Milk ingredients are often included in a wide range of meat products, such as cooked hams and sausages, to improve technological characteristics. However, milk proteins are also important food allergens. The aim of this study was the development of a highly sensitive and specific real-time PCR system targeting the 12S rRNA gene of *Bos domesticus* for the detection and quantification of milk as an allergenic ingredient in processed meat products. The method was able to achieve an absolute limit of detection (LOD) of 6 fg of milk DNA. Using a normalized approach (ΔC_t method) for the detection of milk protein concentrate (MPC), it was possible to obtain sensitivities down to 0.01% (w/w) of MPC in model hams (raw and cooked) and autoclaved sausages, and 0.005% in raw sausage mixtures. The developed systems generally presented acceptable PCR performance parameters, being successfully validated with blind samples, applied to commercial samples, and further compared with an immunochemical assay. Trace amounts of milk material were quantified in two out of 13 samples, but the results mostly infer the excessive practice of the precautionary labeling.

Keywords: Food allergen, real-time PCR, 12S rRNA gene, quantification, meat products, milk.

INTRODUCTION

The meat industry uses a wide range of ingredients with specific technological properties, to improve the appearance, taste and texture of products, as well as their nutritional value. In some cases, the introduction of these ingredients is intended to decrease the production costs or is simply unintentional introduced due to bad manufacturing practices. However, some of these ingredients are a source of major food allergens, such as milk, legumes, egg, celery or cereal gluten proteins, causing a high health risk to consumers suffering from food allergies [1]. Milk proteins are often added to meat products in order to improve their juiciness, texture, and flavor. These proteins can be caseins or whey proteins, such as β -lactoglobulin and α -lactalbumin, which are considered major food allergens. Milk allergy is one of the most common food allergies in early childhood that often tends to resolve with age, although it can also persist through adulthood [2]. People with allergies are forced to implement a complete milk elimination diet in order to avoid adverse reactions, which can vary from mild symptoms, affecting the respiratory or the gastrointestinal tract, to severe symptoms leading to anaphylaxis [3]. To guarantee the safety of 95% of milk-allergic patients, a threshold of clinical reactivity to milk of 30 mg/kg was established by Morisset et al. [4], based on the consumption of 100 g of product. Considering the eliciting dose that protects 99% of the milk-allergic population (ED01), a new reference dose was recently defined as 0.1 mg of milk proteins using proper statistical dose-distribution models. This value represents 3.03 mg of liquid milk per kg of food and 0.28 mg of non-dry fat milk per kg of food, according to the conversion factors available from the U.S. Department of Agriculture [5]. Therefore, analytical methods able to detect and quantify trace amounts of milk allergens in processed foodstuffs are needed to identify their presence as ingredients or as cross-contaminants and verify labeling compliance.

Recently, DNA-based methods have been revealed as promising techniques in the field of food allergen detection, due to the great thermal stability of DNA molecules as compared with proteins, especially upon severe food processing conditions. When analyzing processed foods, such as meat products, polymerase chain reaction (PCR) techniques can be very useful alternatives to immunochemical methods [6]. Until now, the detection of milk allergens in foodstuffs has been mostly performed by protein analysis, relying on mass spectrometry [7–11] and ELISA [12–15]. However, the application of PCR-based methods to detect and quantify milk as an allergenic food is still limited. Xiao et al. [16] performed a real-time PCR assay using a TaqMan minor groove binder probe to recognize the α -lactalbumin gene from the cow. However, the quantitative aptitude of the method was limited since they only quantified the cow's milk DNA, with a rough estimation of milk content as an ingredient and without showing any calibration curve. In such cases, the use of

reference materials or model mixtures is crucial to develop quantitative calibration models for allergen analysis [17]. Moreover, the use of a normalized calibration curve is highly recommended to account for possible amplification differences due to inconsistent DNA recovery and quality and degradation among extracts as a result of food processing [6,18].

Previous to this work, Villa et al. [19] performed an extensive evaluation of molecular markers to assess their suitability for the development of a specific real-time PCR method to detect and quantify milk ingredients in cooked hams. The results highlighted the use of a fragment targeting the 12S rRNA gene of *Bos domesticus* as the most specific and sensitive marker. On the basis of such preliminary findings, this study intends to develop a specific and sensitive normalized real-time PCR assay targeting the 12S rRNA gene to detect and quantify milk protein concentrates in meat products. Additionally, it is intended to evaluate the effects of food matrix and processing using model mixtures, simulating the preparation of cooked hams and sausages (oven cooking for hams and autoclaving for sausages). Finally, the validation of the method with blind mixtures and its further application to analyze commercial meat products was performed to verify their labeling compliance, with the results being further compared with ELISA.

MATERIALS AND METHODS

Reference model mixtures

In the absence of certified reference or testing materials for the specific detection of cow's milk powders in meat products, model mixtures of turkey meat spiked with known amounts of cow's milk protein concentrate (MPC) were prepared. For this study, two independent sets of model mixtures containing 10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005%, and 0.0001% (w/w) of MPC in minced turkey meat were prepared simulating cooked hams and sausages. Turkey meat was previously minced using a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). To simulate ham preparation, 8 g of salt and 4 g of powder sugar were added to 1 kg of meat, while the sausages were prepared, adding 250 g of ice, 20 g of salt, and 375 g of lard to 500 g of turkey meat. To facilitate homogenization, 10 mL of a sterile phosphate-buffered saline solution (0.2 M) was added to both mixtures. The turkey meat (muscle) was acquired at a local retail market (Porto, Portugal) and the MPC was provided by a food additive company (Formulab, Maia, Portugal). The exact protein content of MPC was determined by the Kjeldahl method, corresponding to 83.4% of total milk protein. Accordingly, taking into consideration this value, first mixtures containing 10% of MPC were prepared by adding the required amount of MPC to the ham or sausage raw mixtures (in a total of 200 g).

Each of the two sets of mixtures containing MPC, prepared according to ham and sausage recipes, was divided into two subsets, from which one was immediately stored at -20 °C, while the other was subjected to the respective thermal processing (described in Section “Thermal treatments”).

To avoid contaminations, all mixtures and meat samples were homogenized separately, and all materials and different containers were previously treated with DNA decontamination solution.

Validation mixtures and commercial samples

For method validation, two sets of blind mixtures, for hams and sausages, were prepared similarly to the reference mixtures, in order to contain 4.0%, 0.8%, 0.4%, 0.2%, and 0.002% (w/w) of MPC. Similar to the reference mixtures, both sets of blind mixtures were also divided into two subsets and submitted to the respective thermal treatment. Additionally, 13 commercial food samples of cooked hams ($n = 6$) and sausages ($n = 7$), acquired at local markets, were used to assess the applicability of the method. Validation mixtures and commercial samples were homogenized separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different containers and knives previously treated with DNA decontamination solution and immediately stored at -20 °C after preparation until DNA extraction.

Thermal treatments

Two distinct thermal treatments were applied to the subsets of reference and validation mixtures to evaluate the effect of thermal processing. For cooked ham simulation, the mixtures were oven cooked at 67 °C for 5 h, whereas for sausage simulation, the mixtures were autoclaved (121 °C, 1 bar) for 15 min. The thermally treated mixtures were immediately stored at -20 °C until DNA extraction.

DNA extraction

The NucleoSpin food kit (Macherey-Nagel, Düren, Germany), with some minor modifications according to the manufacturer's instructions, was used to extract the DNA from the reference and validation model mixtures, as well as from commercial samples, with the addition of 2 µL of RNase (2 mg/mL) after the cell lysis step. The extractions were performed at least in duplicate assays using 200 mg of each sample. The extracts were kept at -20 °C until further analysis.

In order to evaluate the yield and purity of DNA extracts, UV spectrophotometric DNA quantification was performed on a SynergyHT multimode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), using a Take 3 micro-volume plate accessory. The

DNA content was assessed by the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA).

Oligonucleotide primers and probes

The oligonucleotide primers and probes used in this work were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and they are presented in Table 1. For the specific detection of bovine milk, primers (916/916-R) and hydrolysis probe (916-P) targeting the bovine region of 12S rRNA gene with NCBI accession no. AY526085.1 were retrieved from [19]. Primers (EUK-F/EUK-R) and hydrolysis probe (S5), designed in the conserved region of the 18S rRNA gene, were used to target a universal eukaryotic sequence of 120 pb as an endogenous control [20]. To evaluate the amplification capacity of extracts, all the samples were amplified by qualitative PCR using 18SRG-F/18SRG-R primers retrieved from Costa et al. [21] targeting a sequence of 113 bp in the same conserved eukaryotic gene (NCBI accession no. HQ873432.1).

Table 1. Key data of primers and probes to target the 12S rRNA bovine gene and two universal eukaryotic regions of the nuclear 18S rRNA gene.

Primers	Sequence (5' → 3')	Amplicon (bp)	Target	Reference
916	GTACTACTAGCAACAGCTTA	121	12S rRNA	[19]
916-R	AGACTGTATTAGCAAGAATTGGTG			
916-P	FAM-TCTAGAAGGATATAAAGCACCGCCAAGT-BHQ1			
EUK-F	AGCCTGCGGCTTAATTTGAC	120	18S rRNA	[20]
EUK-R	CAACTAAGAACGGCCATGCA			
S5	FAM-AGGATTGACAGATTGAG-BHQ2			
18SRG-F	CTGCCCTATCAACTTTTCGATGGTA	113	18S rRNA	[21]
18SRG-R	TTGGATGTGGTAGCCGTTTCTCA			

Qualitative PCR

The PCR amplifications were performed in a MJ MiniTM Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total reaction volume of 25 µL containing 2 µL of template DNA (40 ng), 1x buffer (67 mM of Tris-HCl (pH 8.8), 16 mM of (NH₄)₂SO₄, 0.1% of Tween 20), 200 µM of each dNTP (Grisp, Porto, Portugal), 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM or 1.5 mM of MgCl₂ for 916/916-R and 18SRG-F/18SRG-R primers, respectively, and 200 nM or 240 nM of each primer, 916/916-R and 18SRG-F/18SRG-R, respectively (Table 1). The amplification programs were defined as follows: initial denaturation at 95 °C for 5 min; 40 cycles (for primers 916/916-R) or 33 (18SRG-F/18SRG-R) at 95 °C for 30 s, 56 °C (916/916-R) or 65

°C (18SRG-F/18SRG-R) for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

PCR products were verified by electrophoresis in a 1.5% agarose gel stained with GelRed 1x(Biotium, Inc., Hayward, CA, USA) and carried out in 1x SGTB (Grisp, Porto, Portugal) for 25 to 30 min at 200 V. Agarose gel visualization was performed in a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA), recording a digital image with Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each extract was amplified at least in two independent runs.

Real-Time PCR

For the real-time PCR amplifications, the reaction mixture of 20 µL included 1x SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 240 nM of each primer (916/916-R or EUK-F/EUK-R), 160 nM of each probe (916-P or S5) (Table 1) and 2 µL of DNA extract (40 ng). Each target sequence (eukaryotic and 12S rRNA genes) was amplified in parallel reactions and run simultaneously in a fluorometric thermal cycler CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: 95 °C for 5 min, 50 cycles at 95 °C for 10 s, 56 °C for 10 s, and 72 °C during 30 s, with collection of fluorescence signal at the end of each cycle. The software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) was used to evaluate the data from each real-time PCR run. Cycle of quantification (Cq), also known as cycle threshold (Ct), values were calculated using the software at automatic threshold settings. Real-time PCR trials were repeated in two or three independent runs using $n = 4$ replicates in each one.

ELISA

To validate the quantitative results of analyzed commercial samples by real-time PCR, the RIDASCREEN® FAST Milk ELISA kit was used (R-Biopharm AG, Darmstadt, Germany). This is a sandwich enzyme immunoassay to quantify milk proteins in food using antibodies to specifically detect caseins and β -lactoglobulin of cow's milk in the range of 0 to 67.5 mg/kg. The assay was carried out according to the manufacturer's instructions. Based on the quantitative real-time PCR results, samples #6 and #12 were appropriately diluted (1000-fold and 5-fold, respectively) to ensure their analysis within the range of the calibration curve of the kit. All the remaining samples were not diluted. The absorbency values were plotted against the concentration of standard solutions containing milk proteins. A nonlinear regression function was carried out using a sigmoid four parametric logistic function based on the following expression:

$$Y = \frac{A - D}{1 + \left(\frac{X}{C}\right)^b} + D$$

where Y is the optical density (absorbance), A the maximum absorbance, b the slope of the calibration curve in linear range, C the 50% inhibitory concentration (IC₅₀) (µg/L), D the minimum absorbance, and X the analyte concentration (µg/L). Each sample was analyzed in triplicate.

Statistical analysis

The statistical analysis was performed using the software IBM SPSS Statistics (23.0 package, IBM Corporation, New York, NY, USA). The significance of differences between the ΔC_t values of reference mixtures at the same spiking level was performed using independent samples t-test. Significant differences were considered when $p < 0.05$.

RESULTS AND DISCUSSION

Development of the analytical method

Meat products usually have a very complex composition, containing not only meat and fat, but also several other ingredients that improve their technological properties, nutritional value or flavor. MPC is one of the ingredients often added to meat products [1], but since it is also an allergenic food source, its addition poses a concrete health risk for the milk-allergic individuals. The development of specific and sensitive methods that are able to detect and quantify this type of additive is of general concern, aiming at fulfilling the growing expectations of a global market in providing safe food to consumers. In the specific case of meat products that undergo different processing treatments (thermal, radiation, high pressure or fermentation) during their production, allergenic proteins can be exposed to structural and physical and chemical modifications affecting their detection by immunochemical methods [6,18]. Therefore, DNA-based methods have been applied as reliable alternatives to immunochemical assays for allergen detection. Despite being considered as indirect approaches for allergen analysis, PCR-based methods can enable the detection of specific DNA sequences as unequivocal taxon marker molecules, generally down to the species level. In addition, with the use of appropriate reference materials as calibrants and real-time PCR, the presence of an allergenic species can be estimated. Although real-time PCR does not allow the identification of any specific protein (or allergen), it enables quantifying the amount of an allergenic food within a complex matrix, which is crucial information to verify labelling compliance and the safety of foods [6,18,20,21]. Therefore, a positive DNA result can be directly correlated with the presence of proteins from the allergenic food source. In certain cases where the amount of amplifiable DNA is

limited, the application of PCR-based methods might not be possible. Nevertheless, this could be overcome with the use of appropriate DNA extraction methods, taking advantage of the high sensitivity in detecting trace amounts of degraded DNA. This has been demonstrated in our previous work in which DNA was extracted from milk products [19]. In this case, the choice of the DNA extraction method was crucial to obtain successful amplification results. Accordingly, the NucleoSpin food kit (Macherey-Nagel, Düren, Germany), with the addition of RNase after the cell lysis step, was chosen from our previous results from milk DNA extraction, which provided the best DNA yields, purities, and amplification results [19].

Following the successful DNA extraction, an extensive screening study on mitochondrial and nuclear bovine genes was performed to identify the best candidate markers for MPC detection in processed meat products [19]. The study revealed that it is a challenging task to obtain a sensitive and specific DNA method to detect milk proteins at trace levels. On the one hand, the high similarity among mitochondrial sequences of mammal and avian species interferes when high sensitivity levels are implemented for allergen analysis. On the other hand, the nuclear allergen-encoding genes provide low sensitivity assays, not suitable for allergen analysis. The sequence of the 12S rRNA gene provided the best compromise of sensitivity and specificity, being the selected marker to develop a quantitative method for MPC detection in meat products. In this work, the specific primers and the hydrolysis TaqMan probe previously designed were used to develop a normalized real-time PCR system with raw and heat-treated model mixtures of turkey meat spiked with known amounts of MPC. To evaluate the effect of food matrix and processing, two sets of mixtures were prepared, one simulating cooked ham and the other autoclaved sausage production. At a first stage, a calibration curve for absolute DNA detection of MPC was obtained, followed by the development of four normalized calibration curves for the relative quantification based on the parallel amplification of two sequences, i.e., the bovine target 12S rRNA gene and a universal eukaryotic gene as endogenous control. The method validation was performed using blind mixtures and according to the general guidelines adopted for this type of assay [22,23], since no official requirements are yet defined for allergen testing based on DNA analysis. The validated method was applied to commercial samples and data were further compared with the results of the application of an immunochemical assay. A schematic diagram representing the workflow of this study is presented in Figure 1.

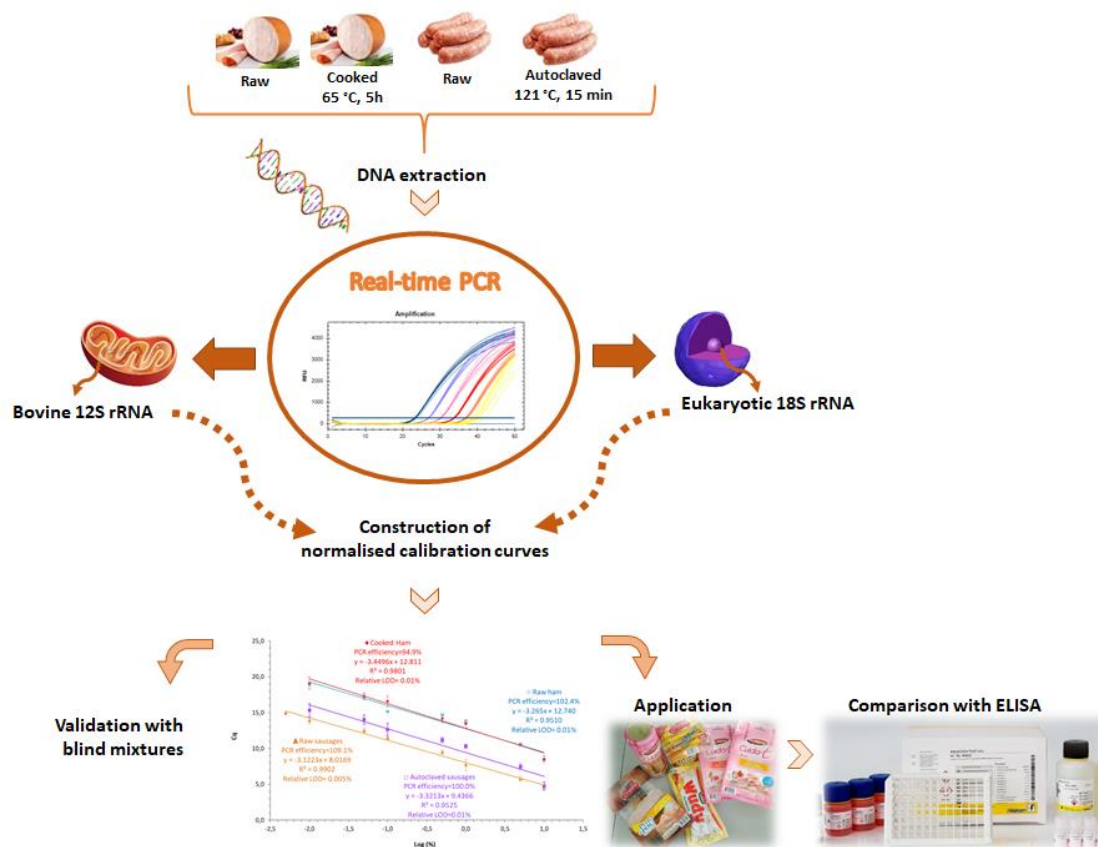


Figure 1. Schematic diagram representing the developed work.

Absolute and relative sensitivity

For absolute detection, DNA extracts of MPC were 10-fold serially diluted to cover six orders of magnitude of the target analyte (from 0.6 ng to 0.6 fg of bovine DNA). The proposed real-time PCR assay enabled the amplification of all replicates (eight/eight) until the level of 6 fg of milk (Figure 2). Therefore, this value was considered the absolute limit of detection (LOD), which is defined as the lowest concentration level of the analyte with positive amplification at least 95% of the times, according to the parameters required for method development and validation [22,23]. Other performance parameters have to comply with the acceptance criteria established for real-time PCR assays. The PCR efficiency should be between 90% and 110%, the correlation coefficient (R^2) equal or above 0.98, and the slope within -3.6 and -3.1 [22,23]. The calibration curve obtained for absolute quantification (Figure 2) is in agreement with these criteria, with a PCR efficiency of 100.0%, a slope of -3.322 , and a R^2 of 0.99. For the relative detection, each subset of model mixtures containing known amounts of MPC (10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005%, and 0.0001%, w/w) in turkey meat, simulating cooked ham and sausages, before and after thermal treatment, were used. All the real-time PCR systems enabled dynamic ranges of four orders of magnitude, with amplifications down to 0.01% (w/w) (100 mg/kg) of MPC in both subsets of hams (raw and cooked) and autoclaved

sausages, whereas in raw sausage mixtures it was down to 0.005% (w/w) (50 mg/kg) of MPC. Therefore, both values were considered as relative LOD for the respective systems since all the replicates were amplified (Table 2, Figure 3).

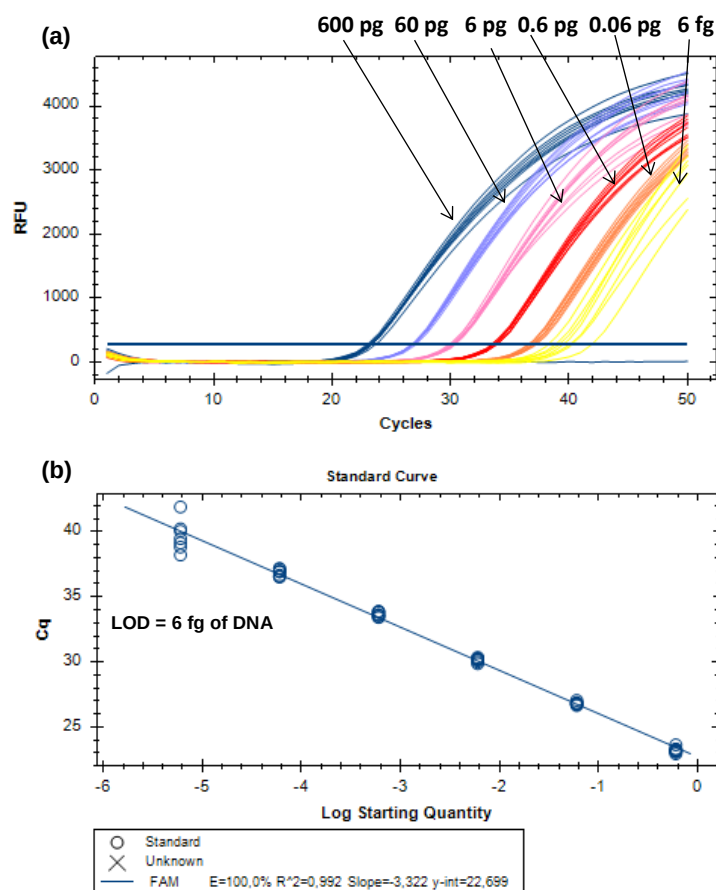


Figure 2. Amplification curves (a) and respective calibration curve (b) obtained by real-time PCR with TaqMan probe targeting the 12S rRNA gene using serially diluted (1/10) DNA extracts from MPC from 0.6 ng to 6 fg ($n=8$ replicates).

Table 2. Calibration curve parameters obtained in normalized quantitative real-time PCR systems using model mixtures of MPC in ham and sausages, with and without thermal treatment.

Parameter	Ham		Sausages	
	Raw	Cooked	Raw	Autoclaved
Correlation coefficient (R^2)	0.951	0.980	0.990	0.961
Slope	-3.265	-3.450	-3.122	-3.203
PCR efficiency (%)	102.4	94.9	109.1	100.0
Relative LOD (%)	0.010	0.010	0.005	0.010

Most studies using DNA-based methods targeting mitochondrial multi-copy genes focus on the authentication of milk and milk products, with sensitivities above 0.1% of cow DNA, not enough in the field of allergen detection [24]. The real-time PCR method developed by Xiao et al. [16] showed an absolute sensitivity of 0.05 ng of bovine DNA, being applied to

42 commercial food samples. However, the authors did not use reference mixtures as calibrants to develop an effective quantitative approach.

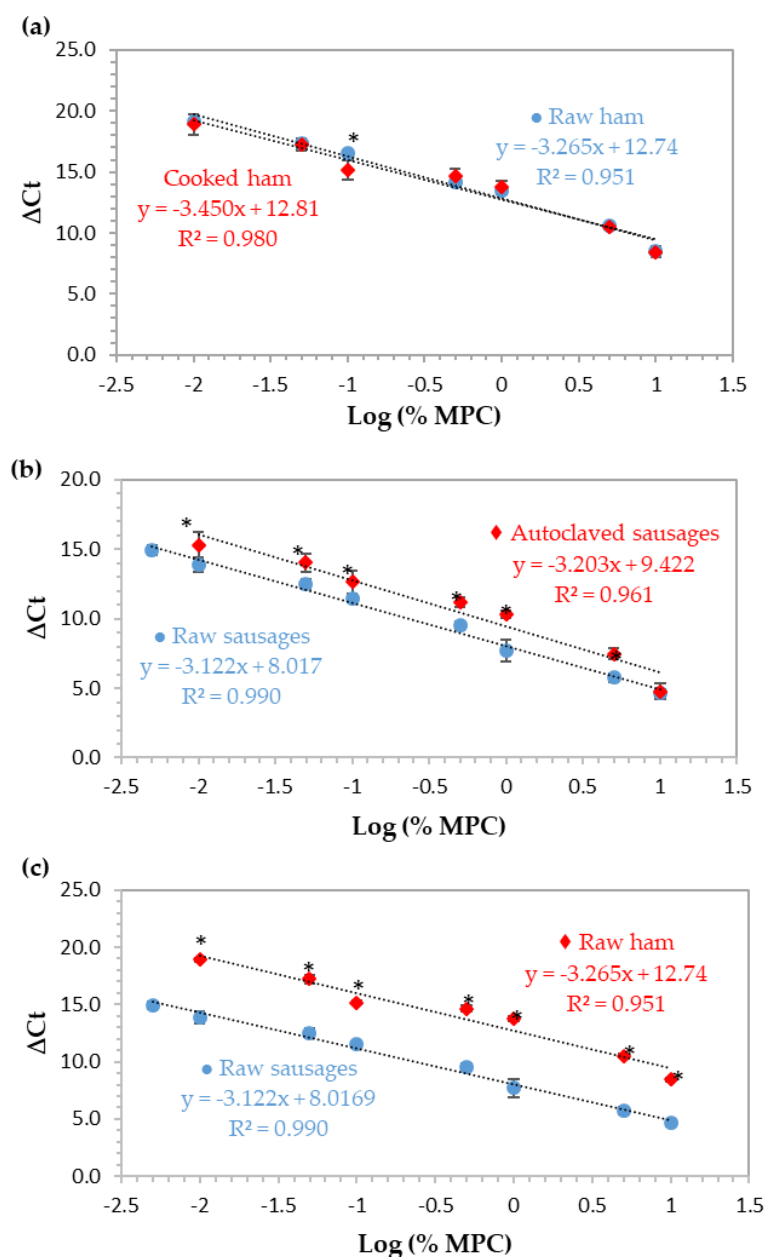


Figure 3. Normalized calibration curves obtained by real-time PCR with TaqMan probe targeting the 12S rRNA gene using ham and sausage model mixtures with 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01% and 0.005% (w/w) of MPC (n=8 replicates) with and without thermal treatment. (a) Raw and cooked-ham reference mixtures; (b) Raw and autoclaved sausage mixtures; (c) Raw mixtures of hams and sausages. *Significant differences ($p < 0.05$) between the ΔC_t values at the same spiking level (independent samples t-test).

Köppel et al. [25,26] reached an absolute sensitivity down to 0.64 $\mu\text{g/mL}$ of bovine DNA, targeting the mitochondrial bovine tRNA-Lys gene, for the detection of cow's milk in processed foods as an allergen, but without its quantification. To the best of our knowledge, the proposed real-time PCR assay enables the highest sensitivity, both absolute and

relative, for the detection of cow's milk ingredients in complex food products, enabling its correct estimation by the use of reference mixtures.

Construction and validation of the normalized quantitative model

For the relative quantification of MPC in cooked ham and sausages, a normalized curve based on the ΔC_t method was constructed for each type and set of model mixtures (ham/sausage and raw/processed). This approach uses the cycle threshold (C_t) values obtained from the parallel amplification of the target sequence (12S rRNA gene of cow) and a universal eukaryotic region (18S rRNA gene as endogenous control) with approximately the same amplification efficiencies, in order to assure the accuracy of results [22]. In this study, ΔC_t values were obtained by the difference between the C_t values of cow and eukaryotic amplifications and plotted against the logarithm of MPC percentage of eight concentration levels (10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, and 0.005%, w/w). The obtained calibration curves, each one constructed with the mean values of two independent real-time PCR runs ($n = 8$), are presented in Figure 3 and the respective PCR performance parameters are summarized in Table 2. All the results are in agreement with the accepted criteria defined for this type of assay [22,23], except for the correlation coefficient of the curves of raw ham and autoclaved sausages (0.951 and 0.961, respectively). During the preparation of ham mixtures, retention of a small quantity of water when they were raw was noticed, which was eliminated after thermal treatment and could affect DNA extraction and PCR amplifications, thus, contributing to a low R^2 value. In the case of autoclaved sausages, the severe thermal treatment might be the main reason to affect this parameter negatively.

In our previous work describing a preliminary real-time PCR trial using known amounts of MPC in raw turkey meat and cooked hams as model mixtures, but without normalization, the C_t values above the threshold of cross-species amplification (38 cycles) were considered negative, setting a relative LOD of 0.05% (500 mg/kg, w/w) of MPC in raw and cooked ham [19]. Comparing the present results with the previous findings, the relative sensitivity increased to 0.01% (100 mg/kg, w/w) in both ham model mixtures, even considering the 38-cycle limit above which the amplification is unreliable due to cross-reactivity with other species, namely chicken and goat [19]. Additionally, regarding cooked ham model mixtures, there was also a clear improvement in the PCR efficiency (from 88.0% to 94.9%). As observed, the use of a reference endogenous gene for normalization had a positive effect on the performance parameters of this system. In complex food matrices, such as meat products, the use of several ingredients and thermal treatments can affect the target gene amplification. Moreover, the DNA degradation, the presence of PCR inhibitors or the differences in the amount and quality of the extracted DNA, but also the differences in target species content can cause amplification variations. These variations in DNA yields

and efficiency of amplification can be controlled by the construction of a normalized calibration curve using an endogenous control [18,27,28].

Table 3. Validation results of normalized quantitative real-time PCR systems applied to blind mixtures of MPC in ham and sausages.

Samples	Milk (% w/w)		SD ²	CV ³ (%)	Bias (%) ⁴
	Actual	Mean predicted ¹			
Raw ham					
A	4	4.7	0.41	8.7	18.2
B	0.8	1.46	0.12	8.1	82.9
C	0.4	0.46	0.08	16.1	15.8
D	0.2	0.07	0.02	16.0	-65.4
Cooked-ham					
E	4	4.5	0.59	1.3	-11.6
F	0.8	0.7	0.12	17.4	-10.8
G	0.4	0.4	0.09	23.7	-5.0
H	0.2	0.17	0.04	19.70	-14.50
Raw sausages					
I	2	1.59	0.30	19.1	-20.4
J	0.4	0.48	0.05	9.5	21.0
K	0.2	0.15	0.03	21.8	-25.5
L	0.02	0.023	0.006	24.9	-15.20
Cooked sausages					
M	2	1.63	0.23	14.4	-18.5
N	0.4	0.46	0.09	19.9	14.8
O	0.2	0.17	0.04	24.5	-15.0
P	0.02	0.025	0.005	19.8	24.3

¹ Mean values of replicate assays (n=8) of two independent runs; ² SD – standard deviation; ³ CV – coefficient of variation; ⁴ Bias = ((mean value - true value)/true value) x 100.

In order to validate the developed quantitative method, different blind mixtures, containing 4.0%, 2.0%, 0.8%, 0.4%, 0.2%, and 0.02% (w/w) of MPC, were used for each subset of model mixtures. Accordingly, several parameters such as trueness, precision, and robustness were assessed [22,23], and the obtained results are presented in Table 3. In terms of trueness, expressed as bias, the majority of the values are between –20.4% and 24.3%, which are within the acceptable bias of $\pm 25.0\%$ of the actual value over the tested dynamic range. However, there are three values that are above the criterion of acceptance, namely, samples B (82.9%), D (65.4%), and K (25.5%). Samples B and D are the raw mixtures of hams that showed, as previously mentioned, a small quantity of water after their preparation, thus affecting PCR amplifications of the target DNA and its subsequent quantification. The ice used in the sausage preparation may have affected, in a similar way, the quantification of MPC in raw sample K. The values of coefficient of variation, expressing the relative standard deviation of results, under repeatability conditions, varied between 1.3% and 24.9%, demonstrating the precision of the method over the tested dynamic range.

($\leq 25.0\%$). Additionally, real-time PCR runs were performed using distinct reference and blind mixtures submitted to two different thermal processes, thus confirming the robustness of the proposed quantification system (Table 3). In summary, the results of method validation and the performance parameters of real-time PCR assays clearly demonstrate the reliability and accuracy of the quantitative method.

Effect of food matrix and thermal treatment

To evaluate the effect of thermal processing and food matrix, two distinct and independent heat treatments were used, simulating the production of cooked hams and autoclaved sausages. The preparation of both formulations was done independently and divided into two subsets, one to be analyzed without processing and the other using the corresponding thermal treatment. In the case of cooked ham, it was cooked in an oven at a temperature of 67 °C for 5 h, in controlled conditions of humidity, while the sausages were autoclaved at 121 °C for 15 min with controlled pressure (1 bar). The normalized calibration curves using MPC in ham and sausages are presented in Figure 3a and b, respectively, comparing, in both cases, the raw with thermally processed mixtures. In Figure 3c, raw mixtures of ham and sausages are compared to evaluate the effect of food matrix. The normalized calibration curves of each set of ham mixtures practically overlapped (Figure 3a) since the slopes and b-intercepts were very close (difference of b-intercept was about 0.1 cycles), with only one significant difference at 0.1% (w/w) level. However, it can be observed that there is a decrease in the correlation coefficient of the method in raw mixtures ($R^2 = 0.951$), suggesting that the excess of water present in these mixtures had a subtle negative effect on the performance of the system, as previously stated. In terms of relative LOD and PCR efficiency, the results suggest that thermal treatment had no effect on the detection of MPC since the same LOD of 0.01% (100 mg/kg) was reached in both ham systems with PCR efficiencies of 102.4% and 94.9% for raw and cooked mixtures, respectively (Figure 3a, Table 2). Regarding sausage mixtures, raw and processed (Figure 3b, Table 2), the values for PCR efficiency, slope, and R^2 were 109.1% or 100.0%, -3.122 or -3.203 , and 0.990 or 0.961, respectively. Comparing the raw with the autoclaved mixtures, a difference of about 1.4 cycles was noticed between calibration curve intercepts, which is in agreement with the significant differences found at all levels of concentration ($p < 0.05$), except for the highest one (10.0%, w/w). In this case, heat processing had a negative effect on the MPC detection since the relative LOD were 0.005% (w/w) and 0.01% (w/w), in raw mixtures and in autoclaved ones, respectively. In addition, the correlation coefficient in autoclaved sausages is slightly out of the acceptable range [22,23]. Comparing both thermal treatments in hams and sausages, the more aggressive autoclaving process seems to slightly affect the detection of MPC, but still maintaining an optimal PCR efficiency.

Food matrix clearly affects MPC detection (Figure 3c) since a difference of about 4.7 cycles between the intercepts of the calibration curves of ham and sausages is obtained, which agrees with significant differences found at all concentration levels ($p < 0.05$) and demonstrates a clear delay in the amplification of DNA from ham mixtures in relation to sausages. Moreover, the LOD of raw sausages is 0.005% (50 mg/kg, w/w) with optimal PCR performance parameters ($R^2 = 0.990$, slope = -3.122 , and PCR efficiency = 109.1%), contrarily to raw ham that achieved a LOD of 0.01% (100 mg/kg, w/w) and a correlation coefficient of 0.951, below the acceptable value of 0.98 (Figure 3c, Table2). The effect of food matrix in food allergen detection by DNA-based methods has been reported by some authors [18,29,30]. The use of reference materials with different calibration models in order to simulate the real food matrices is extremely important. In addition to allowing the correct estimation and quantification of the target, it accounts with all the components of the food matrix, such as fats, carbohydrates, and other metabolites, which can interfere in DNA extraction and lead to a decrement in PCR efficiency, thus, affecting quantitative results [6,18,31].

Analysis of commercial samples

The applicability of the developed methods to real processed meat products (both normalized systems with processed mixtures) was done according to each type of sample, cooked ham or sausage. Thirteen commercial samples of cooked hams and sausages from distinct brands were evaluated for the presence of milk ingredients, being posteriorly analyzed by real-time PCR for quantification purposes. For comparison purposes, the same commercial hams and sausages were tested with ELISA. Table 4 presents the summarized qualitative and quantitative PCR results, as well as ELISA results, together with the relevant label information. The qualitative PCR results show that only four samples are positive to milk, namely #6, #9, #12, and #13, whose labels stated the presence of milk proteins or “may contain traces of milk”. However, in the case of sample #9, there is no label information about the presence of milk, while sample #13 clearly stated that is a “milk-free” product. All the qualitative results were confirmed by real-time PCR, with only one sample (#5) presenting negative amplification. Following our pre-established specificity criteria [19], all amplifications above 38 cycles were considered below the LOD because of the cross-reactive species. Thus, only samples #6 and #12 could be quantified for the presence of milk, obtaining 0.205% (2050 mg/kg, w/w) and 0.014% (140 mg/kg, w/w) of milk content, respectively. All the remaining samples were considered below the stipulated LOD. According to Regulation (EU) no. 1169/2011 [32], the mandatory labelling regarding potentially allergenic ingredients should be complied with.

Table 4. Results of the application of qualitative PCR, normalized quantitative real-time PCR and ELISA to detect and quantify milk ingredients in commercial samples of cooked-hams and sausages.

Samples	Relevant label information	Qualitative PCR			Real-time PCR		ELISA	
		18SRG-F/ 18SRG-R	916-F/ 916-R	EUK-F/EUK-R (Ct ± SD) ¹	916-F/916-R (Ct ± SD) ¹	Estimated milk content (mg/kg) (mean ± SD) ²	Estimated amount (mg/kg) (mean ± SD) ²	
Cooked hams (from pork)								
1	Milk proteins	+	- ⁴	26.60±0.16	40.76 (1/3) ⁵	<LOD ⁶	<LOQ ⁷	
2	No information about milk	+	-	29.57±0.28	39.77 (1/3)	<LOD	<LOQ	
3	No information about milk	+	-	26.83±1.21	45.58 (1/3)	<LOD	<LOQ	
Cooked hams (from turkey)								
4	May contain traces of milk	+	-	21.88±0.62	41.15±1.10 (2/3)	<LOD	3.95±0.10	
5	May contain traces of milk	+	-	25.38±0.08	(0/3)	ND ⁸	<LOQ	
6	Milk proteins	+	+	19.56±0.04	34.19±0.27 (8/8)	2050±320	23300±4722 ⁹	
Sausages (from pork)								
7	No information about milk	+	-	23.75±1.39	40.52±0.90 (3/3)	<LOD	8.02±2.06	
8	No information about milk	+	-	21.31±1.80	40.35±0.27 (2/3)	<LOD	<LOQ	
9	No information about milk	+	+	24.14± 0.40	39.99 ± 0.85 (3/3)	<LOD	<LOQ	
Sausages (from turkey)								
10	May contain traces of milk	+	-	22.49±0.17	39.90 (1/3)	<LOD	<LOQ	
11	May contain traces of milk	+	-	28.67±0.14	41.91±2.07 (3/3)	<LOD	<LOQ	
12	May contain traces of milk	+	+	18.67±0.01	35.77±0.60 (8/8)	140±30	94.5±7.78*	
13	Without milk addition	+	+	20.05±0.04	41.01±1.90 (2/3)	<LOD	<LOQ	

¹ Mean values of cycle threshold (Ct) ± standard deviation (SD) (n = 8) of two independent runs; ² Mean values (mg/kg) ± standard deviation (SD) of replicate assays (n=3); ³ Positive amplification (+); ⁴ No detectable amplification (-); ⁵ Positive replicates and total number of replicates ⁶ <LOD (100 mg/kg); ⁷ <LOQ (2.5 mg/kg); ⁸ ND, not detected; ⁹ Estimated amounts obtained from previously diluted protein extracts.

From samples whose labels stated the presence of milk protein or at traces level, 70% (5/7) were negative to milk by real-time PCR, suggesting the common precautionary labelling to comply with legislation. In the case of samples #6 and #12, which were positive to milk, the estimated amount seems to be in accordance with the label. Thus, all the tested samples were in good agreement with allergen labelling legislation [32].

ELISA and real-time PCR results seem to be well corroborated, comparing both quantitative data for four samples (#4, #6, #7, and #12) (Table 4). Samples #4 and #7 presented trace amounts of milk proteins based on ELISA (3.95 mg/kg and 8.02 mg/kg, respectively), while the results of real-PCR were <LOD, which was defined as 100 mg/kg. Sample #12 exhibited an amount of 140 mg/kg by real-time PCR, which agrees with the value determined by ELISA (94.5 mg/kg). The only difference among results concerned sample #6, whose ELISA result was 10 times higher than the real-time PCR. However, considering that this sample needed a 1000-fold dilution in order to fall within the calibration curve range (2.5 to 67.5 mg/kg), the value might be overestimated. In general, these kits are specialized in evaluating trace amounts of allergenic proteins, not the quantities expressed in this specific sample (#6), which might explain the overestimated value.

CONCLUSIONS

In the present study, we propose the development of four normalized real-time PCR systems targeting the 12S rRNA mitochondrial gene of *Bos domesticus* to detect and quantify trace amounts of milk ingredients (protein concentrate) in complex meat products. Two distinct and independent thermal treatments were applied to two different meat mixtures (hams and sausages) in order to evaluate the effects of food matrix and processing on milk detection and quantification. On the one hand, the ham matrix seems to have a clear effect on milk detection since the sensitivity in hams without thermal treatment was two-fold lower than in raw sausages. On the other hand, the autoclaving process slightly decreases the sensitivity from 0.005% (w/w) in raw sausage mixtures to 0.01% (w/w) in autoclaved ones, while the soft oven-cooking treatment seems to have no effect. The developed methods were validated with blind mixtures and successfully applied to commercial meat samples. The quantitative real-time PCR results of commercial samples were further validated by ELISA, confirming the previous data. The results of commercial samples enabled the quantification of trace amounts of milk in two out of 13 samples, suggesting the excessive practice of the precautionary labeling in most of the samples. With this study, novel, highly specific, and sensitive normalized real-time PCR systems were proposed as accurate and reliable tools for the detection and quantification of milk ingredients in meat products, which could effectively contribute to better manage milk

allergens by the food industry and by the control laboratories, aiming at protecting the health of sensitized and allergic consumers.

Author Contributions: Conceptualization, I.M. and J.C.; methodology, C.V.; validation, C.V.; formal analysis, C.V. and J.C.; investigation, J.C. and I.M.; writing - original draft preparation, C.V.; writing - review and editing, I.M. and J.C.; supervision, I.M.; project administration, I.M.; funding acquisition, I.M.

Funding: This work was supported by UID/QUI/50006/2019 with funding from FCT/MCTES through national funds and by the projects AlleRiskAssess-PTDC/BAA-AGR/31720/2017 and NORTE-01-0145-FEDER-00001. Caterina Villa is grateful to the FCT grant (PD/BD/114576/2016) financed by POPH-QREN (subsidized by FSE and MCTES).

Conflicts of Interest: The authors declare no conflict of interest.

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(Submitted)



nutrients



2.2.3. Effect of autoclaving and *in vitro* gastro-duodenal digestion on the modulation of IgE binding capacity of milk proteins incurred in sausage model food

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ABSTRACT

Food industry commonly uses milk ingredients as technological aids in a wide variety of products. However, milk is a source of food allergens causing severe allergic reactions in sensitized individuals. This work intends to study the effect of autoclaving and *in vitro* digestion on the allergenicity of milk ingredients added to meat products. Protein profiles of raw and autoclaved sausages without and with the addition of 10% of milk protein concentrates were evaluated by SDS-PAGE and LC-MS/MS. Additionally, the final IgE-reactivity were studied by immunoblotting with pooled sera of cow's milk allergic individuals followed by bioinformatic investigation. Results showed that autoclaving lead to an increase in protein fragmentation (higher number of short peptides) and consequently to a higher digestion rate, more pronounced in β -casein. IgE-binding capacity of milk proteins was completely eliminated by autoclaving followed by digestion. This study highlights the importance of using autoclaving as a strategy to produce hypoallergenic formulas.

Keywords: milk allergen, autoclaving, meat products, simulated digestion, IgE-reactivity, LC-MS/MS.

INTRODUCTION

Cow's milk proteins are often used as ingredients in the food industry due to their technological/functional properties, aiming at improving the appearance, taste and texture of products, as well as their nutritional value.¹ However, milk proteins are common food allergens, responsible for one of the most frequent food allergies in early childhood, with a prevalence between 0.6 % and 3% in children under the age of 6 years.² Due to the high number of food products containing milk ingredients as technological aids, accidental exposure to milk proteins is often recurrent, representing a constant threat to allergic individuals. Those patients are forced to implement an elimination diet in order to avoid the occurrence of mild to severe allergic reactions, such as cutaneous, respiratory or gastro-intestinal reactions, and in some extreme cases even systemic anaphylaxis.^{2,3}

Cow's milk proteins are divided in two main groups according to their solubility at pH 4.6 and 20 °C: caseins (α_{S1} -, α_{S2} -, β -, κ -caseins) and whey proteins (α -lactalbumin [ALA], β -lactoglobulin [BLG], bovine serum albumin [BSA], lactoferrin and immunoglobulins [Ig]), accounting for 80% and 20% of the total protein content, respectively.² According to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) list of allergens, cow's milk allergens are classified as: Bos d 8 (name that designates all caseins) or Bos d 9 (α_{S1} -casein), Bos d 10 (α_{S2} - casein), Bos d 11 (β - casein), Bos d 12 (κ -casein) for each individual casein; Bos d 4 (ALA), Bos d 5 (BLG), Bos d 6 (BSA) and Bos d 7 (Ig).⁴ Considering that more than 50% of sera from milk-allergic patients reacts with caseins, ALA, BLG and BSA, these proteins are considered major allergens in cow's milk, while Ig is classified as a minor allergen.²

Milk and milk proteins can be present in several food matrices, being submitted to different types of processing, until they become available to consumers as final products. Food processing, both conventional methods (e.g. heat treatment or fermentation) and novel methods (e.g. high pressure, irradiation or ultra-sound) can induce chemical and physical changes of milk proteins, differently affecting their allergenicity.^{2,5} These alterations may induce the destruction of conformational epitopes, either by denaturation, by aggregation phenomena or by the occurrence of chemical reactions among the different food matrix components (proteins, fat and sugars), limiting the availability of the protein to the immune system and, therefore, causing a reduction in the allergic response.^{6,7} On the other hand, the formation of neo-epitopes and/or a decrease in protein digestibility due to protein-matrix interactions might potentially increase the IgE-binding capacity of proteins.⁸ The high protein content of a food matrix seems to enhance the stability against simulated gastro-intestinal degradation and create a competitive environment for enzyme cleavage, thereby delaying gastrointestinal proteolysis of food allergens.⁹

The effects of food processing and food matrix on milk proteins have been extensively studied in order to control milk allergy.² Different strategies as microwave^{10,11}, fermentation¹², high pressure¹³, pulsed light¹⁴ and ultra-sound¹⁵ have been recently investigated for their potential to alter the intrinsic allergenicity of milk proteins with interesting results. A study developed by Nowak-Wegrzyn et al.¹⁶ demonstrated that about 70% of tested children were able to ingest a muffin containing baked milk without any immediate clinical symptoms. Bavaro et al.¹⁷ compared the IgE-binding capacity of milk baked in the oven with baked milk within a muffin matrix, proving that interactions between milk proteins and food components during oven-heating play a role in the potential reduction of milk allergenicity. Resistance to gastrointestinal digestion proteases is also an important parameter to take into account since the potential of an allergen to trigger an immunoreaction might be enhanced by the preservation of its structural integrity during the digestion process.¹⁸ Several works using *in vitro* digestion models have been performed to study milk protein digestibility, but without the inclusion of the allergen in a food matrix, thus lacking the subsequent evaluation of the interaction between milk proteins and other food components.¹⁹⁻²⁴

This work aimed at studying the effect of autoclaving on the IgE-binding capacity of milk proteins used as technological aids in meat products, after *in vitro* simulated gastroduodenal digestion. Proteomic profiles of digested model mixtures of turkey sausages samples spiked with milk protein concentrates (MPC) were characterized before and after autoclaving treatment and the IgE-binding capacity was tested by immunoblotting experiments with human sera of cow's milk allergic patients (pooled sera) and bioinformatic search.

MATERIAL AND METHODS

Chemicals

Bovine Serum Albumin (BSA), ammonium bicarbonate (AMBIC), iodoacetamide (IAA), dithiothreitol (DTT), along with chemicals for electrophoresis, namely sodium dodecyl sulphate-SDS, glycine, glycerol and Coomassie brilliant blue-G 250 and purified proteins (BLG, total caseins, α - and β -caseins) were provided by Sigma-Aldrich (Milan, Italy). Acetonitrile (Gold HPLC ultragradient), trifluoroacetic acid (TFA) and blue bromophenol were purchased from Carlo Erba Reagents (Cornaredo, Milan, Italy), while ultrapure water was produced by a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Formic acid (MS grade) was purchased from Fluka (Milan, Italy), whilst 0.45 μ m filters in polytetrafluoroethylene (PTFE) and 5 μ m filters in cellulose acetate (CA) were purchased from Sartorius (Gottingen, Germany). Trypsin (proteomic grade) for the in-gel protein

digestion was purchased from Promega (Milan, Italy). As for *in vitro* digestion experiments, potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate (NaHCO_3), sodium chloride (NaCl), magnesium chloride hexahydrate ($\text{MgCl}_2(\text{H}_2\text{O})_6$), ammonium carbonate ($(\text{NH}_4)_2\text{CO}_3$), sodium hydroxide (NaOH), hydrochloric acid (HCl) and calcium chloride (CaCl_2) along with other analytical grade chemicals and enzymes (salivary α -amylase, pepsin, trypsin, chymotrypsin, pancreatic α -amylase, pancreatic lipase plus phospholipid, bile, serine protease inhibitor (PMSF=methyl-phenyl-sulfonyl fluoride)) were obtained from Sigma-Aldrich (Milan, Italy).

Production of model sausages

The mixtures used in this work were prepared following the recipe of industrial sausages incurred with 10% of MPC (Formulab, Maia, Portugal), commonly used as a technological aid in the manufacture of hams and sausages. The real content of proteins in MPC was measured by the Kjeldahl protocol, obtaining a content of 83.4%. The mixture used for sausage preparation was made by the addition of 44% of minced turkey meat, 33% of pork fat, 2% of salt and 21% of crushed ice and grounded in a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). This raw mixture was used as a negative control, named a 0% of MCP addition. The incurred reference mixture containing 10% of MPC was prepared by the addition of 25.2 g of MPC in 184.8 g of the raw mixture taking into account the protein content of MPC measured by Kjeldahl. Model mixtures with 0% and 10% of MPC were divided in two sub-sets each, one to be used as raw material and the other one submitted to an autoclaving processing (121 °C, 15 min, 1 bar) simulating the industrial production of sausages. In summary, the analyzed model mixtures were: a) raw sausage mixture with 0% MPC (SMPC0); b) raw sausage mixture with 10% MPC (SMPC10); c) autoclaved sausage with 0% MPC (PSMPC0); and d) autoclaved sausage with 10% MPC (PSMPC10).

All the mixtures were homogenized and immediately stored at -20 °C until further analysis.

Sera of milk allergic patients

Sera were obtained from a total of 7 milk allergic children according to ethical requirements. Tests were conducted in accordance with the Declaration of Helsinki and all procedures of the study were approved by the local Ethics Committee (code 2018/128). Permission to participate in the study of all children was obtained and the written informed consent was signed by the parents. The allergy symptoms in general ranged from vomit, cough and rhinitis to anaphylaxis. The clinical features of the allergic individuals enrolled in this study are reported in Table 1. Diagnosis of IgE-mediated allergy to cow's milk was

previously confirmed by skin prick test (SPT) and serum-specific IgE (ImmunoCAP, Phadia, Uppsala, Sweden) to cow's milk and cow's milk proteins (sIgE to CM, BLG, ALA, caseins, total serum). All sera were stored at -80°C until further use.

Table 1. The clinical features of the allergic individuals enrolled in this study.

Serum	IgE to Cow's Milk (KU/L)	IgE to Casein (KU/L)	Allergic Reaction Displayed
1	27	6	anaphylaxis
2	79	17.5	cough and rhinitis
3	38	21	anaphylaxis
4	23	21	vomit
5	24	28	anaphylaxis
6	13.9	6	vomit
7	100	100	anaphylaxis

Preparation of digestive fluids

In vitro gastro-duodenal digestion experiments were accomplished according to a standardized static protocol, mimicking chewing, gastric and intestinal compartments as described by Minekus et al.²⁵ Simulated salivary fluid (SSF, pH 7) was prepared in order to include KCl (15.1 mM), KH_2PO_4 (3.7 mM), NaHCO_3 (13.6 mM), $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.15 mM), $(\text{NH}_4)_2\text{CO}_3$ (0.06 mM) and HCl (1.1 mM) for pH adjustment. Simulated gastric fluid (SGF, pH 3) was prepared with KCl (6.9 mM), KH_2PO_4 (0.9 mM), NaHCO_3 (25 mM), NaCl (47.2 mM), $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.1 mM), $(\text{NH}_4)_2\text{CO}_3$ (0.5 mM) and HCl (15.6 mM) for pH adjustment. Simulated intestinal fluid (SIF, pH 7) was prepared with the addition of KCl (6.8 mM), KH_2PO_4 (0.8 mM), NaHCO_3 (85 mM), NaCl (38.4 mM), $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.33 mM) and HCl (8.4 mM) for pH adjustment.

Assessment of protein composition in digestive fluids

With the aim to investigate the composition and the actual amount of proteins solubilized in the digestive fluids and potentially susceptible to enzymatic hydrolysis, raw and processed model sausages without and with MPC addition (SMPC0, SMPC10, PSMPC0, PSMPC10) were submitted to chew (2 min), gastric (2 h) and duodenal (2 h) phases (simulated by the respective fluids), without adding digestive enzymes during the procedure. All steps were carried out in a shaking incubator (170 rpm) at 37°C . Briefly, 1 g of each model sausage was mixed with 995 μL of SSF and 5 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 M) and incubated for 2 min to simulate chew conditions. Then, the obtained mixture was mixed with

1500 μL of SGF and 1 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 M) and the pH adjusted to 3.0 for mimicking the gastric environment. The final volume of 4 mL was completed with ultrapure water (Millipore MilliQ system, Bedford, MA, USA). Final mixture was then left shaking at 37 °C for 2 h. Afterwards, 2200 μL of SIF and 8 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 M) were added to gastric mixture, the pH was adjusted to 7.0 and a final volume of 8 mL was completed by adding MilliQ water in order to simulate duodenal conditions. A last incubation during 2 h at 37 °C was performed. The final mixture was then centrifuged for 15 min at 12,000 rpm at 4 °C and the supernatant was carefully collected and filtered through 5 μm cellulose acetate syringe filter. The total protein content of raw and autoclaved sausages was calculated by Bradford assay (Quick Start™ Bradford Protein Assay, Bio-Rad Laboratories, Segrate, MI, Italy) according to the manufacturer's guidelines. Bovine serum albumin was used as reference protein. All mixtures were stored at -20 °C until use and filtered through 0.45 μm PTFE filters just before electrophoretic analysis.

***In vitro* gastro-duodenal digestion of raw and processed sausages**

Raw and autoclaved model sausages without (0%) and with (10%) MPC addition were submitted to *in vitro* gastro-duodenal digestion according to the standardized protocol described by Minekus et al.²⁵ All steps were carried out in a shaking incubator at 37 °C, at 170 rpm. Oral phase was simulated by adding 620 μL of SSF and 5 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 M) with the addition of 75 U/mL of human salivary amylase (Sigma-Aldrich, St Louis, MO, USA) to 1 g of model sausage which was then kept incubating for 2 min. Afterwards, the chewed mixture was mixed with 1500 μL of SGF and 1 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 M) containing 2000 U/mL of gastric pepsin (Sigma-Aldrich) and 0.17 mM of phospholipids (Sigma-Aldrich) to mimic the gastric digestion. The pH was adjusted to 3.0 with HCl and a final volume of 4 mL was completed with ultrapure water. Mixtures were then incubated for 2 h at 37 °C. For the duodenal phase, a last incubation at 37 °C for 2 h was carried out after incorporating 2200 μL of SIF and 8 μL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3 M) with 100 U/mL of pancreatin (Sigma-Aldrich) and 10 mM of bile salts (Sigma-Aldrich) into the chyme. The pH was adjusted to 7.0 with NaOH and a final volume of 8 mL was completed by adding MilliQ water. Phenyl methane sulfonyl fluoride (PMSF) was added to stop the enzymatic reaction. A centrifugation at 12,000 rpm during 15 min (4°C) was then performed, the supernatant collected and stored at -20 °C until further analysis. Digests were filtrated through 5 μm cellulose acetate syringe filters just before electrophoretic analysis.

SDS-PAGE analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 8–16% polyacrylamide pre-cast gels (8.6 cm × 6.7 cm × 1 mm) using a Mini-

Protean Tetra Cell equipment (Bio-Rad Laboratories, Segrate, MI, Italy). Ten µg of SMPC0, SMPC10, PSMPC0, PSMPC10 proteins extracted by digestive fluids (with or without the addition of enzymes) were separated under reducing conditions. The extracted proteins were mixed with Laemmli buffer (62.5 mM TrisHCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, 100 mM DTT) in a 1:1 proportion and then denatured for 5 min at 95 °C. The electrophoretic separation was performed in a running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) at 100 V until the end. Gels were stained by a Coomassie Brilliant Blue G-250 solution. The bands were detected on a ChemiDoc™ Imaging System (Bio-Rad Laboratories, Segrate, MI, Italy). Precision Plus Protein™ All Blue Standards (10-250 kDa, Bio-Rad Laboratories, Segrate, MI, Italy) were used as protein molecular weight references.

Immunoblot for IgE-binding assay

SDS-PAGE of undigested and digested sausages (corresponding to 10 µg of proteins loaded for both raw and autoclaved samples) under reducing conditions were electroblotted onto a 0.2 µm nitrocellulose membrane (Bio-Rad Laboratories, Segrate, MI, Italy) using a Trans-Blot Cell (Bio-Rad Laboratories, Segrate, MI, Italy) for 7 minutes (1.3 A, 25 V). Immunoblotting experiments were accomplished according to the protocol described by Bavaro et al.¹⁷ As primary antibody, a pooled sera of a total of 7 young allergic patients previously diluted in TBS-T (pH 7.4, 10 mM Tris, 50 mM NaCl, 0.1% Tween 20) at 1/50 ratio was used and kept shaking overnight at 4 °C, while as a secondary antibody a Goat Anti-Rabbit IgG (H+L) HRP Conjugate (Bio-Rad Laboratories) previously diluted 1/5000 (v/v) in TBS-T was added. Final images were acquired on a ChemiDoc™ MP Imaging System.

Purification of gastro-duodenal digestion extracts prior to LC-MS/MS analysis

Gastro-duodenal digests of raw and processed sausage without and with MPC were finally purified by Sep-Pak C18 cartridges (50 mg, 1 mL, Waters spa, Milan, Italy) according to the following protocol: (i) conditioning/equilibration step of column with methanol (3x1 mL) and SIF (3x1mL), (ii) loading of digested sample (1 mL), (iii) washing step (0.5 mL of MilliQ water), (iv) elution step (1 mL H₂O:CH₃CN 10:90 + 0.1% formic acid). Finally, the eluates were filtrated through 0.2 µm PTFE filters and 20 µL were further injected into LC/MS apparatus.

In-gel protein digestion

Selected protein bands from the SDS-PAGE were cut and submitted to in-gel digestion procedure according to De Angelis et al., 2017.²⁶ Each sample was resuspended in 70 µL of H₂O/Acetonitrile, 90/10 + 0.1% formic acid (v/v) and 20 µL were further injected into LC/MS apparatus.

Protein identification by untargeted LC-MS/MS analysis

Protein bands and gastro-duodenal digested sausages were analyzed by using a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer coupled to an Ultra-High-Performance Liquid Chromatography (UHPLC) pump systems (Thermo Fisher Scientific, San José, CA, USA). Peptide mixture was separated on a reversed phase Aeris peptide analytical column (internal diameter 2.1 mm, length 150 mm, particle size 3.6 µm, porosity 100 Å, Phenomenex, Torrance, CA, USA) at a flow rate of 200 µL/mL according to the following conditions: from 0–50 min solvent B increased from 10% to 55%, at 50 min stepwise from 55% to 85%, then kept constant for 15 min, at 65 min down to a constant 10% during 20 min for column conditioning before next injection. As mobile phases, the solution H₂O + 0.1% of formic acid (A) and acetonitrile + 0.1% of formic acid (B) were employed. MS spectra were acquired in the mass range of 200–2000 m/z by running the instrument in data dependent (FullMS-dd2) acquisition mode and only positive ions were considered in this study. Other MS parameters were the same as described in Bavaro et al.²⁷ Final MS raw data were processed via the commercial software Proteome Discoverer™ version 2.1 (Thermo-Fisher-Scientific, San José, CA, USA) and protein identification was achieved by SequestHT searching against a pig, turkey and cow customized database extracted by UniProt DB basing on the taxonomy codes of *Sus scrofa* (ID: 9823), *Meleagris gallopavo* (ID: 9103) and *Bos Taurus* (ID:9913). The sequences of digestive enzymes used for simulating gastro-duodenal digestion were included as well. The identification of tryptic peptides originated by in-gel digestion experiments was accomplished by setting at 5 ppm and 0.05 Da, respectively, the mass tolerance on the precursor and fragment ions. Only trustful peptide-spectrum matches were accepted and in particular a minimum of two or higher were the minimum criteria for protein identification by selecting a high and medium confidence (FDR < 1%, FDR <5%).

Bioinformatic analysis of the residual immunoreactivity of milk-enriched raw and processed sausages after gastro-duodenal digestion

Peptide sequences identified in duodenal digests of raw and processed model sausages with 10% of MPC were searched in IEDB database in order to screen epitope linear sequences surviving to gastro-duodenal digestion. The IEDB results were filtered as follows: linear sequence for epitope structure, substring or exact match for BLAST option, human as host and allergic reaction as disease.

RESULTS AND DISCUSSION

In the current study, the effect of a common industrial food processing, namely autoclaving, was investigated on the final stability and IgE-binding capacity of milk proteins incorporated in a meat product and submitted to *in vitro* digestion procedure, mimicking the human salivary, gastric and duodenal phases. For this purpose, raw and processed sausages without and with the addition of MPC were submitted to standardized *in vitro* digestion. Any changing in milk protein solubility, because of thermal/pressure treatment, was assessed by estimating the sausage protein content with a Bradford assay, as previously reported in other works.²⁷⁻²⁹ In addition, the direct comparison of the SDS-PAGE profiles of raw and processed sausages spiked with MPC, before and after *in vitro* digestion, provided information about the proteins as affected by autoclaving process, both in terms of stability and digestibility. Finally, the effect of autoclaving on the final IgE-binding capacity of milk proteins incorporated into sausages and submitted to *in vitro* digestion was investigated by immunoblotting experiments with pooled sera of young children allergic to cow's milk, followed by bioinformatic investigation to identify milk allergen epitopes surviving to *in vitro* digestion.

Effect of autoclaving on the solubility and digestibility of milk protein fraction in meat samples

Protein content

As first step, the impact of autoclaving on the solubility of milk proteins added to sausage was assessed by the Bradford assay. Specifically, the experiments were carried out only on the proteins solubilized into the digestive biological fluids; this fraction represents the actual amount of proteins potentially susceptible to enzymatic hydrolysis. Thus, realistic results on the behavior of the processed milk proteins during digestion were obtained. In the light of this, raw and autoclaved sausages without and with MPC (10%) addition were submitted to salivary (2 min), gastric (2 hours) and duodenal (2 hours) phases (simulated by the respective fluids), without adding digestive enzymes during the procedure. It could be assumed that this protein fraction represents the protein profile present in undigested extract. As for the simulation of the physiological digestion, the same mixtures were submitted to the original procedure, where all the enzymes specific for each compartment were added. Undigested and digested model mixtures (SMPC0, SMPC10, PSMPC0, PSMPC10) were then analyzed for the protein content, providing information on their solubility as affected by autoclaving. Results are depicted in Figure 1, where the relative protein contents of mixtures are expressed as percentage, comparing raw with their processed counterparts (SMPC0 vs PSMPC0; SMPC10 vs PSMPC10). One-way ANOVA

performed by comparing two by two the protein contents of the four model sausage confirmed that they are all statistically different, both for undigested and digested type. As for undigested mixtures, an increase of 10% and 14% of protein content was observed in autoclaved, comparing with raw, without and with the addition of MPC, respectively, suggesting an increase in protein solubility caused by the applied thermal processing (Figure 1). It could be hypothesized that the combination of pressure and temperature, typical of autoclaving, might promote a displacement of matrix components, leading to a release of proteins previously embedded in the complex. Consequently, an increase of the final protein content in the processed matrix may occur.^{30,31}

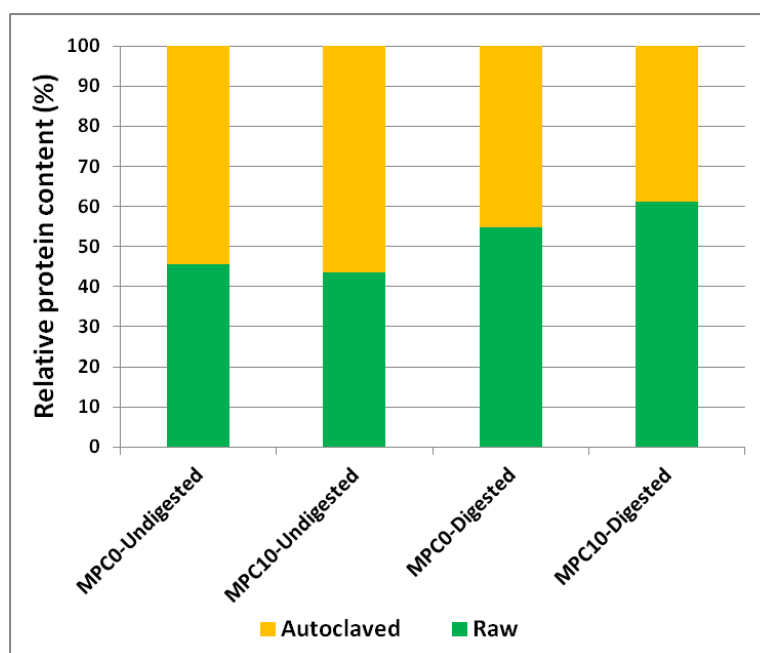


Figure 1. Relative protein content of extracts calculated by Bradford Assay, undigested and digested raw (SMPC0 and SMPC10) and autoclaved (PSMPC0 and PSMPC10) model sausages with and without the addition of 10% of MPC.

Despite the observed in undigested mixtures, protein content decreased after gastro-duodenal digestion by approximately 10% in digested sausages without the addition of MPC and 22% in sausages with MPC (Figure 1). This phenomenon could be ascribed to the high digestion extent occurring by passing from gastric to duodenal tract, with consequent production of small peptides (<3 kDa), which are not detected by the Bradford assay and lead to an apparent protein reduction. This occurrence appears more evident in autoclaved mixtures, likely due to a higher digestibility of proteins that have suffered chemical/structural modifications during food processing. It was already reported that temperatures between 100-120 °C could lead to irreversible aggregation of milk proteins with covalent and hydrophobic interactions causing a higher susceptibility for peptic hydrolysis.³⁰ Heat treatment may also lead to the occurrence of Maillard reactions, which induce radical

formation with subsequent selective attack on the protein backbone.³¹ Meltretter et al.³¹ studied the origin of peptides formed during heat treatment (120 °C) from caseins and whey proteins, showing that some new peptides were originated from caseins due to their fragmentation. Therefore, it can be assumed that autoclaving may facilitate the fragmentation of caseins, specially the β -casein, explaining the increase on protein solubility after the heating process and consequently promoting their higher digestibility.³²

SDS-PAGE

Protein fractions of raw and autoclaved model sausages without and with 10% MPC were then analyzed by SDS-PAGE in order to study the protein stability upon thermal treatment, before and after gastro-duodenal digestion. Results are shown in Figures 2A and 2B. Concerning undigested model mixtures, as appearing from Figure 2A, the addition of MPC (lane 5) to the sausage clearly produces a different protein profile (lane 2) from that of milk-free mixture (lane 1), with new visible proteins, banding approximately in the range 24-31 kDa and comprised between 15 and 20 kDa, which can be assigned to caseins and to whey proteins, respectively. The bands at 25-31 kDa coincide with those obtained in lanes 7, 8 and 9 corresponding to total caseins and purified α - and β -caseins, respectively, while bands between 15 and 20 kDa can correspond to BLG as confirmed by lane 6 representing the purified protein. On the contrary, no defined protein bands appear in processed milk-free sausage profile (Figure 2A, lane 3) with very weak signals visible at 37 kDa, 22 kDa and below 15 kDa, suggesting that autoclaving treatment deeply affects the stability of endogenous proteins of sausage, with almost complete degradation of most of the bands. As for sausage with 10% of MPC (Figure 2A, lane 4), bands around 25 kDa, hypothetically corresponding to casein group, are still strongly visible after autoclaving, while those corresponding to whey proteins (15-20 kDa) are not visible or appear degraded. These results are in good agreement with the principle that whey proteins are largely affected by heat treatment, with the subsequent denaturation of their tertiary and quaternary structures.^{33,35} Additionally, there is also the possibility of increased hydration of protein molecules and/or irreversible aggregation of whey proteins and whey proteins with caseins.^{30,33,35} In this way, whey protein aggregates formed after autoclaving may not be visible because of their high MW (>250 kDa), which hindered their entrance into the gel. These facts may be caused by the occurrence of Maillard reactions between milk proteins and fat and/or lactose present in MPC after thermal treatment.³⁴ Accordingly, Bu et al.³⁵ reported a significant decrease in BLG allergenicity when temperatures above 90 °C were applied. ALA have a similar behavior, but with a greater decrease only at 120 °C during 20 min.

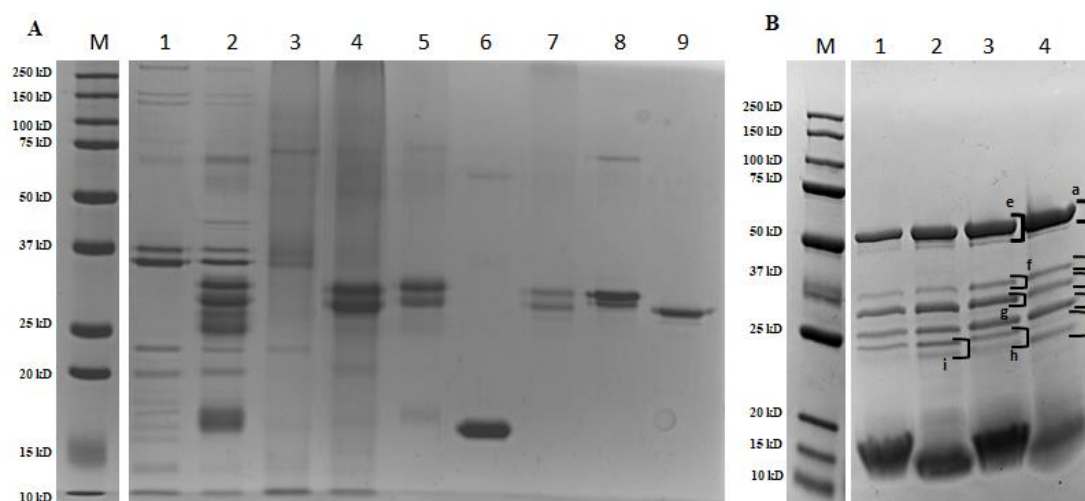


Figure 2. SDS-PAGE run in denaturing conditions comparing protein profiles of undigested (A) and digested (B) raw and autoclaved sausages. Legend: lane 1, SMPC0; lane 2, SMPC10; lane 3, PSMPC0; lane 4, PSMPC10; 5, MPC; 6, BLG; 7, total caseins; 8, α-casein; 9, β-casein; M, Precision Plus Protein™ All Blue Standard (10–250 kDa, Bio-Rad Laboratories, Segrate, Milan, Italy).

After gastro-duodenal digestion, electrophoretic profiles of both raw and processed sausages, without and with MPC addition (Figure 2B, lane 1-4), deeply changed in comparison with the respective undigested samples (Figure 2A, lane 1-4). As appearing from the Figure 2B, all model mixtures (lane 1-4) are characterized by one intense band at approximately 60 kDa and 4 defined bands with MW between 23 and 37 kDa. In addition, one smeared band was detectable in the region of 10 kDa, whose intensity decreased with autoclaving, both in sausages without and with MPC addition (Figure 2B, lanes 2 and 3). In order to deepen the information about the fate of autoclaved milk proteins during digestion, some selected bands (a-j, Figure 2B) were in-gel digested and the resulting tryptic peptides analyzed by LC-MS/MS for protein identification. Bioinformatic investigation revealed that all the analyzed bands were attributed to a mixture of digestive enzymes. This clearly suggests that by passing from chew to gastric and duodenal compartments, all proteins extractable by physiological digestive fluids were almost completely digested by proteolytic enzymes, as also demonstrated by the intense smeared signal banding below 10 kDa, likely produced by coeluting peptides arisen from protein digestion. The lower intensity of the 10-kDa band observable in processed sausage (Figure 2B, lanes 2 and 4) compared with the respective raw mixture could be ascribed to a higher protein fragmentation, likely chemically/structurally altered by autoclaving, with consequent production of low molecular weight peptides (<10 kDa), thus escaping the SDS-PAGE separation. This result is in accordance with the low protein content displayed for autoclaved mixtures by the Bradford assay (see 3.1.1 section).

LC-MS/MS analysis of sausage material upon gastro-duodenal digestion

In order to have complementary information about the digestibility of milk proteins in autoclaved model sausage, raw and treated mixtures with 10% MPC and collected at the end of gastro-duodenal digestion, were loaded on Sep-Pak C18 cartridges for purification purposes and then analyzed by LC-MS/MS. Obtained data were then processed via Proteome Discoverer software and proteins/peptides were identified by searching into a customized database against protein sequences of *Sus scrofa*, *Meleagris gallopavo* and *Bos taurus* species, as well as sequences of digestive enzymes used for simulating gastro-duodenal digestion. Individual proteins identified are summarized in Table 2. In general, all proteins derived from meat or fat used for sausage preparation proved to be fully degraded upon gastro-duodenal digestion, and no proteins were found belonging to turkey or pork species. In the case of milk proteins, some casein peptides appeared to have survived to gastro-duodenal digestion, in fact in both raw and autoclaved mixtures, specifically peptides belonging to β -, k- and α_{s1} -caseins (Table 2) were found. By looking at the corresponding SDS-PAGE profiles (digested SMC10 and PSMC10, Figure 2B, lanes 2 and 4), an extensive digestion of caseins occurred along the transit from gastric to intestinal compartment with likely production of ≤ 4 aa length peptides which may escape by bioinformatic search. Thus, it is reasonable to assume that the lower the number of peptides retrieved by the software for a protein, the higher the probability of its complete digestion. In the light of this, the higher number of peptides identified for β -casein in both mixtures (raw and autoclaved sausages) highlights that this protein was digested at lesser extent than the other caseins, for which very few peptides were retrieved by the software (Table 2).

Table 2. Summary of proteins identified by LC-MS/MS analysis and bioinformatic tool in raw (SMC10) and autoclaved (PSMC10) model sausages after gastro-duodenal digestion. All relevant parameters retrieved by the software were reported.

Sample	Accession	Description	Coverage	Peptides (unique)	PSMs	Score	MW [kDa]
SMC10-Dig	P02666	β -casein OS= <i>Bos taurus</i>	58,04	21 (14)	660	106,64	25,1
	P02668	k-casein OS= <i>Bos taurus</i>	32,11	5 (1)	55	5,42	21,3
	P02662	α_{s1} -casein OS= <i>Bos taurus</i>	41,59	2 (2)	103	4,23	24,5
PSMC10-Dig	P02666	β -casein OS= <i>Bos taurus</i>	62,95	18 (11)	814	108,87	25,1
	P02668	k-casein OS= <i>Bos taurus</i>	34,21	4 (2)	59	3,84	21,3
	P02662	α_{s1} -casein OS= <i>Bos taurus</i>	59,81	2(2)	164	2,63	24,5

Although exhibiting the same protein composition, a different number of peptides was observed for each protein in raw and autoclaved digested sausages. Specifically, a lower number of peptides was identified in processed mixtures for β -casein and k-casein,

suggesting that the chemical/structural changes induced by autoclaving and food matrix could promote enzymatic activity, thus, increasing the digestion rate of these proteins. On the contrary, no significant difference was displayed for α_{S1} -casein in raw and autoclaved mixtures for which 2 peptides were identified in both cases. The extensive fragmentation of proteins induced by digestion and increased by pressure/thermal treatment was also demonstrated by the average length of peptide sequences identified in digested sausages before and after autoclaving for each casein. In Figure 3, all peptides retrieved by the software for SMPC10 and PSMPC10 digested mixtures were grouped according to their length (in three windows, namely 4-6 amino acids (aa), 7-8 aa, and 9-11 aa) and the number of total peptides detected in each mixture. As appearing from the graph, the majority of peptides is made up of short aa sequence (4-6 aa) in digests of raw and autoclaved sausages with added MPC. In particular, a slightly higher number of peptides in this range was recorded for autoclaved mixtures compared to raw ones (Figure 3). In line with this, also the number of longer peptides comprised in the range of 10-11 aa recorded in raw mixtures with MPC was higher than those recorded in autoclaved ones. As expected and already described in section 3.1.1, harsh heat treatments (like autoclave) can promote protein fragmentation and facilitate digestibility, resulting in a higher number of short peptides, principally from caseins.^{31,36} It is also likely to assume that due to the extensive fragmentation induced by this treatment, peptides shorter than 4 aa might not be detected by MS due to the limitation posed by the software and to the constraints imposed for data acquisition.

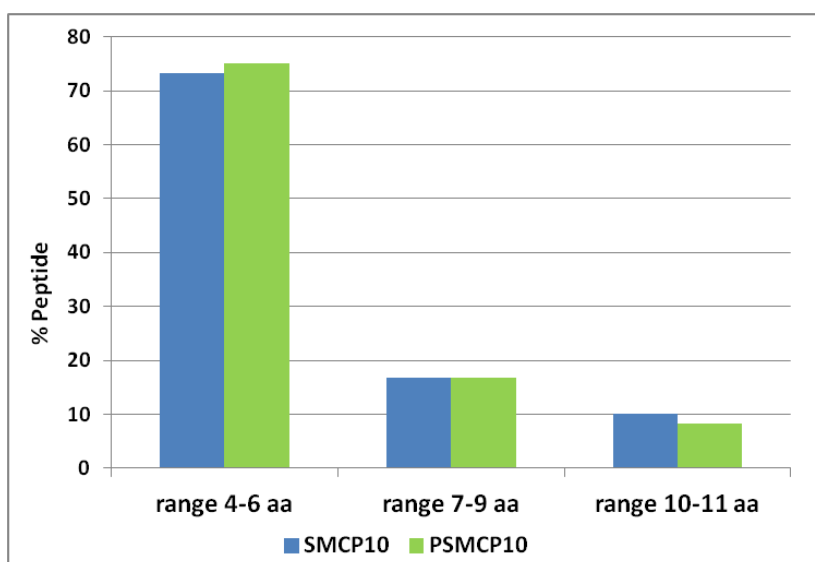


Figure 3. Overview of peptide distribution across three groups differing for sequence length (ranges: 4-6 aa, 7-8 aa, 9-11 aa) and detected in digested SMPC10 and PSMPC10 model sausages.

Assessment of IgE-binding capacity

In order to assess the effects of autoclaving and gastro-duodenal digestion on the final IgE-binding capacity of milk proteins from added MPC used as technological aids in meat products, immunoblotting experiments using sera of cow's milk allergic patients were performed. Specifically, undigested raw and autoclaved model sausages containing 10% of MPC along with their digested counterparts and their respective controls (milk-free) were analyzed. In Figure 4, raw model sausage containing 10% of MPC showed two major reactive bands at approximately 200 kDa and 60 kDa (Figure 4, lane 1), the latter putatively assigned to BSA. Weak IgE-reactivity was observed in bands between 22 and 37 kDa, which correspond to caseins (α_{S1} -, α_{S2} -, β - and κ -casein). No bands corresponding to the major milk allergens BLG and ALA were recognized by the pool of sera in any undigested model sausage. In the autoclaved model sausage, a complete loss of IgE-reactivity of BSA and 200-kDa band could be observed, suggesting that this thermal treatment greatly reduces the IgE-binding capacity of milk proteins (Figure 4, lane 3). As for caseins, the final IgE-binding capacity seems to be reduced at a lesser extent, comparing with the other major allergens, since two weak signals between 22 and 37 kDa are still visible in autoclaved sample (Figure 4, lane 3). Our findings are in accordance with results published by other authors, suggesting that caseins are heat stable proteins with persistent IgE-binding capacity, while whey proteins are thermolabile with substantial reduction of their allergenicity.³⁵ As already reported by Bloom et al.³⁷, sera from milk-allergic subjects remained IgE-reactive to caseins, even after extensive thermal treatment (60 min at 95 °C). On the other hand, regarding BSA, it was demonstrated that after boiling milk at 100 °C for 10 min or baking at 180 °C for 10 min, the protein still maintained strong IgE-binding properties,¹⁷ though our findings showed a clear loss of BSA antigenicity after the autoclaving treatment, possibly because of the harsh conditions applied during this treatment combining elevated temperatures (121 °C) with high pressure (1 bar). As already stated, besides total protein, MPC contain fat and lactose, which may react with milk proteins by heat treatment, possibly affecting their IgE-binding capacity. In this regard, Xu et al.³⁴ found that under heating condition and in the presence of a certain amount of lactose, the Maillard reaction may lead to the loss of linear epitopes of milk allergens, thus reducing their final antigenicity. Lower solubility caused by the formation of high molecular weight aggregates between whey proteins and caseins after thermal treatment, may also lead to a reduction of their antigenicity.³⁵ The involvement of the food matrix in the reduction of IgE-binding capacity needs also to be considered since, during food processing, the formation of complexes between food matrix and milk proteins could occur, thus masking some allergenic epitopes with consequent decrease of casein antigenicity as reported by Bavaro

et al.¹⁷ In the light of this, the contribution of meat matrix to the reduction of IgE-binding with milk proteins should not be excluded.

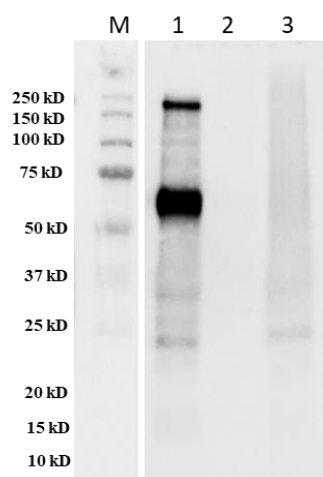


Figure 4. Immunoblot under reducing conditions of undigested raw and autoclaved model sausages. Legend: lane 1, SMPC10; lane 2, PSMPC0; lane 3, PSMPC10; M, Precision Plus Protein™ All Blue Standard (10–250 kDa, Bio-Rad Laboratories, Segrate, Milan, Italy). The immunoblot was carried out on a pool of sera of patients allergic to cow's milk.

Raw and autoclaved sausages submitted to gastro-duodenal digestion were analyzed by immunoblotting experiments with sera of milk allergic patients as well. As expected, due to the extensive digestion of the milk proteins along the gastro-duodenal digestion (see Bradford assay and SDS-PAGE experiments, sections 3.1.1. and 3.1.2), no IgE-binding capacity was observed for all the tested models (data not shown). In order to further confirm the absence of any residual immunogenicity in digested model sausages, the peptide sequences identified by proteomic analysis in raw and autoclaved mixtures with the addition of MPC (SMPC10 and PSMPC10) submitted to gastro-duodenal digestion were screened into IEDB database for retrieving survival milk linear epitopes for *Homo sapiens* host. Results are detailed in Table 3, where the peptide sequences found individually in SMPC10 or PSMPC10 and in both samples were reported along with IEDB results by filtering search on “substring” or “exact match” option. In general, 11 peptides belonging to β -casein were found in SMPC10 and PSMPC10 sausages, highlighting their good resistance to thermal/pressure treatment and gastro-duodenal digestion. On the other hand, 12 peptides were exclusively found in raw sausage probably because they were degraded during treatment. Seven new peptides were instead reported for PSMPC10 sausage, possibly originating from β -casein digestion, previously fragmented by autoclaving. Concerning κ -casein, the majority of peptides were conserved after autoclaving and similar results were obtained for α -casein. This fact suggests that autoclaving has a higher effect on β -casein fragmentation and digestion rate than on α - and κ -caseins, which agrees with the obtained MS data, showing a lower number of β -casein peptides after gastro-duodenal digestion,

though in general this protein is digested at a lesser extent. Interestingly, no intact epitopes reported in IEDB database were found to exactly match the peptides from the digested raw and autoclaved extracts, although almost all of them were found to be originally included into milk allergenic epitopes, as demonstrated by IEDB screening filtered by “substring” option. IEDB results further confirmed that, after gastro-duodenal digestion of milk proteins from sausage matrix, their original immunogenicity seems to be missed.

Table 3. Overview of peptides retrieved by the Proteome Discoverer software for each protein by analyzing raw (SMPC10) and autoclaved (PSMPC10) gastro-duodenal digested sausages. IEDB results by activating “substring” and “exact match” filtering were also reported. Symbol “x” marks sample where individual peptide was found, while “/” stays for “not found”.

Protein (accession)	Peptide sequence	SMPC10 digest	PSMPC10 digest	IEDB Epitope Antigens	ID Epitope (substring)	ID Epitope (exact match)
β-casein OS=Bos taurus (P02666)	PVVVPPFLQPE	x	x	Bos d 11 <i>Bos taurus</i> (bovine)	115393, 115420, 115460, 115907	/
	HQPHQPLPPT	x	x	/	/	/
	HIPLP	x	x	/	/	/
	PVVVPP	x	x	Bos d 11 <i>Bos taurus</i> (bovine)	115393, 115420, 115460, 115907, 115931	/
	PVIGPV	x	x	/	/	/
	PFPGPI	x	x	Bos d 11 <i>Bos taurus</i> (bovine)	59576, 78152, 115246, 115883, 115973, 116025, 229825, 229827, 229828	/
				<i>Bubalus bubalis</i> (domestic water buffalo) protein	229826	
	IPIP	x	x	/	/	/
	YPVEP	x	x	Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115742, 115839, 115865, 229605, 229632, 229673, 229674, 229741, 229759, 229760, 229762, 229767	/
	VYPFPGPI	x	x	Bos d 11 <i>Bos taurus</i> (bovine)	59576, 78152, 115246, 115973, 116025, 229825, 229826, 229827, 229828	/
	PVVVPPF	x	x	Bos d 11 <i>Bos taurus</i> (bovine)	115393, 115420, 115460, 115907, 115931	/
				β-casein <i>Capra hircus</i> (domestic goat)	227136	
	VVPPF	x	x	Bos d 11 <i>Bos taurus</i> (bovine)	115393, 115420, 115460, 115554, 115907, 115931	/
	EMPFPK	x		Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115807, 115823, 229605, 229632, 229673, 229674, 229762, 229767	/
	PQNIPPL	x		Bos d 11 <i>Bos taurus</i> (bovine)	115899, 115969	/
	LNVPGE	x		Bos d 11 <i>Bos taurus</i> (bovine)	53557, 115219, 115466, 115725, 115842	/

Protein (accession)	Peptide sequence	SMPC10 digest	PSMPC10 digest	IEDB Epitope Antigens	ID Epitope (substring)	ID Epitope (exact match)
	MAPK	x		Bos d 11 <i>Bos taurus</i> (bovine)	115216	/
	MHQPHQPLPPT	x		/	/	/
	RGPFPL	x		/	/	/
	RGFPF	x		Bos d 11 <i>Bos taurus</i> (bovine)	115251, 115298, 115763, 116016	/
	PFPK	x		Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115807, 115823, 115865	/
	MFPPQ	x		Bos d 11 <i>Bos taurus</i> (bovine)	38630, 51872, 78287, 115904, 116019	/
				<i>Capra hircus</i> (domestic goat)	227654	
	MPFPK	x		Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115807, 115823, 115865, 229605, 229632, 229673, 229674, 229741, 229762, 229767	/
	EMFPF	x		Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115807, 115823, 229605, 229632, 229673, 229674, 229762, 229767	/
	QPHQPLPPT	x		/	/	/
	PLPPT		x	Bos d 11 <i>Bos taurus</i> (bovine)	51872, 115923,	/
	MFPPQ		x	Bos d 11 <i>Bos taurus</i> (bovine)	38630, 51872, 7828, 115904, 116019	/
				β -casein <i>Capra hircus</i> (domestic goat)	227654	
	PFPK		x	Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115807, 115823, 115865	/
	PPFLQPE		x	Bos d 11 <i>Bos taurus</i> (bovine)	115393, 115420, 115460, 115554, 115895	/
	EMFPFK		x	Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115807, 115823, 229605, 229632, 229673, 229674, 229762, 229767	/
	RGPFPI		x	Bos d 11 <i>Bos taurus</i> (bovine)	115251, 115763	/
	YPVEPF		x	Bos d 11 <i>Bos taurus</i> (bovine)	30533, 115216, 115280, 115742, 115839, 229605, 229632, 229673, 229674, 229741, 229759, 229760, 229762, 229767	/
κ-casein OS=<i>Bos taurus</i> (P02668)	MAIPPK	x	x	Bos d 12 <i>Bos taurus</i> (bovine)	115468, 47842, 115281, 115369, 115379, 115517, 115741, 115853, 161638	/
	IPYP	x	x	/	/	/
	HPHP	x	x	Bos d 12 <i>Bos taurus</i> (bovine)	54078, 115195, 115468, 54079, 115175, 115450, 115517, 115863, 116008	/

	Peptide sequence	SMPC10 digest	PSMPC10 digest	IEDB Epitope Antigens	ID Epitope (substring)	ID Epitope (exact match)
	HPHPH	x		Bos d 12 <i>Bos taurus</i> (bovine)	54078, 115195, 115468, 54079, 115175, 115450, 115517, 115863, 116008	/
	IPPK	x		Bos d 12 <i>Bos taurus</i> (bovine)	115468, 47842, 115281, 115369, 115379, 115517, 115678, 115741, 115853, 161638,	/
	FIPYP		x	/	/	/
α_{S1}-casein OS=<i>Bos taurus</i> (P02662)	HQGLPQ	x	x	Bos d 9 <i>Bos taurus</i> (bovine)	115282, 31145, 109844, 115311, 190448, 229677	/
	VAPFPEV	x	x	Bos d 9 <i>Bos taurus</i> (bovine)	38207, 43705, 44794, 15930, 15931, 67707, 67708, 67709, 69660, 109844, 110049, 115081, 115396, 115436, 115467, 115531, 190478	/
				Other <i>Capra hircus</i> (goats) protein	229748	

In summary, autoclaving followed by simulated gastro-duodenal digestion seems to decrease protein content, possibly because of an increase of their fragmentation, especially within casein fraction. The fragmentation allows the complete degradation of proteins after digestion with the production of low molecular weight peptides (around 10 kDa). Analysis by LC-MS/MS demonstrated that these peptides correspond to caseins (α_{S1} , β - and κ -caseins), being β -casein the most resistant with the production of a high number of peptides after digestion. On contrary, whey proteins were completely degraded by digestion. Their IgE-binding capacity also revealed to be highly affected by autoclaving since this treatment potentiates the formation of aggregates and the interactions between whey proteins and food matrix, with the consequent loss or masking of epitopes. Additionally, autoclaving seems to favor casein fragmentation prior to simulated gastro-duodenal digestion, thus contributing to a generalized reduction in their IgE-binding capacity. However, after simulated gastro-duodenal digestion, no IgE-reactivity was observed for any milk proteins, which was also confirmed by IEDB analysis. The combination of autoclaving and digestion apparently proved to affect at a major extent β -casein than α - and κ -caseins. This was mainly due to the degradation of 12 peptides during autoclaving and the formation of 7 new peptides after digestion probably as a consequence of their higher susceptibility to fragmentation during autoclaving.

Funding

This work was supported by Fundação para a Ciência e Tecnologia under the Partnership Agreement UIDB 50006/2020 and by the projects AlleRiskAssess—PTDC/BAA-AGR/31720/2017.

Acknowledgments

Caterina Villa is grateful to FCT grant (PD/BD/114576/2016) financed by POPH-QREN (subsidized by FSE and MCTES) and to the COST Action FA1402 – ImpARAS – “Improving Allergy Risk Assessment Strategy for New Food Proteins” for the attribution of the travel grant for a STSM (Short-Term Scientific Mission) at ISPA-CNR, Bari, Italy.

Abbreviation Used

aa, amino acids; ALA, α -lactalbumin; AMBIC, ammonium bicarbonate; ANOVA-2, two-way analysis of variance; BLAST, Basic Local Alignment Search Tool; BLG, β -lactoglobulin; BSA, bovine serum albumin; CA, cellulose acetate; DTT, dithiothreitol; HPLC-MS/MS, High-Performance Liquid Chromatography coupled with tandem mass spectrometry; IAA, iodoacetamide; IEDB, Immune Epitope Database; Ig, immunoglobulins; IgE, Immunoglobulin E; MPC, milk protein concentrate; MW, molecular weight, PMSF, methyl-phenyl-sulfonyl fluoride; PSMPC0, autoclaved sausage with 0% milk protein concentrate; PSMPC10, autoclaved sausage with 10% milk protein concentrate; PTFE, polytetrafluoroethylene; SDS-PAGE, sodium dodecylsulfate-polyacrylamine gel electrophoresis; SGF, Simulated Gastric Fluid; SIF, Simulated Intestinal Fluid; SMPC0, raw sausage with 0% milk protein concentrate; SMPC10, raw sausage with 10% milk protein concentrate; SPT, skin prick test; SSF, Simulated Salivary Fluid; TFA, trifluoroacetic acid; WHO/IUIS, World Health Organization and International Union of Immunological Societies;

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CHAPTER 3. Lupine allergens

State-of-the-art

Lupine allergens: clinical relevance, molecular characterisation,
cross-reactivity and detection strategies
Comprehensive Reviews in Food Science and Food Safety, **2020**, 1-29.

Experimental part

Effect of food matrix and thermal processing on the performance of a normalised
quantitative real-time PCR approach for lupine (*Lupinus albus*) detection as a
potential allergenic food
Food Chemistry, **2018**, 262, 251–259

Immunoreactivity of lupine and soybean allergens in foods
as affected by thermal processing
Foods, **2020**, 9, 254

3.1. State-of-the-art

Lupine allergens: clinical relevance, molecular characterisation,
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3.1.1. Lupine allergens: clinical relevance, molecular characterization, cross-reactivity and detection strategies

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ABSTRACT

Lupine is commonly utilized as a technological food and ingredient in a great variety of processed products (snacks, bakery, meat, and dairy products) principally owing to its nutritional value and technological properties. However, its ingestion, even at trace amounts (in the range of mg protein per kg of food), can lead to severe adverse reactions in allergic individuals. Lupine belongs to the Leguminosae family, having the conglutins (α -, β -, δ -, and γ -) as allergens, among other proteins. Cross-sensitization of lupine-sensitized individuals with other legume species, mainly peanut, can occur, but the associated clinical reactivity is still unclear. The protection of the sensitized individuals should depend on an avoidance diet, which should rely on the compliance of food labelling and, as such, on their verification by analytical methods. Food processing, such as heat treatments, has an important influence on the structural properties of lupine proteins, altering their detectability and allergenicity. In this review, different aspects related with lupine allergy are described, namely, the overall prevalence, clinical relevance, diagnosis, and treatment. The characterization of lupine allergens and their potential cross-reactivity with other legumes are critically discussed. The effects of food matrix, processing, and digestibility on lupine proteins, as well as the available analytical tools for detecting lupine at trace levels in foods, are also herein emphasized.

Keywords: allergen, analytical methods, food allergy, *Lupinus* species, protein.

INTRODUCTION

Lupine belongs to the *Lupinus* genus from the Fabaceae or Leguminosae family, which also comprises soybean, peanut, chickpea, and other types of legumes (Villarino, Jayasena, Coorey, Chakrabarti-Bell, & Johnson, 2016). From approximately 450 known species of lupine, four are of agricultural and commercial interest: *Lupinus albus* (white lupine), native from the Mediterranean region and Africa; *Lupinus angustifolius* (blue lupine) from Australia; *Lupinus luteus* (yellow lupine) from Central Europe; and *Lupinus mutabilis* from South America (Jappe & Vieths, 2010; Sanz, de Las Marinas, Fernandez, & Gamboa, 2010). According to FAOSTAT, a total world production of 1,188,213 tonnes of lupine was reached in 2018. Australia is the main producer, with 714,254 tonnes in 2018, accounting to 60.1% of total world lupine production. Europe, Russia and Poland are the main producers, reaching 136,352 and 124,314 tonnes in 2018, respectively, corresponding to 39.9% and 36.4% of total lupine production in Europe, respectively (FAOSTAT [<http://www.fao.org/faostat/en/#home>]).

Currently, owing to the continuing rise of food prices and the high demands for nongenetically modified products and sustainable foods, the use of lupine as food ingredient has augmented over the last years (Villarino et al., 2016). In fact, lupine can be utilized to strengthen the protein content of bread, cookies, pasta, salads, hamburgers, and sausages, and it can substitute soybean and milk proteins as technological agents. Due to its important technological properties, such as high water binding capacity and great emulsifying and foaming abilities, lupine flours or lupine protein isolates and concentrates have been used to formulate baked, meat, and dairy products by the food industry (Carvajal-Larenas, Linnemann, Nout, Koziol, & van Boekel, 2016; Kohajdova, KaroVičová, & Schmidt, 2011). Besides, lupine is appreciated because of its nutritional value, having high contents of protein and dietary fiber, but low levels of fatty acids and carbohydrates. It is rich in antioxidants and vitamins; and, compared to other legumes, it has low antinutritional factors such as trypsin inhibitors or saponins. Lupine does not contain lactose or gluten, being a suitable substitute for individuals with milk intolerance, wheat allergy, or coeliac disease (Jappe & Vieths, 2010; Villarino et al., 2016).

However, lupine can pose some nutritional and health related problems because it contains potentially toxic alkaloids and food allergens. In 1994, it was reported the first case of an immediate allergy reaction after the ingestion of a lupine flour-containing pasta by a 5-year-old child with peanut allergy (Hefle, Lemanske, & Bush, 1994). Since then, the reported incidents of adverse reactions after the ingestion or inhalation of lupine proteins have been constantly increasing. Therefore, in November of 2007, in the European Union (EU), lupine and products thereof were considered as allergenic foods, whose presence

must be stated and emphasized in the list of labeled ingredients of prepackaged foods, irrespective of the quantity (Regulation (EU) No 1169/2011 [European Union, 2011]). Since then, countries like Australia have included lupine as an allergenic food with mandatory labeling in its legislation (FSANZ [<http://www.foodstandards.gov.au/Pages/default.aspx>]). In the United States, the scientific community recognizes lupine as an allergenic food, but there is still a lack of legislation regarding its inclusion in the label of foods (Food Allergen Labeling and Consumer Protection Act) (FDA [<https://www.fda.gov/>]).

Lupine allergy is typically immunoglobulin E (IgE) mediated. Although often used indiscriminately, lupine IgE sensitization is not the same as lupine clinical allergy. The first term only states that the lupine IgE-sensitized individuals have antibodies (IgE) able to recognize one or more lupine allergens (without necessarily exhibiting clinical symptoms), whereas clinical allergy to lupine reflects the manifestation of symptoms. In this manuscript, both terms are used following the above definitions. Lupine allergy can result from two different sensitization pathways: primary sensitization to lupine proteins that can occur directly via ingestion, being the common route for the allergic patients from the Mediterranean regions, where legumes like lupine are part of the diet; or indirectly via inhalation, as a consequence of occupational exposure or in pollen-associated lupine allergy (Bet v 1-birch pollen-related proteins). The exposure to lupine flour through the respiratory tract can be considered as the key reason for the allergic sensitization in bakery/food industry workers, possibly giving rise to occupational asthma and lupine allergy (Campbell, Jackson, Johnson, Thomas, & Yates, 2007; Crespo et al., 2001; van Kampen et al., 2015). Co-sensitization has also been advanced as a common cause of lupine and wheat allergies among bakers due to occupational exposure to both types of flours, highlighting the impact of inhalation as a potent way of sensitization for lupine allergy (van Kampen et al., 2015). Indirect sensitization to lupine proteins can also occur by cross-reactivity with other legumes (lentils, peas, and soybean), but particularly with peanut, in persons with previous diagnosed allergy to one of these legumes (Sanz et al., 2010; Verma, Kumar, Das, & Dwivedi, 2013). The clinical symptoms can be triggered within few minutes after ingestion or inhalation of lupine proteins and they can vary in intensity and severity, including asthma, allergic rhinitis, urticaria, nausea or gastrointestinal pains, and anaphylaxis (Jappe & Vieths, 2010). Like for other food allergies and as a preventive treatment, the patients with lupine allergy must implement an elimination diet, avoiding all foods that declare its presence. Owing to the increased use of this legume in several processed foods, as an ingredient or additive, its elimination can be problematic for the allergic patients, forcing them to a restrictive diet (Sanz et al., 2010).

In this review, a comprehensive overview on lupine allergy is provided, addressing the topics of prevalence, clinical relevance, diagnosis, and treatment, followed by the molecular

characterization of known lupine allergenic proteins and the description of cross-reactivity phenomena of lupine with other legumes, mainly peanut. The advances on the food processing strategies to mitigate lupine allergenicity and on the analytical methodologies to detect lupine allergens in foods are also described and critically discussed.

LUPINE ALLERGY

Prevalence and clinical reactivity

The prevalence of lupine sensitization is unknown, depending on the dietary habits and geographical differences, although it has been reported to grow in parallel with its increasing consumption (Fæste, 2010; Verma et al., 2013). A study developed by Reis et al. (2007), using data of 1,160 individuals from the Mediterranean region (Portugal) with suspicion of food allergy, reported 4.1% of lupine sensitization confirmed by skin prick tests (SPT) among the test population. However, the allergy prevalence could not be established because no systematic tolerance investigation through an oral food challenge was performed. In Finland, the prevalence drops to 1.6%, as demonstrated by a study conducted among 1,522 persons with suspected food allergies, being still considered high, given the sporadic consumption of lupine in this region (Hieta, Hasan, Makinen-Kiljunen, & Lammintausta, 2009). In the specific case of Belgium and France, 14.5% of adults and 17.5% of children from a prospective study with 5,366 of peanut-allergic patients have shown cross-sensitization to lupine (Gayraud et al., 2009). Peeters et al. (2009) reported a sensitization to lupine of 82% in 39 peanut-allergic patients, from which 35% revealed clinical symptoms after food oral challenge. In spite of the high lupine intake in the Netherlands (0.11 kg of lupine flour and 5.5 kg of lupine flour-containing products per capita per year), the prevalence of lupine sensitization in this country is low, varying from 0.27% to 0.81%, considering a test population of 372 patients with suspected food allergy (de Jong et al., 2010). In Germany, the prevalence of lupine allergy was similar or even lower compared to other legume allergies, showing a sensitization rate to lupine in non atopic subjects of 2%, whereas this frequency increased to 6% in atopic subjects (Bähr, Fechner, Kaatz, & Jahreis, 2014). The majority of these studies refer to the prevalence of sensitization to lupine rather than to its demonstrated clinical reactivity. Reports showing the prevalence of clinically relevant lupine allergy are still scarce among the general population. From the existing results, it seems that lupine allergy might not be of high clinical relevance, being possibly caused by cross-sensitization to peanut or birch pollen.

Asymptomatic lupine sensitization is rather frequent, but its real clinical reactivity is less common. Still, the consumption of lupine can induce concrete clinical symptoms in lupine-allergic patients, which can vary in severity, from urticaria and vomiting, to anaphylaxis

(Bansal, Sanghvi, Bansal, & Hayman, 2014; de Jong et al., 2010; Peeters et al., 2009). The first case of an allergic reaction after the ingestion of lupine was reported in 1994, in a 5-year-old girl with peanut allergy, who developed urticaria and angioedema after eating spaghetti fortified with lupine flour (Hefle et al., 1994). The information about the severity of the allergic reactions to lupine is rather scarce, although there are some cases reporting the development of facial swelling, widespread urticarial with asthma and hypotension within minutes of eating foods containing lupine, as well as oral allergy syndrome with associated urticarial and vomiting (Bansal et al., 2014; Brennecke, Becker, Lepp, & Jappe, 2007; Wüthrich, Mittag, & Ballmer-Weber, 2004). Several cases of anaphylaxis to lupine have also been described (Radcliffe, Scadding, & Brown, 2005; Wassenberg & Hofer, 2007; Wüthrich, B., 2008). Although the majority of these cases occur after the ingestion of lupine or lupine-containing products, inhalation was also reported as a cause of recurrent anaphylaxis (Prieto et al., 2010). There are some evidences of the clinical relevance of lupine allergy after its inhalation, describing symptoms of rhinitis, conjunctivitis, and palpebral angioedema, which lead to occupational rhinitis and asthma (Campbell et al., 2007; Parisot, Aparicio, Moneret-Vautrin, & Guerin, 2001). In some cases, after stopping handling lupine flour, the symptoms disappeared (Parisot et al., 2001). An 8-year-old, asthmatic child with peanut allergy developed an asthma attack after the inhalation of lupine (Moreno-Ancillo, Gil-Adrados, Domínguez-Noche, & Cosmes, 2005), whereas a 3-year-old child with lupine allergy showed asthma, rhinitis, conjunctivitis, cough, dyspnea, and cyanosis after playing with a lemon tree manured with lupine dust (Novembre et al., 1999). Adverse reactions (conjunctivitis, rhinoconjunctivitis, and asthma) were also reported in employees working with lupine flour, but without preexistent allergy to peanut or other legumes (Crespo et al., 2001). Some patients with lupine allergy are also allergic to pollen, although there are poor evidences about sensitization through pollen inhalation in lupine allergic patients (Jappe, & Vieths, 2010). Moneret-Vautrin et al. (1999) reported patients with IgE specific to lupine pollen and flour, and the existence of cross-reactions between lupine pollen and peanut with associated symptoms in the ocular, nasal, and bronchial mucosa. They suggested a possible primary sensitization to lupine pollen, which could evolve toward sensitization to lupine seed and flour or even to cross-reactivity to peanut. However, the information regarding sensitization through lupine pollen is still scarce and requires more investigations.

Diagnosis and treatment

Like for other food allergies, lupine allergy diagnosis starts with the evaluation of the clinical symptoms observed upon the ingestion of lupine or lupine-containing products. After correlating the clinical symptoms with a convincing history of IgE-mediated reactions in relation to lupine, *in vitro* and *in vivo* diagnostic tests can be applied (Matricardi et al., 2016).

SPT are among the most common tests implemented for the diagnosis of lupine allergy, which can be performed using raw lupine flour, ground raw lupine seed, or even a commercially available lupine seed prick test solution (Phadia, Uppsala, Sweden). Serum-specific IgE testing via a fluorescent enzyme immunoassay (ImmunoCAP, Phadia, Uppsala, Sweden) using lupine and lupine pollen material is also commercially available. Component-resolved diagnosis (CRD), the serum-specific IgE against molecular components, has an important role as a second-level diagnostic step, but in lupine allergy a commercial CRD is still not available (Ciccarelli, Calabrò, Imperatore, & Scala, 2014).

Lately, the basophil activation test (BAT) (functional assay using live basophils from allergic patients) has been regarded as an excellent tool to determine the capacity of IgE to mediate the activation of basophils upon allergen stimulation (Santos & Brough, 2017). It is still not used in routine analysis, but its implementation as a more reliable diagnostic test has been increasing. De las Marinas, Cojocariu, Escudero, Pardo, and Sanz (2007) reported the use of BAT to confirm the diagnosis of lupine allergy in a patient exhibiting general malaise, nasal blockage, coughing attacks, palmoplantar pruritus, vomiting, and wheal and flare lesions on limbs upon consumption of lupine-containing food. BAT also evidenced strong response to other legumes (soybean, chickpeas, and beans), although the patient was previously tolerant to those legumes (De las Marinas et al., 2007). In the case of a positive outcome, other legumes (such as peanut and soybean) should also be tested to determine possible cross-reactivity phenomena. To confirm or reject the clinical significance of lupine sensitization, oral challenges (in open, closed, or blind formats) could be considered, except for the cases with already documented anaphylaxis (Jappe & Vieths, 2010; Sanz et al., 2010). Besides being a confirmatory test for clinically relevant allergy, the double-blind placebo-controlled food challenge (DBPCFC) is similar to a clinical trial and it can allow the determination of threshold levels, depending on the challenge protocol.

Until now, only few studies have been conducted to establish an allergy-eliciting dose for lupine, though with inconsistent results. Peeters et al. (2009) reported the lowest lupine eliciting dose, responsible for mild symptoms in peanut-sensitized/allergic individuals, as 0.5 mg of lupine flour. The VITAL 2.0 program of the Allergen Bureau of Australia and New Zealand (ABA) recommended 4.0 mg of lupine protein as the reference dose for this allergenic food (Allergen Bureau, 2012; Taylor et al., 2014), value that was recently revised to 2.6 mg of lupine protein according to the new VITAL 3.0 program (Allergen Bureau, 2019; Remington et al., 2020). This value refers to milligrams of protein from an allergenic food below which only the most sensitive (1% for VITAL 3.0 and between 1% and 5% for VITAL 2.0) allergic patients are likely to experience adverse reactions (Allergen Bureau, 2019). Compared to the eliciting dose (ED₀₁) of 0.7 mg for peanut, the same value for lupine is almost four times higher, suggesting that peanut is a much more potent allergenic food than

lupine (Remington et al., 2020). Still, it is also important to highlight that the ED₀₁ for lupine was calculated on the basis of 25 individuals (Remington et al., 2020), meaning that more studies with much more individuals are needed.

In the case of confirmed clinical allergy to lupine, the individual must stop the ingestion of lupine or products susceptible of containing lupine, but without avoiding other previously tolerated legumes. Despite the high immunologic cross-reactivity among different species of legume family, the correlation with clinical hypersensitivity is not reported, therefore lupine-allergic patients should not avoid the ingestion of other legumes (Bernhisel-Broadbent, Taylor, & Sampson, 1989). In the case of accidental exposure to lupine, antihistamines can be prescribed to treat the adverse immunological response, but in the case of anaphylaxis, adrenaline, either administered intramuscularly or intravenously, constitutes the first line of treatment, followed by intravenous corticosteroids combined with antihistamines (Ramanujam, Fiocchi, & Smith, 2016). However, for each case, the best line of treatment should always be carefully considered based on the severity of the clinical manifestations.

MOLECULAR CHARACTERIZATION OF LUPINE ALLERGENS

Lupine generally contains about twice of the amount of proteins found in other legumes, such as lentil, pea, or soybean, commonly consumed by humans. This amount can vary from 28% to 48% among different lupine species and cultivars because of soil types and growing conditions (Kohajdova et al., 2011). The major protein classes in lupine are the globulins (80.0% to 94.0% of the total protein fraction) and the albumins (5.0% to 15.4%) (Carvajal-Larenas et al., 2016), whereas other fractions, namely, glutelins and profilins, are detected in small amounts, similarly to other legumes (Gulewicz et al., 2008). According to the allergen list made available by the Allergen Nomenclature Subcommittee of the World Health Organization and International Union of Immunological Societies (WHO/IUIS [<http://www.allergen.org/>]) and the ALLERGOME (<http://www.allergome.org/>) databases, lupine allergens are classified into different families of proteins, namely, globulins (α -, β -, and γ -conglutin), 2S albumins (δ -conglutin), and some minor fractions, such as pathogenesis-related (PR)-10 proteins (Lup a 4 and Lup I 4), nonspecific lipid transfer proteins (nsLTP) (Lup an 3), and profilins (Lup a 5). ALLERGOME database includes all the allergenic proteins registered in the WHO/IUIS list of allergens, but also proteins with a proved IgE-mediated disease (anaphylaxis, asthma, atopic dermatitis, conjunctivitis, rhinitis, and urticaria) published by international scientific journals and from web-based resources, since the early 60s (Marietal., 2006). A summary of the known lupine allergens, their biological functions, and databases' accession numbers is presented in Table 1.

α -Conglutin (Lup a α -conglutin and Lup an α -conglutin)

α -Conglutin is a legumin-like lupine globulin, belonging to the 11S globulin family. According to ALLERGOME nomenclature, 2 α -conglutins have been identified in different lupine species, one in *L. albus* (Lup a α -conglutin) and one in *L. angustifolius* (Lup an α -conglutin). They are storage proteins, located in the storage vacuoles of the cotyledon cells. In both species, this fraction shows molecular masses between 330 and 430 kDa, representing 35% to 37% of the total globulins in lupine (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008). Another α -conglutin was also identified in the *L. mutabilis*, being considerably different, both in composition and structure, from its counterpart in *L. albus* (Santos, Ferreira, & Teixeira, 1997), but it is not classified as an allergenic protein, most likely because this species is not frequently consumed and, consequently, not extensively studied. In *L. albus*, α -conglutin is an oligomeric protein consisting of hexamers of disulfide-linked basic and glycosylated acidic trimers (typical structure of legumins), constituted by four subunits of 50 to 60 kDa (Carvajal-Larenas et al., 2016; Duranti et al., 2008; Fæste, 2010). In immature subunits, the predominant form of the protein is a trimer (Adachi et al., 2003), but after pro-polypeptide cleavage, the mature subunits are divided into acidic (heavier) (38 to 54 kDa) and basic (lighter) (19 to 22 kDa) polypeptide chains (Duranti et al., 2008; Santos et al., 1997). In *L. angustifolius*, α -conglutin contains four subunits, ranging from 55 to 80 kDa. The subunits are noncovalently linked and contain a disulfide-bound moiety of 20 kDa. Upon reduction, the larger subunits are split into polypeptides of 36 to 63 kDa (Fæste, 2010). For legumins, the acidic polypeptides, in relation to the basic ones, are mostly positioned in the outer part of the molecule with significantly lower hydrophobicity. In *L. albus*, the polarity of the legumin seems to be attributed to polypeptide composition and structure because the assembly of its subunits was likely shaped by the polarity allocation within the polypeptide. Despite the high homology among α -conglutins from distinct lupine species, some variations in their primary sequences and respective conformational structures can be expected (Carvajal-Larenaset al., 2016). Guillamón, Rodríguez et al. (2010) showed that a 20-kDa band of *L. albus* strongly reacted with sera from patients diagnosed with lupine allergy, which corresponds to the basic subunit of α -conglutin. This fact was also described by Magni, Ballabio et al. (2005), who demonstrated that the GQL(I/L)VVPQNFVV sequence of α -conglutin is very conserved in lupine, peanut, and soybean allergens (Ara h 3 and Gly m 6), and it can be a possible cross-reactive IgE epitope in all species. On the other hand, Holden, Sletten, Lindvik, Faeste, and Dooper (2008) reported that two proteins of approximately 40 and 43 kDa of *L. angustifolius* were IgE reactive in five out of six lupine-allergic patients, corresponding to the acidic subunits of α -conglutin.

Table 1 - Identification of lupine allergens according to their biochemical classification, biological function and their respective accession numbers.

Biochemical classification	Protein superfamily	Biological function	Allergen	Isoforms	MW (kDa)	Nucleotide (NCBI)	Protein (NCBI)	Protein (UniProt)
α -conglutin	Cupin (11S legumin)	Seed storage protein	Lup a α -conglutin		330 to 430	AJ938034.2	CAI83773.2	Q53154
						AJ938033.1	CAI83770.1	Q53155
			Lup an α -conglutin			HQ670406.1	AEB33709.1	F5B8V6
						CM007365.1	AAC49787.1	
β -conglutin	Cupin (7S vicilin)	Seed storage protein	Lup a 1	Lup an 1.0101	60 to 75 (major polypeptide chains)	U74384.1		
						AJ966470.2	CAI84850.2	Q53HY0
						AY500372.2	AAS97865.1	Q6EBC1
						DQ142920	ABB13526.1	
						EF455724.1	ABR21771.1	B0YJF7
						EF455725.1	ABR21772.1	B0YJF8
			Lup an 1			HQ670409.1	AEB33712.1	F5B8V9
						HQ670410.1	AEB33713.1	F5B8W0
						HQ670411.1	AEB33714.1	F5B8W1
						HQ670412.1	AEB33715.1	F5B8W2
						HQ670413.1	AEB33716.1	F5B8W3
						HQ670414.1	AEB33717.1	F5B8W4
δ -conglutin	Prolamin (2S albumin)	Seed storage protein	Lup a δ -conglutin		14	HQ670415.1	AEB33718.1	F5B8W5
						EU352876.1	ACB05815.1	B8Q5G0
						AM156845.1	CAJ42100.1	Q333K7
						AM160790.1	CAJ43922.1	
						HQ670418.1	AEB33721.1	F5B8W8
	Cupin (7S vicilin)	Aspartic protease	Lup an δ -conglutin		200	X53523.1	CAA37598.1	Q99235
						HQ670419.1	AEB33722.1	
						AJ297568.2	CAC17729.2	Q9FEX1
						AJ297490.1	CAC16394.1	Q9FSH9
						X65601.1	CAA46552.1	Q42369
PR-10 proteins	Bet v 1 like	Plant development and defence system	Lup a 4	PR-10	17	HQ670416.1	AEB33719.1	
						AJ000108.1	CAA03926.1	O2401
						AB070618.1	BAB63949.1	Q93X10
						X79974.1	CAA56298.1	P52778
	Lup I 4			Lup 18A		X79975.1	CAA56299.1	P52779
						AY303549.1	AAF57943.1	Q7XZT8
						AY288355.1	AAF37978.1	Q7Y1W5
						AF322226.1	AAK09429.1	Q9AXK1
	Lup 10.2C			Lup 10.2B		AF322225.1	AAK09428.1	Q9AXK2
						AF170092.2	AAF77634.1	Q9LLQ2
						AF170091.1	AAF77633.1	Q9LLQ3
						AF180941.1	AAD55099.1	Q9SPB2
	Profilin	Structural function of the cell	Lup a 5	Lup a 5.0101	12-15	FG090100.1	-	-
						-	-	-
Non-specific lipid transfer protein	Prolamin (nsLTP)	Transport of lipids	Lup an 3	Lup an 3.0101	11	-	XP_019446786.1	-

A 43-kDa band of *L. albus* was also identified by the IgE of five out of six peanut-allergic patients in a study developed by Moneret-Vautrin et al. (1999), highlighting that this allergen is an important cross-reactive protein for peanut allergic individuals. These results might suggest a different contribution for the allergenicity of α -conglutin, depending on its acidic or basic subunits, being both involved in cross-reactivity phenomena with other legumes.

β -Conglutin (Lup a 1 and Lup an 1)

β -Conglutin is the most abundant lupine seed protein, accounting for 44% to 45% of total protein fraction. β -Conglutins were already identified and proposed as food allergens in the four lupine species. They are vicilin-like globulins belonging to the 7S globulin family (presenting quaternary structures), located at the cotyledon protein bodies and with storage and development functions. In *L. albus*, the β -conglutin is a noncovalently associated heterogeneous trimeric protein, containing more than 20 polypeptide chains with 25 to 80 kDa, having glycosylated subunits and no disulfide bonds (Carvajal-Larenas et al., 2016; Duranti et al., 2008; Fæste, 2010; Nadal, Canela, Katakis, & O'Sullivan, 2011; Santos et al., 1997). In *L. mutabilis*, *L. luteus*, and *L. angustifolius*, β -conglutin composition is quite different from the one identified in *L. albus*, being composed of major polypeptide chains (50 to 75 kDa), two polypeptide chains with 33-38 kDa, and several low-molecular-size polypeptides (Jimenez-Lopez et al., 2018; Santos et al., 1997). The vicilins show a self-association into micelle arrangements due to the high hydrophobicity at protein surface. This hydrophobic pattern can be different according to the composition of vicilins in each lupine species (Carvajal-Larenas et al., 2016). Both α -conglutin (legumins) and β -conglutin (vicilins) belong to the cupin superfamily of proteins, which are characterized by a common β -barrel structural core domain. They can be divided into δ -conglutin (Lup a δ -conglutin and Lup an δ -conglutin) single- and two-cupin domains, the latter including the 7S (vicilins) and the 11S (legumins) seed storage proteins (Fæste, 2010). β -Conglutin was characterized in 2008 as a major allergen in *L. angustifolius* (Goggin, Mir, Smith, Stuckey, & Smith, 2008), being added to the allergen list as Lup an 1 (WHO/IUIS [<http://www.allergen.org/>]). Lup a 1 is not yet recognized as a lupine allergen by the WHO/IUIS, but it was added to the ALLERGOME (<http://www.allergome.org/>) database as an allergenic β -conglutin from *L. albus*. The β -conglutin polypeptide of 34.5 kDa originated from post-translational modifications in the seed and was considered as a major allergen in *L. albus* (Guillamón, Rodríguez et al., 2010). Ballabio et al. (2013), Goggin et al. (2008), Holden et al. (2008), and Jimenez-Lopez et al. (2018) also characterized this protein as a major allergen in *L. albus* and *L. angustifolius*. Different studies indicate that 38-kDa and 65-kDa proteins from β -conglutin might also have considerable allergenic potential (Dooper, Plassen, Holden, Lindvik, & Faeste, 2009; Faeste, Lovik, Wiker, & Egaas, 2004; Peeters et al., 2007).

δ-Conglutin (Lup a δ-conglutin and Lup an δ-conglutin)

δ-Conglutin is a protein from the 2S sulfur-rich albumin family, accounting for 10% to 12% of total protein fraction in *L. albus*, *L. luteus*, and *L. angustifolius* (Carvajal-Larenas et al., 2016). The 2S albumins are seed storage proteins of the prolamin superfamily, characterized as the legume water-soluble protein fraction. They present an α-helical globular domain, with a preserved pattern of six or eight cysteine residues, respectively, forming three or four intramolecular disulfide bonds (Carvajal-Larenas et al., 2016; Fæste, 2010). δ-Conglutin is a monomer of 14 kDa, containing two disulfide-linked polypeptides of 4 and 9 kDa, rich in cysteine residues. The covalent dimerization of monomeric units is potentially increased by the existence of an odd cysteine residue, contributing to create a stable dimer with 23 to 25 kDa, which, at neutral pH and low ionic strength, assembles reversibly to a 56 kDa oligomer (Duranti et al., 2008). Some reports have classified these two aggregated forms of δ-conglutin, as δ₂- and δ₁-conglutins, respectively (Duranti et al., 2008; Nadal et al., 2011). Owing to the high amounts of glutamic acid, δ-conglutin is the most acidic globulin in lupine seed, which influences the behavior of the protein by increasing its hydrophilicity (Carvajal-Larenas et al., 2016). This class of proteins is commonly involved in the storage function of the seeds, but due to some similarities with the plant cereal inhibitor family, it may also have a defense role (Duranti et al., 2008). Some studies have demonstrated no IgE-binding toward δ-conglutin by immunoblot analysis (Foss, Duranti, Magni, & Frokiaer, 2006; Magni et al., 2005). However, Holden et al. (2008) reported different rates of IgE-binding using immunoblotting or indirect enzyme-linked immunosorbent assay (ELISA), suggesting that this protein can present a less stable structure or conformational epitopes that can be destroyed by the denaturing conditions in sodium dodecyl sulfate (SDS). Others authors evidenced the IgE cross-reactivity between lupine δ-conglutin and Ara h 2 (peanut 2S albumin), thus confirming its allergenic potential (Dooper et al., 2009; Moneret-Vautrin et al., 1999). Presently, they are included in the ALLERGOME database as Lup a δ-conglutin and Lup an δ-conglutin from *L. albus* and *L. angustifolius*, respectively (ALLERGOME [<http://www.allergome.org/>]).

γ-Conglutin (Lup a γ-conglutin and Lup an γ-conglutin)

γ-Conglutin is a basic 7S globulin of 200 kDa, having monomers of 42 to 43 kDa composed of two different disulfide-linked subunits of 17 and 29 kDa, being the latter glycosylated with covalently linked mannose and glucosamine units (Duranti et al., 2008; Santos et al., 1997). It accounts for about 4% to 5% of lupine seed proteins (Nadal et al., 2011) and, at neutral pH, the prevalent form of this protein is a tetramer or a hexamer. Lupine γ-conglutin is predominantly placed in the protein bodies of developing lupine seeds,

being also found in the extracellular apoplastic regions of cotyledons in germination. The biological function of the protein was not yet completely clarified, but *in vitro* tests with proteolytic enzymes and its extravacuolar location suggest that it is not a storage protein (contrarily to vicilins), being classified as an aspartic protease, a catalytic type of protease (Mane, Johnson, Duranti, Pareek, & Utikar, 2018). It also binds divalent metal ions, including Zn^{2+} and Ni^{2+} , which have been shown to promote the refolding of the denatured protein (Duranti et al., 2008). Lupine has been widely correlated with antihyperglycemic properties, which have been mainly attributed to the γ -conglutin (Bertoglio et al., 2011; Lovati et al., 2012; Muñoz, Luna-Vital, Fornasini, Baldeón, & Gonzalez de Mejia, 2018), although recently this bioactive role has also been identified in the β -conglutin (Lima-Cabello et al., 2017). According to Magni et al. (2005), γ -conglutin is considered as a major lupine allergen. A number of studies demonstrated that some polypeptides were IgE reactive in the sera of peanut-sensitized patients, especially one with a molecular mass of 43 kDa, which could correspond to γ -conglutin in its unreduced form (Magni et al., 2005; Poltronieri et al., 2002). However, some authors suggest that this protein is more likely to be the α -conglutin rather than the unreduced γ -conglutin because most of patients' sera used for their studies did not react with this protein (Dooper et al., 2009; Holden et al., 2008). Apparently, it seems to occur a huge discrepancy in the IgE-binding pattern of lupine proteins among patients, which could probably be associated with the exposure to different lupine species. Recent publications show that all of the α -, β -, γ -, and δ -conglutin subunits are potential allergens, being α -, β -, and γ -conglutins the most allergenic ones (Dooper, Holden, Faeste, Thompson, & Egaas, 2007; Goggin et al., 2008; Guillamón, Rodríguez, et al., 2010; Jimenez-Lopez et al., 2018; Nadal et al., 2011). Lup a γ -conglutin and Lup an γ -conglutin from *L. albus* and *L. angustifolius*, respectively, were included in the ALLERGOME database (<http://www.allergome.org/>).

PR-10 (Bet v 1 like) (Lup a 4 and Lup I 4)

The PR-10 proteins are a family of relatively small molecules (154 to 163 amino acids) with masses around 17 kDa, being encoded by multigene families. They are slightly acidic and resistant to proteases, presenting a cytosolic location (Pasternak et al., 2005). This protein family has a characteristic fold consisting of a highly curved β -sheet and α -helices assembled in a large hydrophobic cavity in the core of the protein (Fernandes, Michalska, Sikorski, & Jaskolski, 2013). They are mainly involved in plant development and defense mechanisms, being synthesized in response to various types of pathogens (viruses, bacteria, and fungi) and to biotic/abiotic stresses. Sikorski et al. (1999, 2000) identified two gene families encoding 5 PR-10 homologues belonging to two different subclasses: LIPR10.1 (156 amino acid residues) and LIPR10.2 (158 amino acid residues). Posteriorly,

Pinto, Ribeiro, Regalado, Rodrigues-Pousada, and Ricardo (2005) reported the expression of homologue proteins in *L. albus*, presenting high sequence identity (>93%) with LIPR10.2 of *L. luteus*, confirming the existence of similar proteins in different lupine species. Likewise, Pasternak et al. (2005) demonstrated that the structure of PR-10 protein (*L. luteus*) from the LIPR10.2 shared the same folding pattern (rigid β -sheet wrapped around a variable helix α 3) of other PR10 members, whose elements seem to be crucial for the functioning of this family of proteins. PR-10 proteins of *L. albus* present high sequence and structural homologies with other allergens, namely, Ara h 8 (peanut) and Glym 4 (soybean), which are members of the PR-10 family of proteins (Berkner et al., 2009; Guarneri, Guarneri, & Benvenga, 2005). Potential cross-reactivity between the PR-10 protein (white lupine) and the allergens, Ara h 8(peanut) and Bet v 1 (birch pollen), has been advanced by *in silico* and homology modeling approaches (Guarneri et al., 2005), although this fact has not yet been proved experimentally. Lup a 4 and Lup l 4 were added to ALLERGOME database (<http://www.allergome.org/>) as allergens for *L. albus* and *L. luteus*, with two and nine isoforms, respectively (Table1).

Profilin (Lup a 5)

Profilins encompass a huge family of proteins in plants with molecular masses of 12 to 14 kDa, participating in the reorganization of the cytoskeleton and regulating the activity of the microfilament system and the intracellular calcium levels (Asturias et al., 2002). Their structure comprises three α -helices, seven β -strands, and 10 turns that form two hydrophobic cores separated by a central six-stranded β -sheet (Krishnan & Moens, 2009). Profilins are often classified as panallergens due to their high structural similarity among distantly related species (Hauser, Roulias, Ferreira, & Egger, 2010), being recognized as allergens in a wide range of pollens, such as olive and sunflower, as well as in foods such as apple, tomato, and soybean (WHO/IUIS [<http://www.allergen.org/>]). In 2017, the *L. albus* profiling (15kDa) was recognized as a food allergen (Lup a 5), according to a study where six subjects with convincing history of lupine allergy (1/6 proven by food challenge) or peanut allergy with a suspicion on lupine allergy (5/6) were IgE reactive to a recombinant lupine profilin (WHO/IUIS [<http://www.allergen.org/>]). Despite its official recognition as a lupine food allergen, the information is limited to the one reported by the WHO/IUIS database, which is expected to be further detailed in the near future.

Non-specific lipid transfer protein (Lup an 3)

Like the 2S albumins, the nsLTP are small extracellular molecules (around 10 kDa), being part of the Prolamin superfamily. These proteins are rich in cysteine residues and present a compact and stable globular conformation (Salcedo, Sanchez-Monge, Barber, &

Diaz-Perales, 2007). They are typically located in the outer layers of the peel of fruits and seeds and their allergenic potency can be reduced, when the tissues rich in these proteins are removed, such is the case of peach (Salcedo et al., 2007; Zuidmeer & van Ree, 2007). nsLTP have different biological functions, but the most well-known property regards their capacity to bind distinct types of lipids in order to facilitate their transport (Liu et al., 2015). Recently, a nsLTP (with 11 kDa and 116 aminoacids) was officially recognized by the WHO/IUIS as an allergenic food protein in *L. angustifolius* (blue lupine), being designated as Lup an 3 (Table 1). Data seem to indicate that the sera from six out of nine lupine- and/or peanut-allergic individuals were IgE reactive to this protein. From those patients, three developed clinical symptoms upon oral challenge with lupine flour, whereas from other four subjects with convincing history of peanut allergy and suspicion of lupine allergy, only one presented clear IgE binding to the nsLTP under reducing conditions (WHO/IUIS [<http://www.allergen.org/>]). Like in the previous case, the information for Lup an 3 is only limited to the one reported by the WHO/IUIS database, highlighting the need for more detailed data regarding this particular lupine allergen.

LUPINE POTENTIAL CROSS-REACTIVITY

Until now, it was not possible to identify the allergens exclusively responsible for lupine sensitization or those involved in parallel and/or cross-sensitization with other legumes. Moreover, it is not known which lupine proteins are correlated with the severity of clinical reactions (Sanz et al., 2010). Despite the high sequence and structural homologies among their proteins, in most cases, the cross-reactivity between lupine and peanut, soybean, lentil, or pea does not seem to be of high clinical relevance. Lupine allergy seems to coexist more often with peanut allergy than with of soybean or other legumes, but their clinical cross-reactivity is still unknown, with only a low percentage of peanut-allergic individuals having confirmed lupine allergy (Jappe & Vieths, 2010). This issue requires further investigations regarding the identification and characterization of lupine allergens, their clinical relevance, and routes of sensitization.

Legumes have structurally homologous proteins that share common epitopes, making natural cross-reactivity likely to occur. High sequence and structural homologies of lupine allergens with other legume allergens, as well as the existence of common linear and conformational epitopes, seem to support the cross-reactivity among them. Recently, it has been demonstrated that lupine β -conglutins are structurally related to Len c 1 (lentil) and more distantly close to the Gly m 5 (soybean). Additionally, the presence of several T- and B-cell epitopes was responsible for cross-allergic reactions among legume proteins (Jimenez-Lopez, Lima-Cabello, Melser, Foley, & Singh, 2015). Table 2 summarizes several soybean and peanut proteins reported to be cross-reactive with lupine proteins. Lupine

allergy can manifest itself individually or in parallel with other legume allergies, such as peanut, lentil, soybean, chickpea, bean, or pea (Jappe & Vieths, 2010). The sensitization to a particular allergen is normally evaluated by serological IgE testing (performed by IgE-inhibition immunoassays, either in simplex or multiplex formats), thus providing a measure of patient's sensitization profile (Matricardi et al., 2016). Multiple sensitizations to cross-reactive allergens, identified either by SPT and/or by *in vitro* testing, are not sporadic (such as multiple sensitization to different legume allergens) (Sicherer, 2001). However, the interpretation of these results must be carefully performed because it is crucial to differentiate a clinically relevant cross-reaction from an asymptomatic one (Worm et al., 2014). Despite the high rates of immunological cross-reactivity among legumes, they have low clinical relevance (such as the case of lupine with soybean or lentils). In opposition, the cross-reactivity between lupine and peanut appears to be of higher clinical significance (Cabanillas, Jappe, & Novak, 2018; Jappe & Vieths, 2010). In a study developed by Moneret-Vautrin et al. (1999), 44% of the peanut-allergic subjects were sensitized to lupine, although relevant clinical allergy (symptoms in the ocular, nasal, and bronchial mucosa) to lupine was observed in 28% of those patients. Similarly, Shaw, Roberts, Grimshaw, White, and Hourihane (2008) reported that 34% of peanut-allergic children and teenagers were sensitized to lupine and 4% of those were clinically allergic to lupine presenting objective respiratory symptoms, urticaria, and itchy mouth. An Italian study showed that two out of 12 peanut-allergic children, included in a DBPCFC clinical trial, showed clinical symptoms (anaphylactic shock, asthma, angioedema, urticaria, allergic rhinitis, and gastrointestinal symptoms) when tested with lupine-enriched pasta (Fiocchi et al., 2009). Afterward, the same research group demonstrated that lupine β - and γ -conglutins were involved in the *in vitro* and *in vivo* cross-reactivity with peanut proteins in the same group of allergic children, whereas the role of α -conglutin in the cross-reactivity between lupine and peanut proteins seems to be mainly related to its basic subunit (Ballabio et al., 2013).

The lupine α -conglutin has structural homology with peanut Ara h 3 (legumin) by alignment studies, displaying a homology of 55% to 100% and an identity of 67% to 88% (Barre, Borges, Culerrier, & Rouge, 2005; Holden et al., 2008; Moneret-Vautrin et al., 1999; Sirtori, Resta, Arnoldi, Savelkoul, & Wichers, 2011). Legumin basic subunits of lupine and peanut present large sequence homology in the proximity of the proteolytic processing site, a region that is highly preserved in these proteins, thus contributing to their cross-reactivity (Magni et al., 2005). Moreover, when comparing peptides from reactive regions, it seems that β -conglutin (lupine vicilin) exhibits considerable similarity to the Ara h 1 (peanut vicilin), presenting an identity between 50% and 76% and a homology between 63% and 93% (Guarneri et al., 2005; Sirtori et al., 2011). A sequence identity of 39.2% and similarity of 66.6% were also found between β -conglutin and Ara h 1 (Guillamón, Rodríguez, et al.,

2010). Dooper et al. (2009) suggest that β - (vicilin) and δ -conglutins (2S albumin) crossreact with Ara h 1 and Ara h 2 (peanut vicilin and 2S albumin), respectively. Sirtori, Resta, Brambilla, Zacherl, and Arnoldi (2010) showed that *L. angustifolius* and *L. albus* δ -conglutins have sequence identities of 39.1% and 40.9% or 41.7% and 44.4% with Ara h 2 or Ara h 6 (peanut 2S albumins), respectively. Cross-reactivity between α -conglutin (lupine legumin) and Ara h 2 (peanut 2S albumin) was also demonstrated, whereas between γ -conglutin (lupine 7S globulin) and Ara h 3 (peanut legumin) was observed at a lesser extent (Dooper et al., 2009). Goggin et al. (2008) stated that, despite the structural similarities between Ara h 1 (peanut vicilin) and β -conglutin (lupine vicilin), none of the lupine-allergic individuals clinically responding to β -conglutin reacted to peanut. Individuals reacting to lupine and peanut, compared with lupine responders only, are reactive to distinct IgE epitopes of β -conglutin or even to different lupine allergens (such as α -conglutin), which may be related to the primary sensitizer (lupine or peanut) (Goggin et al., 2008). In spite of the absence of sequence similarities between γ -conglutin (lupine 7S globulin) and Ara h 3 (peanut legumin), some studies reported its strong cross-reactivity with peanut allergen-specific IgG and IgE (Magni et al., 2005; Moneret-Vautrin et al., 1999), which is probably related to potential homologies in their conformational structures.

Table 2 – Cross-reactivity among lupine allergens and other legume proteins.

Lupine allergens (<i>L. albus</i> , <i>L. angustifolius</i> , <i>L. luteus</i>)	Peanut allergens (<i>Arachis hypogaea</i>)	Soybean allergens (<i>Glycine max</i>)	Plant family proteins (Food allergens)
α -Conglutin	Ara h 3	Gly m 6	Legumin-like protein (11S)
β -Conglutin	Ara h 1	Gly m 5	Vicilin-like proteins (7S)
δ -Conglutin	Ara h 2/Ara h 6	Gly m 8	2S albumin (Prolamin superfamily)
γ -Conglutin	Ara h 3	Not reported	Vicilin-like protein (7S)
PR-10 of <i>L. albus</i>	Ara h 8	Gly m 4	PR-10 protein (Bet v 1 superfamily)
Lupine profilin	Ara h 5	Gly m 3	Profilins

Schiarea, Arnoldi, Fanelli, De Combarieu, and Chiabrando (2013) reported the possible involvement of N-glycans of lupine γ -conglutin as a source of potential cross-reactivity. The glyco-epitopes (core β 1,2-xylose and core α 1,3-fucose) attached to γ -conglutin and known as cross-reactive carbohydrate determinants (CCD) induce the production of antibodies in humans specific for the carbohydrate target, but not for the carrier protein. This fact led to cross-reactivity in *in vitro* tests with non homologous (but CCD-carrying) glycoproteins (Schiarea et al., 2013). Later, Jappe, Kull, Opitz, and Zabel (2018) reported the case of a patient with confirmed peanut allergy that suffered angioedema, dyspnea, gastrointestinal symptoms, and loss of consciousness, upon the consumption of a vanilla ice cream that

was proved to contain lupine. After evaluating the serum of this patient, strong IgE reactivity was verified with the CCD of lupine γ -conglutin. Still, the allergic potential and clinical relevance of CCD is unclear and needs further investigations.

Some reports have also shown clinically relevant cross-reactivity of lupine with other legumes, such as lentil, bean, chickpea, pea, and soybean, as well as tree nuts, namely, almond. Holden et al. (2008) reported a possible clinical cross-reactivity between α -conglutin and a 14-kDa almond protein, although the identity of almond protein was not yet clarified. The authors also suggested that amandin (prunin or Pru du 6) could be a possible cross-reactive protein with α -conglutin due to its conserved β -barrel domains that might allow IgE binding, but oral provocation tests in almond- and lupine-allergic patients should be investigated. In a population study of peanut-sensitized subjects, different sensitization patterns were observed with related legumes, namely, 87% to soybean, 82% to lupine, and 55% to pea, whereas clinical relevancetosoybean,lupine,andpeawas perceivedin33%, 35%, and 29%, respectively (Guillamón, Rodriguez, et al., 2010; Peeters et al., 2009). The amino acid sequence of β -conglutin (vicilin) from *L. albus* and *L. angustifolius* was found to be highly homologous to that of Pis s 1 (pea vicilin) and Len c 1.01 (lentil vicilin) (approximately 40% and 70% of sequence identity and similarity, respectively). The homology was lower for Gly m Bd 28K (soybean vicilin), evidencing 26.1% and 58.8% of sequence identity and similarity, respectively (Guillamón, Rodriguez, et al., 2010). In a test population of 1,160 individuals with suspicion of some type of food allergy, 4% were sensitized to lupine, whereas 75% or 82.1% of the patients were co-sensitized to lupine and other legume, or to lupine and pollen, respectively (Reis et al., 2007). The Gly m 4 (soybean Bet v-1 homologous protein) is responsible for triggering severe adverse immunological responses to food, but whose primary sensitizer is the pollen allergen (birch Bet v 1) (Mittag et al., 2004). Additionally, Gly m 4 is particularly similar to the PR-10 proteins of yellow lupine (*L. luteus*) with sequence similarities between 74% and 84% (Berkner et al., 2009). Clinical and immunological cross-reactivity between lupine and fenugreek was also reported by Vinje, Namork, and Løvik (2012) using mice as a food allergy model. The authors showed that 12.5% of the lupine sensitized mice developed serious anaphylaxis after challenge with fenugreek, suggesting the involvement of IgE and IgG instead of intestinal mast cells. However, more studies involving the allergic response in humans need to be performed.

EFFECT OF PROCESSING, FOOD MATRIX AND DIGESTIBILITY ON LUPINE PROTEINS

Lupine is a widely utilized ingredient in many food applications. Lupine flour and lupine protein isolates or concentrates are excellent materials for supplementing different food products owing to their high protein content and technological characteristics (Kohajdova et

al., 2011). Lupine flour and products thereof are frequently used by the bakery industry, as a substitute or in addition to wheat, in order to increase the content and nutritional value of protein, and the amount of dietary fiber of wheat-based products (bread, cookies, muffins, brownies, instant noodles, and pasta) or in gluten-free foods. Moreover, its addition influences the technological aspects of bread production in terms of efficiency, dough quality, and organoleptic acceptability. Lupine is frequently utilized as egg and/or butter replacer owing to its emulsifying properties and yellow color (Villarino et al., 2016). Lupine can also be utilized as a substitute of fat and vegetable protein extender in a range of meat products, such as frankfurters and sausages, as well as in dairy products (ice cream and fermented milk) (Kohajdova et al., 2011; Villarino et al., 2016).

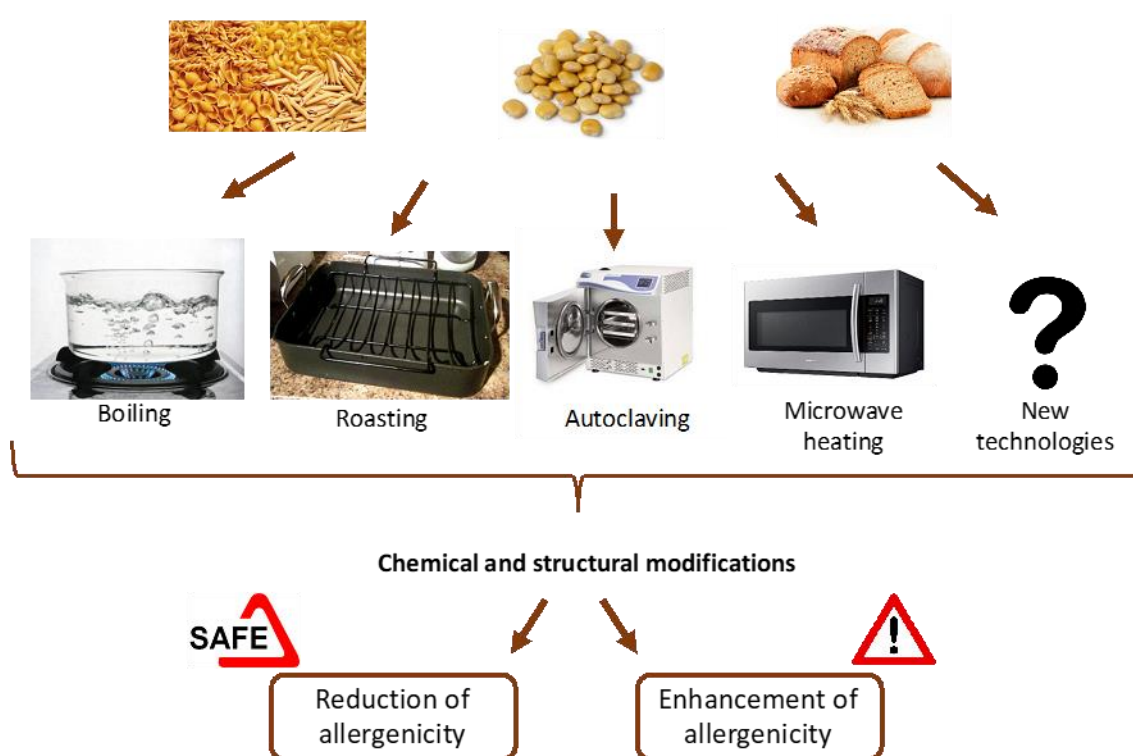


Fig. 1 – Schematic representation of the effect of food processing on lupine allergenicity.

Accordingly, most of the lupine-containing foods have been submitted to some kind of technological processing. Food processing can induce several chemical and structural modifications in lupine proteins, affecting their allergenicity by the alteration of some epitopes or the exposure of new ones (Figure 1) (Loza & Lampart-Szczapa, 2008). Similarly, the occurrence of chemical reactions among proteins, fat, and sugars, in the food matrix, can restrict the protein availability to interact with the immune system, reducing their allergenicity, but it can also decrease protein digestibility (preserving the existing epitopes), potentially increasing protein allergenicity (Costa et al., 2020; Villa, Costa, Oliveira, & Mafra, 2018). Although there are some studies reporting the influence of food processing, food

matrix, and digestibility on the allergenicity of lupine proteins (summarized in Table 3), the existing information is still insufficient.

First reports about the effect of thermal treatments suggest that lupine proteins are heat resistant after boiling (Alvarez-Alvarez et al., 2005; Duranti, Sessa, Scarafoni, Bellini, & Dallochio, 2000; Rojas-Hijazo, Garces, Caballero, Alloza, & Moneo, 2006). The results from Alvarez-Alvarez et al. (2005) suggest that autoclaving (138 °C for at least 20 min) can significantly affect the integrity and structure of lupine proteins, with a reduction in IgE-binding capacity and consequent relevant decrease of their overall allergenicity. After autoclaving treatment, only two bands of 23 and 29 kDa had IgE-binding capacity, whereas with 30 min at the same temperature no IgE-binding was observed. However, a new IgE-reactive band of 70 kDa seems to be generated during the autoclaving, probably as a consequence of the formation of aggregated structures, suggesting the production of a neoallergen. The same study revealed that boiling, microwave, and extrusion did not induce any modifications on lupine allergenicity (Alvarez-Alvarez et al., 2005). Rojas-Hijazo et al. (2006) investigated several manufactured foods (soups, chicken bouillon cubes, and cookies) using the serum of a monosensitized lupine-allergic patient. Different IgE-binding proteins ranging from 14 to 48 kDa were identified, from which the 14-kDa protein was verified as highly resistant to boiling (20 min) and roasting (170 °C, for 20 min). Guillamón et al. (2008) tested an innovative processing technique used in food technology, named as instantaneous controlled pressure drop, on the IgE-binding capacity of lupine proteins. Several pressure and time conditions were assayed, indicating significant modifications on protein pattern by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). The IgE-binding capacity of most proteins was reduced or even abolished with increasing pressure and duration treatments, although two major bands of 26 and 29.5 kDa and some <20 kDa were still detected by SDS-PAGE with the most severe pressure processing treatment (6 bars, 3 min). These results presented similar patterns to the ones reported by Alvarez-Alvarez et al. (2005) in the case of autoclave processing. Therefore, Guillamón et al. (2008) suggested that the combination of heat and steam pressure can drastically decrease lupine *in vitro* immunoreactivity and, as a result, instantaneous controlled pressure drop treatment could reduce lupine allergenicity, which still needs to be confirmed by oral food challenges. In order to increase the use of thermally treated lupine ingredients, as an attempt to reduce the risk of occurrence of allergic reactions, the same group tested the effect of their addition on the breadmaking properties (Guillamón, Cuadrado, et al., 2010). Supplementing wheat flour with thermally treated lupine flour showed similar physical dough properties, bread structure, and sensory characteristics to the untreated lupine, allowing its use in baking.

As in the study of Holden et al. (2008), Rojas-Hijazo et al. (2006) also used lupine-containing foods mimicking food processing mixtures, contrarily to Alvarez-Alvarez et al. (2005) and Guillamón et al. (2008) who tested lupine seeds and lupine cotyledons, respectively. Holden et al. (2008) processed *L. albus* seeds in the form of a tofu-like product (Lopino), which were soaked and blended, and the obtained filtrate boiled and pressed. The majority of patients' sera showed less IgE-binding capacity to heat-treated lupine (Holden et al., 2008), whereas the results of Alvarez-Alvarez et al. (2005) indicated an effect only after prolonged autoclaving. These findings indicate that allergenic lupine proteins seem to be protected by the matrix components, demanding severe processing conditions for reducing their allergenicity. Additionally, the induction of an IgE response depends on their intrinsic properties, as well as the matrix in which the proteins are administered (Foss et al., 2006; Holden et al., 2008). More recently, Villa, Moura, Costa, and Mafra (2020) reported a higher reduction in the immunoreactivity of γ -conglutin in model breads containing lupine than in raw mixtures (model flour mixtures of rice or wheat with lupine), which agrees with previous findings (Alvarez-Alvarez et al., 2005; Holden et al., 2008; Rojas-Hijazo et al., 2006).

The digestion of lupine allergens by proteolytic enzymes has also been studied to evaluate its effect on their allergenicity (Capraro, Magni, Scarafoni, & Duranti, 2009; Czubiński, Dwiecki, Siger, Neunert, & Lampart-Szczapa, 2014; Czubiński, Siger, & Lampart-Szczapa, 2016; Pinto, Neves, & Machado de Medeiros, 2009). Czubiński et al. (2016) showed that lupine globulins are completely digested by pepsin, using a double digestion model (pepsin followed by pancreatin) and chymotrypsin. However, pancreatin and trypsin do not entirely hydrolyze lupine globulins due to the high specificity of their cleavage sites (e.g., trypsin cleaves peptide chains mainly at the carboxyl side of lysine or arginine). γ -Conglutin is resistant to the action of these enzymes, retaining its antigenic properties after simulated intestinal digestion with pancreatin and trypsin. This fact can be due to the protective effect of complexes with flavonoids cleaved from other proteins during digestion or as result of the relatively low number of trypsin cleavage sites found in γ -conglutin. Moreover, it seems that pH can also affect the digestion of lupine allergens, especially the γ -conglutin that suffers pH-dependent oligomerization from monomer at pH < 4.5 to hexamer at pH > 7.0 (Czubiński et al., 2016).

Table 3 – Recent studies about the effect of processing techniques and enzymatic proteolysis on lupine proteins.

Allergen	Treatment	Allergenicity/Protein structure	Digestibility	Matrix	References
γ -conglutin	Thermal treatment	60 °C, 3 h, pH 7.5 – no changes in aggregation pattern; Stability of γ -conglutin not affected by heat treatment at neutral pH.	-	Purified protein	Duranti et al. (2000)
Lupine proteins	Boiling, autoclaving, microwave heating, extrusion cooking	Autoclaving (138 °C, 20 min) – reduced allergenicity; Boiling, microwave heating and extrusion cooking – unaffected allergenicity.	-	Lupine seeds	Álvarez-Álvarez et al., (2005)
Lupine proteins	Thermal treatment (boiling and roasting)	Roasting and boiling did not destroy the recognition of some IgE-binding proteins; 14 kDa protein was highly resistant to heat.	-	Lupine extracts, soups, chicken bouillon cubes and cookies.	Rojas-Hijazo et al. (2006)
Lupine proteins	Instantaneous controlled pressure drop (DIC)	DIC at 6 bar, 3 min – reduced allergenicity	-	Lupine cotyledons	Guillamón et al. (2008)
Lupine conglutins	Thermal treatment	Heat-treated lupine – less intense IgE binding bands	-	Lopino (processed protein from <i>L. albus</i> seeds)	Holden et al. (2008)
γ -conglutin	Oven cooking (3h, but with maximum temperature at 180°C during 25 min)	Decrease in the immunoreactivity of γ -conglutin assessed by rabbit anti- γ -conglutin antibody	-	Model wheat breads containing lupine and raw binary mixtures of lupine in rice or wheat	Villa et al. (2020)
γ -conglutin	Proteolysis (pepsin, pancreatin, bromelain)	-	Resistance to proteolytic attack at pH greater than 4.0; acidic pH renders the protein susceptible to proteases.	Purified protein	Capraro et al. (2009)
Lupine globulins	Proteolysis (pepsin, trypsin)	Pepsin – after 30 min, reduced antigenicity; Trypsin – after 30 min, antigenic epitopes completely destroyed.	Pepsin – complete hydrolysis; Trypsin – partial hydrolysis.	Seeds of <i>L. albus</i>	Pinto et al. (2005)
Lupine proteins	Thermal treatment, mechanical process and high pressure	100 °C, 30 min and 200 °C, 5 min - decrease in native β -conglutin; 200 °C, 15–30 min – decrease in native α - and β -conglutin; Mechanical stress and pressure (100 bar) – unaffected proteins.	β -conglutin peptides more resistant to thermal processing and tryptic digestion	Lupine protein isolate (<i>L. angustifolius</i>)	Sirtori et al. (2010)
Lupine globulins	Proteolysis (pancreatin, trypsin, chymotrypsin)	-	γ -conglutin resistant to pancreatin and trypsin; globulins almost complete digestion with chymotrypsin	Purified proteins (<i>L. angustifolius</i>)	Czubinski et al. (2014)
Lupine globulins	Proteolysis (pepsin, pancreatin, trypsin, chymotrypsin)	Retained allergenicity after hydrolysis	Pepsin – complete digestion of globulins; Pancreatin and trypsin – partial digestion of globulins	Purified proteins (<i>L. angustifolius</i> and <i>L. luteus</i>)	Czubinski et al. (2016)

Likewise, Capraro et al. (2009) reported that at pH above 4.0, γ -conglutin is less prone to suffer proteolysis, but at acidic conditions the protein is more susceptible to protease activity due to a transitional state in the protein native structure. Pinto et al. (2009) showed that pepsin gradually destroyed the epitopes of sweet lupine major globulins, inhibiting their antigenicity in 77% after 30 min. In the case of trypsin, the antigenic epitopes present in the hydrolysates were completely destroyed after 30 min. Despite the described relevant findings on how lupine allergens are affected by different food processing techniques, diverse food matrices, and digestion conditions, they are not fully supported by direct clinical studies. It is important to highlight that lupine allergenicity has been mostly assessed by indirect analysis rather than by direct methods, such as oral food provocations or DBPCFC. Indirect analysis (immunoblotting, inhibition ELISA, among others) is based on the use of patients' sera, meaning that this assessment is more prone to bias because a patient positive to specific IgE does not necessary lead to clinical allergic symptoms. Patients' sera are affected by several intrinsic (age, sex, presence of concomitant diseases, genetic heritage, among others) and environmental (such as geographical origin) factors, which cannot be ruled out when evaluating the allergenic potential of different proteins (Costa et al., 2020). Regarding lupine allergens, it became clear that some molecules are more prone to lose their allergenicity upon processing and/or digestion (such as α -conglutin and β -conglutin), whereas others may preserve their clinical reactivity (such as γ -conglutin).

ANALYTICAL METHODS FOR LUPINE DETECTION IN FOODS

Since the first reported case of lupine allergy, the concern about the presence of lupine as a hidden allergen in processed foods has been increasing. As previously referred, lupine is a technological aid often used in the preparation of a great number of foods, from bakery to dairy or meat products (Carvajal-Larenas et al., 2016). Lupine flours, protein isolates, or concentrates are normally prepared from the "sweet lupine" species (*L. albus*, *L. luteus*, and *L. angustifolius*) because they contain low levels of bitter-tasting and potentially toxic alkaloids. According to EU legislation, lupine is considered as an allergenic food that must be declared and highlighted in the ingredient list of foods labels, irrespective of its amount (Regulation (EU) No 1169/2011 [European Union, 2011]). However, despite the strong regulatory measurements to protect allergic individuals, unintentional contact with hidden allergens in foods caused by cross-contamination during processing or mislabeling represents a concrete health risk for this part of the population (Costa, Fernandes, Villa, Oliveira, & Mafra, 2017; Villa, Costa, Oliveira, et al., 2018). Even with a proper labeling stating the presence of lupine as ingredient, peanut allergic patients, who are not informed about the possibility of cross-reactivity with lupine allergens, can still be at risk of suffering adverse immunological reactions. Therefore, analytical strategies able to detect and

quantify lupine at trace levels in foods are critical, not only for helping food manufacturers managing the allergenic foods, but also for protecting the allergic population. Numerous approaches have been advanced to screen, identify, and quantify lupine in foods, which include protein-based methods relying on immunoassays and mass spectrometry (MS), DNA-based methods using real-time polymerase chain reaction (PCR), and more recently, biosensors (Figure 2). Table 4 presents the resumed information about the recent reported analytical methods for lupine allergen detection in foods.

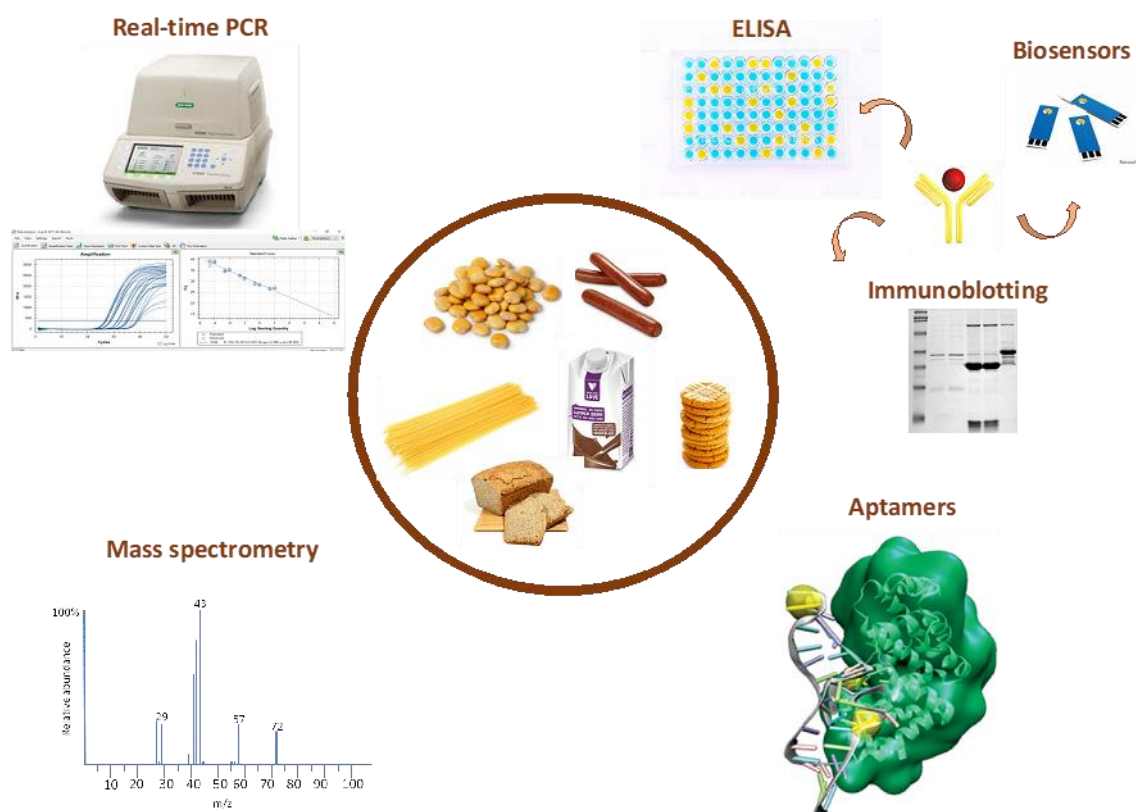


Fig. 2 – Representative analytical methods used for the detection and quantification of lupine allergens in processed foods.

Protein-based methods

Immunoassays

Immunochemical methods are based on the specific interaction of antibody/antigen (allergen or protein marker), such as lateral flow devices, ELISA, immunosensors, and other relevant immunoassays (such as immunoblotting). For the specific detection of lupine in foods, the most representative and widely used method is the ELISA. The availability as commercial kits is one main advantage of ELISA. Besides, it provides simple, rapid performance and versatile, reproducible, quantitative, and sensitive analysis of allergens, without requiring advanced equipment and specialized personal. Nevertheless, ELISA results can be influenced by the food matrix due to cross-reactivity of antibodies with

nontarget compounds, leading to false positive results, and by food processing that induces conformational changes on target proteins, leading to false negative results (Costa, Fernandes, et al., 2017).

Owing to the increasing demand for specific allergen detection, a wide range of commercial kits for lupine detection has become available in the market and summarized in Table 5. Moreover, in-house developed-systems are frequently reported in the literature (Table 4) (Ecker & Cichna-Markl, 2012; Ecker, Ertl, Pulverer, et al., 2013; Ecker, Ertl, & Cichna-Markl, 2013; Holden, Faeste, & Egaas, 2005; Holden, Moen, Sletten, & Dooper, 2007; Kaw, Hefle, & Taylor, 2008; Koeberl et al., 2018; Lima-Cabello, Alché, & Jimenez-Lopez, 2019; Revák, Golian, Židek, Capla, & Zajác, 2014; Röder, Kleiner, Sachs, Keil, & Holzhauser, 2013). The majority of these systems relies on polyclonal antibodies (IgY from hen's eggs or IgG from rabbit) targeting lupine proteins from one or more *Lupinus* spp., normally providing suitable specificity for all the sweet lupine species used as food ingredients or technological aids. Nonetheless, these assays might cross-react with other plant species (specially legumes or nuts) (Ecker, Ertl, Pulverer, et al., 2013; Holden et al., 2005; Holden et al., 2007; Kaw et al., 2008). The limit of detection (LOD) is normally around 1 mg of lupine protein per 1 kg of matrix, which is enough to detect trace amounts of lupine that might present a risk for the allergic subjects (Holden et al., 2007). Holden et al. (2007) reported that in a sandwich ELISA, the combination of polyclonal-monoclonal antibody could significantly improve the sensitivity because they observed an enhanced recovery of both processed and native proteins from two different lupine species. The comparison of different ELISA kits from distinct manufacturers is quite difficult because each one has their own generated antibodies, conjugates, standards, and calibrants (Table 5). Koeberl et al. (2018) performed an interesting comparison of three different ELISA kits to detect lupine protein or lupine flour protein from one or more species, realizing that the specificity of the antibodies used in the kits was not available, which is an important information for the detection capability to lupine and cross-reactivity phenomena. The estimated concentration of lupine in foods differed among the kits due to the use of different standards and cross-reactivity was observed with some legumes and nuts. This study highlighted the need to standardize the available ELISA kits in order to obtain comparable and consistent results.

Mass spectrometry

The recent progresses in MS technology and equipment, combined with effective bioinformatic tools, have prompted the era of proteomics, providing the identification, characterization, and quantification of food allergens (Costa, Fernandes, et al., 2017; Monaci & Visconti, 2009). MS offers a fast analysis of proteins with high sensitivity, accuracy, specificity, and reproducibility, allowing a large degree of freedom in the selection

of target analytes as markers of food allergens (Johnson et al., 2011; Picariello, Mamone, Addeo, & Ferranti, 2011). In addition, MS is independent from biological interaction of antibody/allergen (marker protein), contrarily to immunoassays, overcoming the problems of cross-reactivity phenomena and enabling accurate identification of analyzed molecules (Monaci & Visconti, 2009). Despite the ability for multi-target analysis, it is a time-consuming technology and requires high cost equipment and specialized personnel (Johnson et al., 2011). Moreover, the effects of food matrix and processing must be considered in the selection of marker peptides to avoid compromising the performance of the method (Costa, Fernandes, et al., 2017).

Regarding the detection of lupine as an allergenic food in processed products, several MS-based methods have been developed (Table 4). Hoffmann, Münch, Schwägele, Neusüß, and Jira (2017) established a sensitive multi-target method for the identification of lupine (*L. angustifolius*), soybean (*Glycine maxima*), and pea (*Pisum sativum*) in meat products applying a high-performance liquid chromatography coupled to tandem MS detection (HPLC–MS/MS) technology. The method reached a sensitivity down to 2.0 mg/kg of lupine protein in sausages using a peptide marker for β -conglutinin from *L. angustifolius*, being comparable to those of PCR or ELISA, which ranged between 1 mg/kg (Holden et al., 2005; Holden et al., 2007; Kaw et al., 2008) and 10 mg/kg (Ecker, Ertl, Pulverer, et al., 2013; Waiblinger, Boernsen, Naumann, & Koepfel, 2014). Similarly, other works using HPLC–MS/MS and UPLC–MS/MS reported sensitivities between 0.05 and 24.0 mg/kg in pasta, cookies, and beverages (Huschek, Bonick, Lowenstein, Sievers, & Rawel, 2016; Locati, Morandi, Zanotti, & Arnoldi, 2006; Mane, Bringans, Johnson, Pareek, & Utikar, 2017; Mattarozzi, Bignardi, Elviri, & Careri, 2012; Resta, Brambilla, & Arnoldi, 2012). It is also important to highlight that MS-based methods go beyond the detection/quantification of lupine, allowing to further confirm its species identity, representing an additional advantage over the immunoassays.

DNA-based methods

Lately, the methods relying on DNA analysis have expanded their application in food allergen detection. Real-time PCR has been the technique of choice owing to its suitable setup cost, reasonable running time, and high specificity and sensitivity. A relevant advantage of applying these methods to processed foods is related with the relatively high heat stability of DNA molecules (Costa, Amaral, Grazina, Oliveira, & Mafra, 2017; Costa, Oliveira, & Mafra, 2013), in comparison with proteins. Moreover, the specific design of PCR assays makes cross-reactivity phenomena less prone to occur, being independent from biological effects, such as the related with antibody-antigen interaction (Costa, Fernandes, et al., 2017; Costa, Ansari, Mafra, Oliveira, & Baumgartner, 2014). DNA targets are usually

genes coding allergens or other species-specific regions. Regarding allergen analysis, DNA-based methods are considered as indirect methods because they do not target the offending protein, thus being a topic of controversy among researchers. In spite of that, some governmental agencies have already established real-time PCR assays to detect several food allergens, reinforcing their high utility reliability (Holzhauser, 2018).

In the case of lupine detection (Table 4), the majority of reported DNA-based methods target the nuclear region of the internal transcribed spacer 1 (ITS1) (multicopy) of lupine, achieving sensitivities of lupine in food in the range of 0.1 to 10 mg/kg (Demmel, Hupfer, Busch, & Engel, 2012; Demmel, Hupfer, Busch, & Engel, 2011; Demmel, Hupfer, Ilg Hampe, Busch, & Engel, 2008; Haase, Brüning, Matissek, & Fischer, 2013; Waiblinger et al., 2014). It has to be pointed that these values are expressed as milligrams of allergenic food per kilogram of food. According to Waiblinger and Schulze (2018), these limits can be converted to milligrams of total lupine protein per kilogram of food using a conversion factor of 2.5. Other works target genes encoding allergenic proteins (typically unicopy genes), such as conglutins (Galan, Brohée, Scaravelli, van Hengel, & Chassaigne, 2010; Revák et al., 2014; Scarafoni, Ronchi, & Duranti, 2009), or mitochondrial genes, namely, tRNA-MET (Galan, Brohée, de Andrade, van Hengel, & Chassaigne, 2011), with sensitivities down to 10.0 and 2.0 mg/kg, respectively. In the last years, multicopy genes have been targeted by real-time PCR to detect allergenic foods because of providing enhanced sensitivity and results comparable to ELISA or MS techniques. Similar to MS platforms, the DNA-based methods also have the potential of being species-specific, allowing discriminating among the different species of sweet lupine.

As in other analytical methods, the food matrix and processing are key issues in allergen analysis by DNA-based methods. Villa, Costa, Gondar, Oliveira, and Mafra (2018) assessed the effect of matrix on the detectability of lupine in food products, showing that the sensitivity in wheat flour was 20 times lower than in rice flour. The baking process lead to a slight decrease (mostly influenced by high temperatures of baking for an extensive period of time approximately 3 hr) in the sensitivity of lupine detection in bread, compared with the non-processed rice or wheat flour matrices. The comparison of the performance of real-time PCR methods with other protein methods, namely, ELISA, has been done in the case of lupine detection, showing that the sensitivities of the former are slightly lower than the latter (both in-house-developed and ELISA kits) (Ecker, Ertl, Pulverer, et al., 2013; Revák et al., 2014; Röder et al., 2013). Despite this finding, the sensitivities of real-time PCR assays are within the recommended range for allergen analysis (down to 1 mg/kg), considering the levels of elicitation doses.

Table 4 – Analytical methods for the detection of lupine in different food products.

Method	Matrices	Target	LOD/LOQ	Reference
Immunoblotting	Cookies, chicken bouillon cubes and chicken soups	Lupine allergen of 14 kDa (serum from sensitized patients)	Not reported	Rojas-Hijazo et al. (2006)
Sandwich ELISA	Hot-dogs, vegetarian sausages, pasta and chocolate	<i>L. albus</i> (polyclonal rabbit anti-lupine)	LOD = 1.0 mg/kg	Holden et al. (2005)
Sandwich ELISA	Cakes, bread, pasta, chocolate spread, biscuits, flour and chips	<i>L. albus</i> and <i>L. angustifolius</i> (polyclonal-monoal rabbit antibody)	LOD = 1.0 mg/kg	Holden et al. (2007)
Sandwich ELISA	Frankfurter and corn muffins	<i>L. albus</i> (polyclonal antisera from rabbit)	LOQ = 1.0 mg/kg	Kaw et al. (2008)
Sandwich ELISA	Biscuits, vegetarian patties, breads, rusk and noodles	<i>L. albus</i> , <i>L. angustifolius</i> and <i>L. luteus</i>	LOD = 0.4–2.3 mg/kg; LOQ = 1.9–6.2 mg/kg	Ecker & Cichna-Marx (2012)
Competitive ELISA	Vegetarian patties, breads and rusk	<i>L. albus</i> , <i>L. angustifolius</i> and <i>L. luteus</i> (anti-lupine IgG from rabbits and anti-lupine IgY from hen)	LOD = 11.0–126.0 mg/kg LOQ = 16.0–295.0 mg/kg	Ecker et al. (2013)
Indirect ELISA	Lupine seeds, lupine flours, biscuits, toasted bread, pickled lupine, Bolognese sauce, lupine burger, spreads, lupine drink, tofu, boiled lentils, boiled chickpea and boiled fava bean.	<i>L. angustifolius</i> , <i>L. albus</i> and <i>L. luteus</i> (β -conglutinin (Lup an 1) using anti-IgG β -conglutinin antibody)	Not reported	Lima-Cabello et al. (2019)
ELISA, commercial kits	Lupine and other legumes and nuts	<i>L. albus</i> , <i>L. angustifolius</i> and <i>L. luteus</i>	Not reported	Koeberl et al. (2018)
Sandwich and competitive ELISA (IgG and IgY) and Real-time PCR with TaqMan probe	Bread, biscuits, rice patties and noodles	<i>L. albus</i> , <i>L. angustifolius</i> and <i>L. luteus</i> (anti-lupine IgG from rabbits and anti-lupine IgY from hen); tRNA nucleotidyltransferase (CCA1) mRNA from <i>L. albus</i> (U15930.1)*	LOD (IgG) = 5.0–20.0 mg/kg LOD (IgY) = 3.0–34.0 mg/kg LOD (sandwich) = 0.1–0.6 mg/kg LOD (Real-time PCR) = 50.0–384.0 mg/kg LOQ (IgG) = 26.0–68.0 mg/kg LOQ (IgY) = 4.0–79.0 mg/kg LOQ (sandwich) = 0.5–1.7 mg/kg	Ecker et al. (2013)
ELISA, commercial kits, and Real-time PCR with TaqMan probe	Different cultivars of <i>L. albus</i> , <i>L. angustifolius</i> and <i>L. luteus</i> seeds	Lupine proteins (kit ELISA); Lup an beta-conglutinin gene (HQ670415.1, EF455724.1, DQ142920.1)*	LOD (ELISA A) = 0.6 mg/kg LOD (ELISA B) = 0.2 mg/kg LOD (Real-time PCR) = 1.0 mg/kg	Roder et al. (2013)
ELISA, commercial kits, and Real-time with TaqMan probe	Lupine samples	Lupine proteins (ELISA kit); α -conglutinin (U74384 and U743844)* and δ -conglutinin (X53523 and X53523)* genes from <i>L. albus</i> and <i>L. angustifolius</i>	LOD (ELISA) = 2.0–30.0 LOD (Real-time PCR) = 10.0 mg/kg	Revák et al. (2014)
Real-time with TaqMan probe	Bakery products, fruit-flavored gums, and model mixtures of ice cream	ITS1 gene from <i>L. angustifolius</i> (Z72202)*	LOD = 0.1 mg/kg	Demmel et al. (2008)

Table 4 – Analytical methods for the detection of lupine in different food products (continued).

Method	Matrices	Target	LOD/LOQ	Reference
Real-time with SYBR Green	Bread, biscuits and snacks	<i>L. albus</i> CyA32 gene (Lup a gamma conglutinin) (CAC16394)*	7 pg of lupine DNA <0.1% of lupine flour in foods	Scarafoni et al. (2009)
Real-time with TaqMan probe	"Tofu-like" products, vegetarian sausage, breads and cookies	α - (U74384)* and δ -conglutinin (X53523)* genes from <i>L. angustifolius</i>	1 pg of lupine DNA 10 mg/kg	Galan et al. (2011)
Real-time with TaqMan probe	Bread, pastry, cookies, vegetarian products	mitochondrial tARN-MET (X04377)* from <i>L. angustifolius</i>	1 pg of lupine DNA 2 mg/kg	Galan et al. (2011)
Real-time with TaqMan probe	Mixtures of lupine in wheat flour	ITS1 gene from <i>L. angustifolius</i> (Z72202)*	LOQ/LOQ = 1.0-10.0 mg/kg	Demmel et al. (2012)
Bar-HRM Real-time PCR	Model mixtures of lupine and soybean	DNA barcoding region trnL from lupine	LOD = 1% in soybean flour	Madesis et al. (2012)
Real-time with SYBR green	Marzipan mixtures	ITS1 from <i>L. albus</i> (AF007481)*	LOD = 10.0 mg/kg	Haase et al. (2013)
Real-time with TaqMan probe	Rice cookies, wheat cookies and sauce hollandaise powder	ITS1 gene from <i>L. angustifolius</i> (Z72202)*	LOD = 10.0 mg/kg	Waiblinger et al. (2014)
Real-time with TaqMan probe	Cookies, breads, pastry, wafers	Lup a 4 from <i>L. albus</i> (J000108.1)*	1 pg of lupine DNA LOD/LOQ (rice flour) = 5.0 mg/kg LOD/LOQ (wheat flour) = 100.0 mg/kg LOD/LOQ (bread) = 500.0 mg/kg	Villa et al. (2018)
MLPA	Mixtures of lupine in Jytte flour	ITS1 gene from <i>L. angustifolius</i> (Z72202)*	0.09 ng of lupine DNA LOD = 0.001% in Jytte flour	Mustorp et al. (2018)
Aptamers (competitive ELONA and SPR)	Not reported	Lup an 1, β -conglutinin	LOD = 153.0 nM	Nadal et al. (2012)
Aptamers (FRET-based dimeric aptamer probe)	Not reported	Lup an 1, β -conglutinin	LOD = 150.0 pM	Mairal et al. (2014)
Aptamers (Apta-PCR and SPR)	Not reported	Lup an 1, β -conglutinin	LOD = 2.0 nM (100.0 ng/mL)	Pinto et al. (2014)
Aptamers (Apta-PCR)	Lupine flour	Lup an 1, β -conglutinin	LOD = 85.0 pM (25.0 ng/mL)	Svobodova et al. (2014)
Aptamers (Lateral Flow)	Not reported	Lup an 1, β -conglutinin	LOD (competitive lateral flow) = 55.0 pM LOD (competitive-amplification lateral flow) = 9.0 fM	Jauset-Rubio et al. (2016)
Aptamers (Apta-RPA)	Not reported	Lup an 1, β -conglutinin	LOD = 3.5 fM	Jauset-Rubio et al. (2017)
iSPR Biosensor (combined with antibody array)	Cookies and dark chocolates	<i>L. albus</i> (polyclonal rabbit anti-lupine)	LOD (cookies) = 0.8 mg/kg LOQ (cookies) = 3.4 mg/kg LOD (chocolates) = 0.6 mg/kg LOQ (chocolates) = 2.3 mg/kg	Raz et al. (2010)

Table 4 – Analytical methods for the detection of lupine in different food products (continued).

Method	Matrices	Target	LOD/LOQ	Reference
HPLC/ESI-MS/MS	Lupine beverage	Lupine proteins	LOD = 0.05 mg/mL	Locati et al. (2006)
HPLC-Chip-MRM	Not reported	γ -conglutinin from <i>L. albus</i>	LOD = 5.0 ng/ μ L	Resta et al. (2012)
HPLC-ESI-MS/MS	Pasta and biscuits	Lupine proteins	LOD (pasta) = 1.0–13.0 mg/kg LOQ (pasta) = 4.0–42.0 mg/kg LOD (biscuits) = 1.0–24.0 mg/kg LOQ (biscuits) = 4.0–80.0 mg/kg	Mattarozzi et al. (2012)
UPLC-MS/MS	Mixtures of lupine in wheat flour, cookies and soft bread	β -conglutinin from <i>L. albus</i>	LOD/LOQ = 20 mg/kg	Huschek et al. (2016)
HPLC-MS/MS	Sausages	<i>L. angustifolius</i> proteins	LOD = 2 mg/kg	Hoffmann et al. (2017)
HPLC-MS/MS	Not reported	γ -conglutinin from <i>L. angustifolius</i>	LOD = 2.68 μ g/mL LOQ = 8.12 μ g/mL	Mane et al. (2017)

Other DNA-based techniques have also been used in allergen analysis, namely, high-resolution melting (HRM) analysis coupled to DNA barcodes and ligation-dependent probe amplification (LPA). HRM is a new alternative method consisting of a closed-tube post-PCR analysis based on the shape of melting transitions of real-time PCR products, which can be combined with DNA barcode markers in allergen analysis (Costa, Fernandes, et al., 2017). Madesis, Ganopoulos, Anagnostis, and Tsaftaris (2012) showed the applicability of HRM analysis coupled to the universal plant DNA barcode region *trnL* to identify bean species, including *Lupinus* spp. Although HRM analysis is merely a qualitative tool that allows the differentiation of closely related species, the authors proposed a rough quantitative model to estimate lupine (*Lupinus* spp.) as an adulterant in soybean (*Glycine max*) (Table 4).

In LPA, the amplicons are formed from the specific hybridization of bipartite probes and one primer pair targeting several DNA sequences (Costa, Fernandes, et al., 2017). It is considered a highly specific technique due to the requirement of two different probes that hybridize next to each other on the target sequence (Mustorp, Dromtorp, & Holck, 2011). Mustorp et al. (2011) developed a multiplex LPA (MLPA) to detect eight allergens in different foods, namely, sesame, soybean, hazelnut, peanut, celery, gluten, mustard, and lupine. The achieved LOD was in the range of 5 to 400 gene copies and, its application to several foods spiked with mustard, celery, soybean, or lupine flour and enabled a relative sensitivity within 1% to 0.001%, depending on the allergen. In the case of lupine, the selected target region was the ITS1, which allowed reaching sensitivities of 0.09 ng of lupine DNA and 0.001% (10mg/kg) of lupine flour in spiked food.

Biosensors

Recently, biosensors have been considered as innovative and attractive immunochemical tools in the detection of food allergens, providing high potential for full automation, fast, repeatable, and highly sensitive methods (Prado et al., 2016). In brief, in biosensors a signal is produced by a transducer upon recognizing the interaction between a probe or antibody (receptor) and the target molecule (DNA or protein). Surface plasmon resonance (SPR), applied in optical biosensors, is the most used device, which relies on measurements of the refractive index changes that occur upon binding of antibody to target protein (Zhou et al., 2019). Raz, Liu, Norde, and Bremer (2010) used a SPR-based biosensor coupled to an antibody microarray to directly target 13 food allergens, including lupine, with a sensitivity up to 0.6 mg/kg of lupine in dark chocolate.

Table 5 – Commercially available ELISA kits for lupine allergen detection/quantification in foods.

Commercial kit (brand)	Matrix	Type of assay (cat no)	Analytical range (mg/kg)	LOD (mg/kg)	Performance time (min)
Lupine ELISA kit (ABNOVA, Taoyuan City, Taiwan)	Food products	ELISA (KA3310)	0-30	0.2	60
Lupine ELISA kit (Arigo laboratories, Hsinchu City, Taiwan)	Food products	ELISA (ARG80804)	2-30	0.2	20
BioAssay™ ELISA Kit (Lupinek et al.) (US Biologicals, Salem, Massachusetts, USA)	Food products	ELISA (L6000-01)	0-30	0.2	60
Lupine ELISA kit (BioFront technologies, Tallahassee, FL, USA)	Food products	ELISA (LU2-EK-96)	1-40	0.13	30
Lupine ELISA (Demeditec Diagnostics GmbH, Kiel, Germany)	Food products	ELISA (DELUPE01)	2-30	0.2	20
ELISA Systems® (ELISA Systems™, Queensland, Australia)	Food products and environmental samples	ELISA (ESLFP-48)	0.5-5.0	Not available	Not available
RIDASCREEN®FAST Lupine (r-Biopharm AG, Darmstadt, Germany)	Food products	Sandwich ELISA (R6102)	0-27	0.7	50
AgraQuant® ELISA Lupin (Romer Labs Div., Getzersdorf, Austria)	Food products	Sandwich ELISA (COKAL1548)	2-30	Not available	Not available
PriboFast® ELISA Kit (PriboLab, Singapore)	Food products	ELISA (EKT-LU10)	2-30	Not available	Not available

Aptamers are artificial oligomers or nucleic acids with appropriate secondary structures capable of binding to a wide range of targets, which have emerged as new analytical reagents in different biosensing transduction systems (Jauset-Rubio et al., 2016; Zhou et al., 2019). They present some advantages that make them ideal components for the development of sensors, namely, high affinity and binding specificity for their targets, low production cost, and ease labeling with different reporter molecules (Amaya-González, de los-Santos-Alvarez, Miranda-Ordieres, & Lobo-Castañón, 2013). Table 4 summarizes some works using aptamers for the specific detection of the major lupine allergen, Lup an 1. The first report using aptamers enabled detecting Lup an 1 at concentrations higher than 153 nM in aqueous media, demonstrating high specificity without cross-reactivity with other flour components or with other conglutin fractions of lupine (Nadal, Pinto, Svobodova, Canela, & O'Sullivan, 2012). Since then, several efforts have been made to develop highly specific and sensitive aptamers for the detection of β -conglutin, reaching sensitivities ranging from 3.5 fM to 2.0 nM (Jauset-Rubio et al., 2017; Jauset-Rubio et al., 2016; Mairal, Nadal, Svobodova, & O'Sullivan, 2014; Pinto et al., 2014; Svobodova, Mairal, Nadal, Bermudo, & O'Sullivan, 2014).

CONCLUSION

In the last years, the concern about lupine allergy has significantly increased because more cases of relevant clinical manifestations have been reported after the ingestion of this legume. Currently, several dietetic and healthy foods have lupine as an ingredient due to its nutritional properties. Furthermore, lupine can be applied as a substitute for wheat in gluten-free products and as a technological aid in meat or dairy products. Therefore, lupine allergic individuals should avoid all foods that state the presence of lupine in their ingredients. Specific food legislation has been created with the main objective of protecting the health of allergic individuals, and in most cases, is adapted to the reality of most countries/regions where the legislation is implemented. Owing to the fact that lupine is highly consumed in several countries, including EU and Australia, this food was added to a preexisting list of allergenic foods that should be emphasized on food label, regardless of their amount. In countries such as United States, lupine is not as extensively consumed, which probably precludes its inclusion as part of the priority list of food allergens. Still, when looking at the increasing globalization, enhancing migratory movements, rising global trades, and changes in food habits and culinary practices, the trend of food allergies is also shifting, which means that the legislation might also change in accordance with this trend. Besides, cross-contamination or mislabeling can still occur, posing a concrete risk for accidental exposure in allergic consumers. Consequently, the development of analytical methodologies with adequate performance for quantifying trace amounts of lupine in

processed foods is extremely important to support allergen management in the food industry. The available techniques have relied mainly on immunochemical assays, such as ELISA, MS, and real-time PCR, which all enable adequate sensitivities for lupine detection as a food allergen. However, the specificity might be compromised in the case of immunochemical assays due to their high susceptibility to cross-react with other nontarget proteins. Regardless of the method, the effects of food matrix and processing should be carefully considered and particularly assessed during method development and validation.

The major allergens of the *Lupinus* species are storage proteins, namely, conglutins, but other protein families such as the profilins, the nsLTP, and the PR-10 proteins are also classified as important allergens. According to the literature, two routes are mainly responsible for lupine sensitization: the direct sensitization by the ingestion of lupine as part of diet and the indirect pathway by inhalation of lupine proteins or other cross-reactive proteins (such as Bet v 1). Lentil, chickpea, pea, soybean, and, particularly, peanut are reported as cross-reactive legume species in lupine sensitization, but the clinical relevance associated with the ingestion of related legumes is still unclear. Lupine allergy was described in some peanut-allergic patients, suggesting not only cross-reactivity between both legumes, but also a potential co-sensitization to both peanut and lupine. The prevalence of lupine allergy is not clear because such studies have not been conducted in general population. Data on lupine sensitization ranging from 0.27% to 4.1% have been advanced in different European countries, but these values might not reflect the true prevalence of lupine allergy. Data from clinical tests, such as oral food challenges, are much needed to determine the true prevalence of lupine allergy, mainly in countries where the consumption of lupine is high, namely, in Australia and within the EU.

Food processing has an important influence on the structural properties of lupine proteins, stimulating the development of studies to assess its effect on lupine allergenicity. Lupine allergens seem to be relatively stable toward thermal processing. However, chemical and structural modifications can still occur after processing that, allied with some interactions occurring with other compounds from the food matrix, may induce changes in lupine proteins. These changes can greatly influence their susceptibility to gastrointestinal digestion, absorption kinetics, and, subsequently, their allergenicity. In this context, more research based on *in vitro* and *in vivo* studies is needed to further exploiting different and innovative processing technologies aiming at developing hypoallergenic formulas for the allergic population.

In summary, this review shows that vast information about lupine allergy, allergen characterization, and allergen detection has been generated during the past years. Nonetheless, more studies about lupine allergy prevalence are particularly needed to give

a global perspective and more research about application of food processing is required to provide more insights on the changed structural features and allergenicity.

Acknowledgments

This work was supported by Fundação para a Ciência e Tecnologia (FCT) under the Partnership Agreement UIDB 50006/2020 and by the project AlleRiskAssess—PTDC/BAA-AGR/31720/2017. Caterina Villa is grateful to FCT grant (PD/BD/114576/2016) financed by POPH-QREN (subsidized by FSE and MCTES).

Author contributions

Caterina Villa wrote the manuscript with critical input and corrections by Isabel Mafra, and Joana Costa. Isabel Mafra did the final editing. All authors contributed to locating and to interpreting the literature sources.

Conflict of interests

The authors declare no conflict of interests.

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3.2. Experimental part

Effect of food matrix and thermal processing on the performance of a normalised quantitative real-time PCR approach for lupine (*Lupinus albus*) detection as a potential allergenic food

Food Chemistry, **2018**, 262, 251–259

Immunoreactivity of lupine and soybean allergens in foods as affected by thermal processing

Foods, **2020**, 9, 254



3.2.1. Effect of food matrix and thermal processing on the performance of a normalised quantitative real-time PCR approach for lupine (*Lupinus albus*) detection as a potential allergenic food

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ABSTRACT

Lupine is widely used as an ingredient in diverse food products, but it is also a source of allergens. This work aimed at proposing a method to detect/quantify lupine as an allergen in processed foods based on a normalised real-time PCR assay targeting the Lup a 4 allergen-encoding gene of *Lupinus albus*. Sensitivities down to 0.0005%, 0.01% and 0.05% (w/w) of lupine in rice flour, wheat flour and bread, respectively, and 1 pg of *L. albus* DNA were obtained, with adequate real-time PCR performance parameters using the ΔC_t method. Both food matrix and processing affected negatively the quantitative performance of the assay. The method was successfully validated with blind samples and applied to processed foods. Lupine was estimated between 4.12 and 22.9% in foods, with some results suggesting the common practice of precautionary labelling. In this work, useful and effective tools were proposed for the detection/quantification of lupine in food products.

Keywords: Lupine allergens, real-time PCR, food analysis, food matrix, baking, ΔC_t method, quantification.

INTRODUCTION

Lupine is a legume from the Leguminosae family, comprising approximately 200–600 species, from which only four are of agronomic interest and frequently used for food and feed (Prusinski, 2017). Native from the Mediterranean region, the *Lupinus albus* (white lupine) is the most widely cultivated lupine species in this region. The *Lupinus angustifolius* (blue or narrow-leaved lupine) and the *Lupinus luteus* (yellow lupine) are from Australia and Southern Europe respectively, and the less known *Lupinus mutabilis* (pink or Andean lupine) belongs to South America. The first three lupine species are known as “sweet lupines” due to their low levels of potentially toxic alkaloids. Like soybean and milk, lupine is considered as a highly functional food, being commonly used by the industry as technological aids (ingredients) in all kinds of food products (bakery, confectionary, snacks). Due to their nutritional properties, lupine species are also used as feed (Ramanujam, Fiocchi, & Smith, 2016; Sanz, De Las Marinas, Fernández, & Gamboa, 2010). In 2014, Australia was the main producer of lupine with 625.600 tonnes, followed by Poland and Russia (FAOSTAT, 2017). In Europe, lupine is a common substitute for milk and soybean in bakery and dietary products, being commonly used as a functional ingredient in gluten-free foods (Scarafoni, Ronchi, & Duranti, 2009).

Since the first reported case of adverse immunological reactions to lupine in 1994 (Hefle, Lemanske, & Bush, 1994), the allergy to lupine has been referred with an increasing prevalence (Sanz et al., 2010). Clinical manifestations can vary from urticaria, angioedema, respiratory and abdominal symptoms to fatal reactions (anaphylactic shock). Although lupine allergy can emerge by primary sensitisation, in most of the cases it occurs owing to cross-reactivity in individuals allergic to other legumes, principally peanut and soybean (Sanz et al., 2010). The lowest eliciting dose for allergic reactions to lupine, responsible for inducing mild symptoms in peanut-sensitised patients, was 0.5 mg of lupine flour as reported by Peeters et al. (2009). More recently, the VITAL (Voluntary Incidental Trace Allergen Labelling) program of the Allergen Bureau of Australia and New Zealand (ABA) established 4 mg of protein as the reference allergenic dose for lupine (Taylor et al., 2014).

As consequence of the significant rise of the reported cases of lupine allergy, this legume seed was included in European Union regulations as a food susceptible of inducing allergies and whose presence must be declared and highlighted in the list of labelled ingredients of prepackaged foods, regardless of its amount (Regulation (EU) No. 1169/ 2011). The application of the present labelling legislation, while protecting the health of sensitised consumers, it can also restrict the range of available/safe foods if the producers excessively use the precautionary labelling. This demands highly sensitive and quantitative methods in order to identify cross-contamination that justifies the precautionary labelling or the

presence of lupine as an ingredient. Therefore, analytical methods able to detect and quantify lupine as an allergenic food with high specificity and sensitivity are of great importance to help food industry in allergen management, and to guarantee the life quality of sensitised/allergic individuals.

Different methods have been developed for the detection of lupine allergens in processed foodstuffs, targeting the allergenic proteins by enzyme-linked immunosorbent assay (ELISA) (Ecker & Cichna-Markl, 2012; Ecker et al., 2013; Holden, Faeste, & Egaas, 2005; Holden, Moen, Sletten, & Dooper, 2007; Kaw, Hefle, & Taylor, 2008) or mass spectrometry (MS) platforms (Hoffmann, Münch, Schwägele, Neusüß, & Jira, 2017; Mattarozzi, Bignardi, Elviri, & Careri, 2012). As alternative to protein analysis, the indirect identification of DNA encoding sequences of allergens takes advantage of the high stability of the target analytes to processing and the ubiquity of nucleic acids in the cells. Therefore, the number of studies focused on the development of DNAbased methods in allergen detection has rapidly evolved in recent years (Costa, 2013; Costa, Fernandes, Villa, Oliveira, & Mafra, 2017). For the specific detection of lupine, some quantitative real-time PCR approaches with specific hydrolysis probes (Demmel, Hupfer, Busch, & Engel, 2011, 2012; Demmel, Hupfer, Hampe, Busch, & Engel, 2008; Galan, Brohée, de Andrade Silva, van Hengel, & Chassaigne, 2011; Galan, Brohée, Scaravelli, van Hengel, & Chassaigne, 2010; Köppel, van Velsen-Zimmerli, & Bucher, 2012; Röder, Kleiner, Sachs, Keil, & Holzhauser, 2013) or with binding dyes (Scarafoni et al., 2009) have been proposed. Most reports used mitochondrial DNA markers of *Lupinus* spp., except the method described by Röder et al. (2013) that targeted a DNA sequence encoding for the β -conglutin allergen of *L. angustifolius*. Besides, all of them describe the absolute quantification of lupine DNA, with a rough estimation of lupine content as an ingredient.

In this work, a real-time PCR method targeting a sequence of Lup a 4 encoding gene of *L. albus* and using different model mixtures of lupine as calibrators was developed in order to detect and quantify lupine at trace amounts, and to assess the effect of food matrix and thermal processing on the analytical performance of the assay. A normalised approach was proposed to achieve a reliable and accurate estimation of the relative content of lupine by the use of a reference endogenous gene to minimise the effects associated with food matrix and processing. The successfully validated approaches were further applied to detect and quantify lupine as a potential allergenic ingredient in commercial foods and to verify the compliance with the labelled information.

Materials and methods

Sampling

Seeds of different species of *Lupinus* genus, namely *L. albus* (accessions no. 91-0035D and 91-0023D), *L. luteus* (accessions no. 90-0578D and 90-0579D), *L. mutabilis* (accessions no. 90-0580D and 90-0581D) and *L. angustifolius* (accession no. PI167938, PI615407, PI606487, PI180708, PI237721 and PI 660698) were gently provided by the Bank of Germplasm of University of Arizona (Boyce Thompson Arboretum, AZ, USA) and by the National Genetic Resources Program (NGRP) (Washington State University, Pullman, WA, USA). Flours of *L. luteus* and *L. albus* were gently provided by the company Germisem (Coimbra, Portugal) or acquired at local markets.

For method specificity testing, a total of 71 different species used as food were assayed: tree nuts (macadamia, pine nut, chestnut, almond, hazelnut, brazil nut, pecan nut, cashew nut, walnut and pistachio nut) and peanut; fruits (tomato, cherry, plum, apricot, blackberry, strawberry, nectarine, peach, mango, pineapple, orange and bitter orange); meats (boar, duck, partridge, hare, quail, pheasant, deer, rabbit, chicken, turkey, lamb, goat, ostrich, cow, horse and pig); fishes (European seabass, whiting pout, Atlantic horse mackerel, tadpole codling, rose fish, rock ling, Pacific mackerel); spices/condiments (onion, garlic, parsley, pepper, bay leaf, sweet chilli, chilli, oregano, basil, coriander and turmeric); cereals and other edible crops (fava bean, colza, sunflower, oat, barley, rye, maize, rice, pumpkin seeds, wheat, soybean, potato and cassava).

For assay applicability, a total of 26 commercial foods whose labels stated the presence of lupine (cookies, cupcakes, cakes, wafers and bakery products) were acquired at different Portuguese retail markets.

Model mixtures and sample preparation

In the absence of certified reference or testing materials for the specific detection of lupine in food products, two independent sets of binary model mixtures containing 50.0%, 10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001% (w/w) of lupine flour (*L. albus*, Biosagesse, France) in rice or in wheat flours were prepared. The first spiked reference mixture, containing 50% of lupine, was prepared by the addition of 200 g of lupine flour to 200 g of rice flour or wheat flour. All the following spiking levels of lupine were made by successive additions of each matrix flour until the proportion of 0.0001% of lupine. For method validation, blind samples were prepared similarly to reference mixtures, but independently, adding 25%, 2.5%, 0.8%, 0.25% and 0.025% (w/w) of lupine flour to rice or wheat flours and further analysed as unknown samples.

For the preparation of model bread, starting from binary mixtures of lupine in wheat flour, 180 g of water, 3 g of bread improver, 4.5 g of salt and 6 g of baker's yeast were added to a final amount of 300 g of the respective mixture. Dough was cooked in a bread machine Moulinex OW6101 during 3h. After cooling, breads were cut in the middle to remove slices, which were ground in the laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). All materials and different blender containers were previously treated with DNA decontamination solution to avoid contaminations. All samples were immediately stored at -20°C until further analysis. Commercial foods were also minced/homogenised separately in a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). Due to their high hardness, seeds of *L. albus* and *L. luteus* were ground in a mill suitable for flours with granulometry of 0.1 mm (MFC-90D MicroHammer Mill, Culatti, Zurique, Switzerland), while seeds and grains of *L. angustifolius* and *L. mutabilis* were soaked in distilled water and homogenised separately in a mortar.

DNA extraction

DNA was extracted using the Wizard method as described by Mafra, Silva, Moreira, Ferreira da Silva and Oliveira (2008) and/or NucleoSpin food kit (Macherey-Nagel, Düren, Germany), according to manufacturer instructions, using 200 mg of each sample. The extractions were performed in duplicate for each binary mixture and food. The extracts were kept at -20°C until further analysis. UV spectrophotometric DNA quantification on a SynergyHT multimode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), using a Take 3 micro-volume plate accessory, was performed to evaluate the yield and purity of DNA extracts. The DNA content was assessed by the nucleic acid quantification protocol in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA) with sample type defined for double-strand DNA.

Oligonucleotide primers and probes

DNA sequences available at NCBI database (<http://www.ncbi.nlm.nih.gov/>) corresponding to the gene encoding the allergenic PR-10 protein of *L. albus* (Lup a 4) (accession no. AJ000108.1 and AB070618.1) and *L. luteus* (accession no. AF170091.1, AF322226.1, AY288355.1, AF322225.1 and AF170092.2) were aligned using the software BioEdit V7.2.5. (Ibis Biosciences, Carlsbad, CA, USA). Common regions between sequences were selected for the design of three sets of new primers (La4-F1/La4-R1; La4-F2/La4-R2 and La4-F3/ La4-R3) for the specific detection of *Lupinus* spp. (Table 1). The design of primers was performed with the software Primer3 Output designing tool (<http://frodo.wi.mit.edu/primer3/>). The hydrolysis probe (La4-P) was also designed based on a specific region for *L. albus* in the same gene (accession no. AJ000108.1), labelled with

FAM as fluorescent reporter and BHQ-1 as quencher (Table 1 and Fig. S1A, supplementary material). For specificity purposes, *in silico* analysis of the nucleotide and primer sequences was performed using the basic local alignment search tool BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), respectively. OligoCalc software (<http://www.basic.northwestern.edu/biotools/oligocalc.html>) was also used to check primer properties and to ensure the absence of primer hairpins and self-hybridisation.

Supplementary Figs. S1–S3 associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.04.079>.

The amplification capacity of extracts was performed with universal eukaryotic primers (EG-F/EG-R) targeting a highly conserved 18SrRNA nuclear region (NCBI accession no. AF412275), acting also as an endogenous control gene (Villa, Costa, Oliveira, & Mafra, 2017). For the development of the normalised real-time PCR systems, a probe for the same region (EG-P) was also designed. The primers and probes listed in Table 1 were synthesised by STABVIDA (Lisbon, Portugal).

Table 1. Key data of primers and probe designed to specifically target DNA sequences of the Lup a 4 encoding gene of lupine and a conserved eukaryotic region (nuclear 18S rRNA).

Primers	Sequence (5' → 3')	Amplicon (pb)	NCBI (Accession no.)	Reference
<i>Lupinus</i> spp				
La4-F1	GGTGGCCCTGGAACCATTAAGA	134	AJ000108.1	This work
La4-R1	GGTAACCCAACTCCACCAACTA			
La4-F2	AACCAAAGGAGATGCTAAACCTAA	124	AJ000108.1	This work
La4-R2	GAGTTGAGTTTAGTTGTAATCAGG			
La4-P	FAM-AAGAGGGTAAAGCTGCTAAGGCTAGA-BHQ-1			
La4-F3	TAGTTGGTGGAGTTGGGTACC	133	AJ000108.1	This work
La4-R3	TTAGGTTTAGCATCTCCTTTGGTT			
Eukaryotes				
EG-F	TCGATGGTAGGATAGTGGCCTACT	109	AF412275	Villa et al. (2017)
EG-R	TGCTGCCTTCCTTGGATGTGGTA			
EG-P	FAM-ACGGGTGACGGAGAATTAGGGTTCGATTC-BHQ-1			This work

Qualitative PCR

In order to optimise PCR conditions, different concentrations of MgCl₂ and temperatures of annealing were tested for each primer pair (Table 1). Using the optimised PCR conditions, the reactions were performed in a MJ Mini™ Gradient Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 25 µL containing 2 µL of template DNA (100 ng), 1 × buffer (67 mM of Tris-HCl (pH 8.8), 16 mM of (NH₄)₂SO₄, 0.1% of Tween

20), 200 mM of each dNTP (Grisp, Porto, Portugal), 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM (for primers La4-F1/La4-R1, La4F2/La4-R2 and EG-F/EG-R) or 3.5 mM (for primers La4-F3/La4-R3) of MgCl_2 and 200 nM of each primer (except for primers EG-F/EG-R, for which 280 nM were used) (Table 1). The amplification programs were defined as following: initial denaturation at 95 °C for 5 min; 35 (for primers EG-F/EG-R) or 40 cycles (for all the other primers) at 95 °C for 30 s, 59 °C (for primers La4-F2/R2), 62 °C (for primers La4-F1/R1 and La4-F3/R3) or 63 °C (for primers EG-F/EG-R) for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

PCR products were verified by electrophoresis in a 1.5% agarose gel stained with GelRed 1 × (Biotium, Inc., Hayward, CA, USA) and carried out in 1 × SGTB (Grisp, Porto, Portugal) for 20-25 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was recorded using Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each extract was amplified at least in two independent runs.

Real-time PCR

For the real-time PCR amplifications, the reaction mixture of 20 µL included 1 × of SsoFast Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 300 nM of each primer (EG-F/EG-R or La4-F2/La4R2), 200 nM of each probe (EG-P or La4-P) for eukaryotic or lupine genes (Table 1), respectively, and 2 µL of DNA extract (100 ng). Each target sequence (eukaryotic and lupine genes) was amplified simultaneously in parallel reactions, using a fluorometric thermalcycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the following conditions: 95 °C for 5 min, 50 cycles at 95 °C for 15 s and 60 °C for 45 s, with collection of fluorescence signal at the end of each cycle. The software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) was used to evaluate the data from each real-time PCR run. Cycle of quantification (Cq), also known as cycle threshold (Ct), values were calculated using the software at user defined settings. Real-time PCR trials were repeated in two independent runs using $n=4$ replicates in each one.

For the determination of absolute limit of detection (LOD) and limit of quantification (LOQ) a series of 10-fold serially diluted lupine DNA extracts were prepared (10 ng–1 pg), while the binary mixtures (0.0001–50%) allowed the determination of the relative LOD and LOQ of lupine flour in food matrix. The LOD was considered as the lowest amplified level for 95% of the replicates and the LOQ was established as the lowest amplified level within the linear or dynamic range of the calibration curve (ENGL, 2015).

Sequencing

DNA from *L. albus*, *L. luteus*, *L. angustifolius* and *L. mutabilis* were amplified with primers La4-F3/La4-R2 to produce fragments of 233 bp for sequencing. The following amplification conditions were used: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 60 °C for 45 s and 72 °C for 60 s; and a final extension at 72 °C for 5 min. PCR products were purified with GRS PCR & gel band purification kit (GRISP, Porto, Portugal) to remove contaminants such as proteins, divalent cations, unincorporated nucleotides and enzyme inhibitors. The purified products were sent to a specialised research facility (GATC Biotech AG, Constance, Germany) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences with very good quality. Data were analysed using the available BioEdit v7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and FinchTV (Geospiza, Seattle, WA, USA) softwares and further compared with consensus sequence of *L. albus* (NCBI accession no. AJ000108.1).

RESULTS AND DISCUSSION

DNA extracts

DNA extracts used to test the specificity of the method were obtained with the Wizard method described by Mafra et al. (2008), presenting a wide range of yields (6.0–1608.4 ng/μL) and respective purities (1.6–2.2). *Lupinus* spp. specimens, reference mixtures and commercial samples were extracted with NucleoSpin food kit (Macherey-Nagel, Düren, Germany), providing DNA yields that varied between 9.1 and 1705.1 ng/μL with purities of 1.6–2.3. Prior to any amplification with specific primers and in order to ensure the absence of any possible false-negative results, the amplification capacity of all DNA extracts was tested with universal primers targeting the eukaryotic nuclear 18S rRNA gene (Table 1), revealing positive results for all samples.

Selection of primers

To evaluate the suitability of the designed new primers (La4-F1/ La4-R1, La4-F2/La4-R2 and La4-F3/La4-R3) (Table 1) for lupine detection by PCR, sensitivity and specificity tests were performed. The sensitivity was assayed by PCR amplification of 10-fold serially diluted lupine (*L. albus*) DNA extracts starting from 100 ng, while specificity was tested using a wide range of plant and animal species commonly used as food (as described in “Sampling” section) to assure the absence of any possible cross-reactivity. The PCR assay with primers La4-F1/La4-R1 revealed strong cross-reactivity with soybean from the 71 investigated species (Fig. S2A and S2B, supplementary material), which invalidated their further use. In

addition, the obtained PCR fragments show a size much higher than the expected (134 bp), suggesting that the region contains an intron that was probably spliced during the transcription process and, therefore, not reported in the target sequence of *Lupinus albus* mRNA available at NCBI database (accession no. AJ000108.1). La4-F3/La4-R3 primers were able to amplify the expected fragment of 133bp of lupine DNA (Fig. S2E and S2F, supplementary material), but, again they reacted with soybean, thus invalidating their use. The best results were obtained with La4-F2/La4-R2 primers, whose sensitivity was down to 10 pg of lupine DNA, with an expected fragment of 124 bp and no observable cross-reactivity with any other non-target species (Fig. S2C and S2D, supplementary material). Specificity of the PCR was further assessed with the other *Lupinus* species besides *L. albus*, namely *L. luteus*, *L. angustifolius* and *L. mutabilis* (Fig. S3A, supplementary material), which all produced the expected PCR fragment, showing that the assay is *Lupinus* genus specific. Lupine and soybean are species from the same legume family (Leguminosae), sharing structural similarities between their respective allergens. Serologic cross-reactivity between other members of the Leguminosae family and lupine is often common, although it does not always translate in clinically relevant symptomatic allergies (Berkner et al., 2009; Peeters et al., 2007; Ramanujam et al., 2016). The soybean allergen Gly m 4 is a member of the superfamily of Bet v 1 homologous proteins, the major birch pollen allergen, and according to the structural classification of proteins, they are both members of the PR-10 family. In this context, Berkner et al. (2009) demonstrated sequence similarities of 74–84% between Gly m 4 (soybean) and the PR-10 proteins (lupine) responsible for lupine allergy. This fact explains the cross-reactivity obtained in our results (Fig. S2B and S2F, supplementary material).

Development of the quantitative real-time PCR method

Specificity

For the real-time PCR method, the use of a fluorescent probe rather than a universal dye was selected to enable high sensitivity, avoiding unspecific amplification products. The hydrolysis probe was designed based on the alignment with the available PR-10 sequences at NCBI of *L. albus* (accession no. AJ000108.1 and AB070618.1) and *L. luteus* (accession no. AF170091.1, AF322226.1, AY288355.1, AF322225.1 and AF170092.2), in order to be specific for *L. albus* combined with the La4-F2/La4-R2 primers (Fig. S1A, supplementary material). The choice of developing a method specific for *L. albus* was due to the fact that it is the most often cultivated lupine species in Mediterranean region for human nutrition and, consequently, with high probability on being present in foodstuffs as a potential allergen (Prusinski, 2017). The real-time PCR results with the designed new hydrolysis

probe (La4-P) using the different *Lupinus* species confirmed that the assay was specific for *L. albus* (Fig. S3B, supplementary material). Contrarily to the qualitative PCR results, in which all the four lupine species were amplified (Fig. S3A, supplementary material), the hydrolysis probe allowed increasing the specificity of the proposed real-time PCR system.

In order to identify the nucleotide differences among the species in the target Lup a 4 sequence, the 124 bp PCR products (obtained with primers La4-F2/La4-R2) of *L. albus*, *L. luteus*, *L. angustifolius* and *L. mutabilis* were sequenced. Firstly, the sequencing results (Fig. S1B, supplementary material) of *L. albus* showed four differences in the target fragment of 124 bp, one of them in the probe region (G→A), compared with the consensus sequence from the NCBI (accession no. AJ000108.1). This difference was common to all species, suggesting possible nucleotide mistakes in the deposited sequence at the database or no effect on the probe binding to the target region in the case of *L. albus*. In the same region of the probe, *L. angustifolius* and *L. mutabilis* showed five nucleotide differences in comparison with sequenced *L. albus*, while *L. luteus* evidenced only two nucleotide differences. In all cases, the nucleotide differences among species were sufficient to affect the efficiency of probe ligation, thus explaining the absence of amplification of *L. luteus*, *L. angustifolius* and *L. mutabilis* by real-time PCR (Fig. S3B, supplementary material). Moreover, the sequence of the forward primer in *L. angustifolius*, *L. mutabilis* and *L. luteus* presents two differences (C→A and C→T), which did not affect primer binding by qualitative PCR (Fig. S3A, supplementary material).

Sensitivity

The absolute LOD of the real-time PCR method was established by the use of 10-fold serially diluted *L. albus* DNA extracts to cover the amplification of 5 orders of magnitude of the target analyte (10 ng to 1 pg). According to the parameters required for method development and validation, the LOD has to be defined as the lowest concentration level of the analyte with positive amplification at least 95% of the times (Bustin et al., 2009; ENGL, 2015). In this study, the LOD was determined assuming the lowest amount of *L. albus* DNA with positive amplification in all replicates (8/8), which corresponded to the level of 1 pg of lupine DNA, equivalent to approximately 1.7 genomic DNA copies (Fig. 1). The estimation of the amplified DNA copies was calculated using the available prime estimate value of *L. albus* genome size (0.6 pg), obtained from the Plant DNA C-value database (Bennett, & Leitch, 2012), assuming that the targeted sequences are single copy genes. Since the lowest amplified level (1 pg) is within the linear range of the calibration curve, it is also considered as the LOQ. With the proposed real-time PCR system, the absolute sensitivity was improved when compared with other reports that targeted allergen encoding sequences of *L. angustifolius* (500 pg and 50 pg for Lup an α -conglutinin and Lup an δ -

conglutin genes, respectively) (Galan et al., 2010). The obtained absolute sensitivity (1 pg) was also higher than the one (7 pg) reported by Scarafoni et al. (2009) using a real-time PCR system based on SYBR Green dye to detect Lup a γ -conglutin encoding sequence of *L. albus*. The TaqMan real-time PCR method reported by Galan et al. (2011) was able to reach a sensitivity of 1 pg of *L. angustifolius* DNA, but targeting a mitochondrial gene (tRNA-MET), which is a multicopy gene, in opposition to the allergen encoding sequences that are single-copy genes.

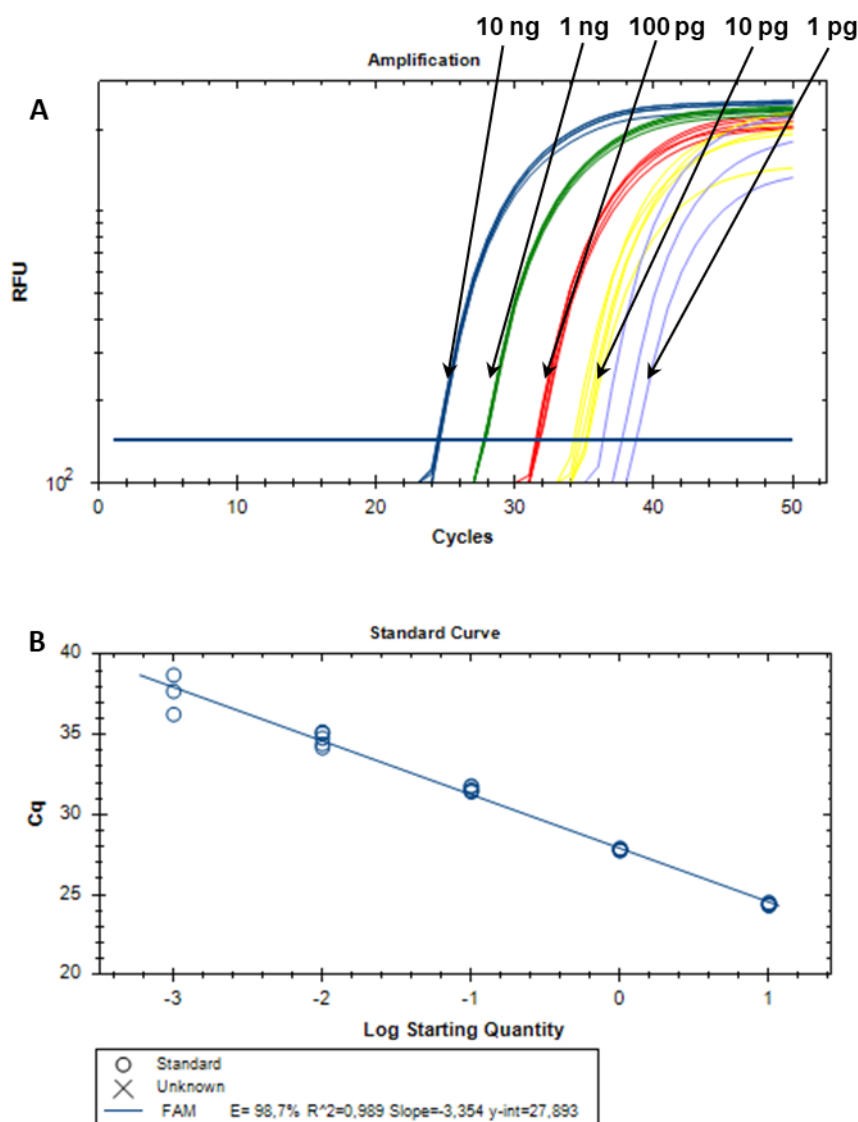


Fig. 1. Amplification curves (A) and respective calibration curve (B) of a real-time PCR assay with a hydrolysis probe targeting the Lup a 4 gene. The amplified extracts were obtained from 10-fold serially diluted lupine DNA from 10 ng to 1 pg (n=4 replicates). Cq (cycle of quantification) is also known as Ct (cycle threshold).

According to the general guidelines described by Bustin et al. (2009) and ENGL (2015), other performance parameters of real-time PCR methods have to comply with the acceptance criteria established for this type of assay, namely PCR efficiency that should

range between 90 and 110%, slope within -3.6 and -3.1 and correlation coefficient (R^2) above 0.98. Fig. 1 shows the amplification curves and the respective calibration curve, as an example run, highlighting its adequate performance in terms of R^2 (0.991), PCR efficiency (101.0%) and slope (-3.298).

Binary model mixtures containing known amounts of *L. albus* flour (50.0%, 10.0%, 5.0%, 1.0%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001%, 0.0005% and 0.0001%, w/w) in rice flour were used for the relative quantification of lupine. The real-time PCR system enabled a dynamic range of 5 orders of magnitude, covering the amplification down to 0.0005% (w/w) (5 mg/kg) of lupine in rice flour, which was within the linear range of the calibration curve, thus being considered as the relative LOD and LOQ (Fig. 2A). This value is comparable to the relative sensitivities reported by Röder et al. (2013) (1 mg/kg), Galan et al. (2010, 2011) (10 mg/kg and 2.5 mg/kg, respectively) and Demmel et al. (2008, 2011, 2012) (0.1-10 mg/kg), all using real-time PCR methods with TaqMan probes. However, it should be highlighted that the reports of Demmel et al. (2008, 2011, 2012) and Galan et al. (2011) targeted mitochondrial DNA sequences (multi-copy genes), which enable achieving higher sensitivities than the nuclear sequences such as the allergen encoding genes. It is also important to refer that the sensitivity reported by Röder et al. (2013) (1 mg/kg) was established based on an estimate of serially diluted lupine DNA and not using model mixtures, including much more interfering compounds and non-target DNA. Accordingly, the highest absolute and relative sensitivity for lupine detection using real-time PCR with TaqMan probes, targeting allergen-encoding sequences, was reached in the present work.

Model construction and validation

For the relative quantification of lupine, two strategies were evaluated using: i) a non-normalised real-time PCR calibration curve and ii) a normalised curve based on the parallel amplification of the target lupine sequence (specific amplification) and a universal eukaryotic region (reference gene) with approximately the same amplification efficiencies. Both calibration curves were constructed and the respective performance parameters were evaluated and compared. Non-normalised Ct values from the amplification of the binary reference mixtures targeting *Lup a 4* gene were plotted against the logarithm of lupine content, providing a calibration curve with a slope of -2.76 ± 0.07 , a R^2 of 0.972 ± 0.002 and a PCR efficiency of $130.2 \pm 4.2\%$. Similarly, to improve the performance parameters of method, a normalised calibration curve was constructed using the ΔCt values from the amplification of the target lupine sequence and a eukaryotic conserved DNA region of nuclear 18S rRNA as endogenous control, considering the following expression:

$$\Delta Ct = Ct_{\text{lupine}} - Ct_{\text{euk}}$$

where Ct_{lupine} and Ct_{euk} are the Ct values for lupine and eukaryotic sequences, respectively. It can be noted that method normalisation leads to a clear improvement of the analytical performance since the parameters of PCR efficiency ($103.3 \pm 2.7\%$), R^2 (0.989 ± 0.001) and slope (-3.24 ± 0.07) were all within the acceptance criteria for this type of assays (Bustin et al., 2009; ENGL, 2015). Fig. 2A represents the normalised and non-normalised calibration curves, each one constructed with mean values of two independent real-time PCR runs ($n=8$).

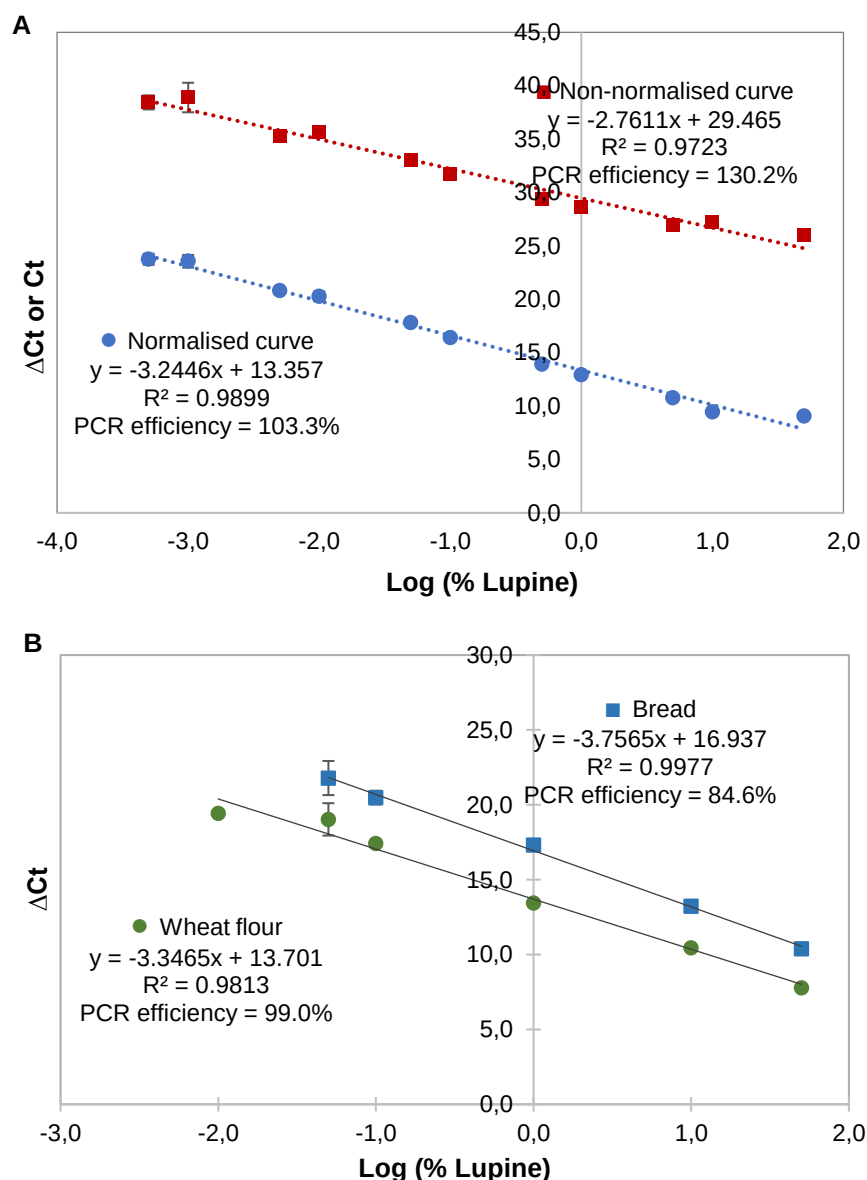


Fig. 2 Calibration curves obtained by real-time PCR targeting the *Lup a 4* encoding gene of lupine. (A) Normalised and non-normalised calibration curves using the binary mixtures of lupine in rice flour (50%, 10%, 5%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, 0.001% and 0.0005% (w/w)) in both models; (B) normalised calibration curves of lupine in wheat flour and breads (50%, 10%, 1%, 0.1%, 0.05% and 0.01% (w/w)). The normalised ΔCt method was performed by the parallel amplification of a eukaryotic gene (18S rRNA) as reference, using data from two independent real-time PCR runs ($n=8$ replicates).

To assess the quantitative performance of both non-normalised and normalised models, a set of blind mixtures containing 0.025, 0.25, 0.8 and 2.5% (w/w) of lupine in rice flour was used. The obtained lupine estimates and comparative analysis with the actual values are presented in Table 2. All the estimates exhibited adequate values of coefficient of variation (CV), expressing the relative standard deviation of results that varied between 7.6% and 21.6%, which demonstrated acceptable precision ($\leq 25\%$) of both non-normalised and normalised methods over the tested dynamic range. In terms of trueness (bias), the estimates using the non-normalised curve exhibited bias between -76.8% and 168.6% (Table 2), which are greatly above the criterion of acceptance of $\pm 25\%$ of the actual value (ENGL, 2015). In opposition, with the normalised calibration curve, the accuracy data ranged from -4.63% to 20.0% for the three highest levels, which are within the acceptable bias. The mixture of 0.025% (w/w) of lupine in rice, revealed a bias higher than $\pm 25\%$, but at these low concentration levels the deviations from the true value are more likely to occur. Therefore, the method can provide acceptable quantitative results in the range of 0.25-2.5% of lupine in rice flour. The obtained results from the method validation and the performance parameters of real-time PCR assays clearly demonstrate that the normalisation improves the reliability and accuracy of the quantitative method. This fact is due to the use of a reference endogenous gene that accounts for possible amplification differences due to inconsistent DNA recovery and quality/degradation among extracts, which might affect PCR efficiency (Villa et al., 2017; Costa, Amaral, Grazina, Oliveira, & Mafra, 2017).

Table 2. Validation results obtained in normalised and non-normalised quantitative real-time PCR systems with model mixtures of lupine in rice flour.

Blind mixture	Actual value (%)	Normalised			Non-normalised		
		Estimated value ^a (%)	CV ^b (%)	Bias ^c (%)	Estimated value ^a (%)	CV ^b (%)	Bias ^c (%)
1	2.50	2.38 $\pm$ 0.18	7.6	-4.63	4.51 $\pm$ 0.41	9.0	80.3
2	0.80	0.96 $\pm$ 0.11	11.66	20.0	2.15 $\pm$ 0.17	8.1	168.6
3	0.25	0.28 $\pm$ 0.03	9.1	13.4	0.40 $\pm$ 0.04	9.1	61.1
4	0.025	0.012 $\pm$ 0.003	21.6	-53.4	0.006 $\pm$ 0.001	18.5	-76.8

^a Mean values $\pm$ standard deviation (SD) (n=8) of two independent runs; ^b CV – coefficient of variation; ^c Bias=((mean estimated value-true value)/true value $\times 100$).

Effect of food matrix and processing

To evaluate the effect of food matrix and processing on the performance of the proposed method, the model mixtures of wheat flour and breads spiked with 50.0%, 10.0%, 1.0%, 0.05%, 0.01% and 0.001% (w/w) of lupine flour were used. Regarding the wheat flour models, the real-time PCR system enabled the amplification down to 0.01% (w/w) (100 mg/kg) of lupine in wheat flour, which can be considered as the relative LOD and LOQ. The normalised calibration curve constructed by plotting the ΔC_t values against the logarithm of

lupine content provided a slope of -3.35, a R^2 of 0.981 and a PCR efficiency of 99.0% (Fig. 2B). On the other hand, the application of the normalised real-time PCR system to model breads enabled the lupine flour amplification down to 0.05% (w/w) (500 mg/kg). The mean values for PCR efficiency, slope and R^2 were 84.6%, -3.76 and 0.998, respectively (Fig. 2B). Comparing both calibration models, a difference of about 3.2 cycles can be noticed between the curve intercepts, which demonstrates a clear delay of the amplification of DNA from bread mixtures in relation to wheat flour.

Food matrix clearly affected the sensitivity of the method since the LOD of the assay using the wheat flour model mixtures was 20-fold higher than the one using rice flour mixtures. Waiblinger, Boernsen, Näumann and Koeppel (2014), who studied the application of a multiplex real-time PCR to detect different allergenic foods, including lupine, in a ring-trial involving several laboratories, reported the importance of matrix effect. The ring-trial results obtained by the analysis of different food matrices (rice cookies, wheat cookies and sauce hollandaise powder) spiked with known amounts of the allergen, showed that the effect of matrix cannot be neglected (Waiblinger et al., 2014). The presence of components such as fats, carbohydrates or other plant metabolites might hamper the efficient DNA extraction from foods, which leads to a decrement on PCR efficiency, subsequently affecting the quantitative results (Costa, Melo, Santos, Oliveira, & Mafra, 2015). Thus, the assessment of different calibration models is fundamental for the adequate development of a quantitative method.

The influence of processing, more specifically baking, seems to have a negative effect on lupine detection demonstrated by the decrease of sensitivity of lupine detection in bread compared with rice or wheat flour models and by PCR efficiency (84.6%) that is slightly out the acceptable range (Bustin et al., 2009; ENGL, 2015). Thermal treatment may alter the structural properties of food components, namely the protein fraction (including allergens), representing one of the major problems for their analysis by immunochemical methods. DNA is more stable to this type of treatment, but high temperatures during long time may negatively affect the PCR performance, depending on the extent of DNA degradation (Costa, 2013; Costa, Amaral, et al., 2017; Costa, Oliveira, & Mafra, 2013).

A validation test to assess the quantitative performance of the two systems as affected by food matrix and processing was also performed with a set of blind mixtures (25%, 2.5% and 0.25%, w/w of lupine flour in wheat flour or breads). Table 3 shows the obtained results, which demonstrate the high precision and accuracy of the two methods since both displayed adequate values of coefficient of variation (CV) and bias within the criterion of acceptance of $\pm 25\%$ of the actual value (ENGL, 2015).

Table 3. Validation results based on the application of the normalised quantitative realtime PCR approach to blind mixtures of lupine flour in wheat flour and in breads.

Samples	Lupine (% w/w)		CV (%) ^b	Bias (%) ^c
	Actual value (%)	Estimated value ^a (%)		
Wheat flour				
A	25	23.5 ± 3.1	13.3	-6.0
B	2.5	3.08 ± 0.25	8.0	23.1
Bread				
C	2.5	2.79 ± 0.18	6.4	11.6
D	0.25	0.258 ± 0.060	23.4	3.2

^a Mean values ± standard deviation (SD) (n=8) of two independent runs; ^b CV – coefficient of variation; ^c Bias=((mean estimated value-true value)/true value×100).

Analysis of commercial samples

In order to evaluate the application of the developed method to real foods, several commercial samples labelled with lupine as an ingredient or at trace levels were tested for its presence. The summarised PCR results, together with the corresponding label information of samples, are presented in Table 4. Based on the qualitative PCR results, it is possible to verify that, from the 14 samples declaring the information “may contain traces of lupine”, only two tested positively for lupine (#2 and #15) by qualitative PCR. These results were further confirmed by real-time PCR, with estimated concentrations <0.25%, suggesting the common practice of the precautionary labelling. The samples declaring “sweet lupine flour” and presenting positive amplification by qualitative PCR were also tested by real-time PCR (#4, #25 and #26). Two samples (#4 and #25) showed low estimates (<0.25%), while in sample #26 the presence of lupine was not confirmed by real-time PCR, though with the universal primers the result was positive (Table4). This finding might be due to the addition of sweet lupine flour as a minor ingredient and to the fact of being highly processed and complex products (cookies and wafers), which makes it very difficult or even disables lupine DNA amplification. Besides, the designation of “sweet lupine” is attributed to domesticated lupine species that contain low levels of bitter-tasting and potentially toxic alkaloids, which in practice refers to the three main species (*L. albus*, *L. luteus* and *L. angustifolius*). Therefore, the very low estimated content or undetectable lupine might be due to the specificity of the probe for *L. albus* species, which can be present at low quantities or even absent in sweet lupine flour. In the 6 samples stating “lupine flour” or “lupine protein” in their labels, lupine was detected, with the exception of sample #23 (Panzerottini), possibly because of being added at minor amounts and being a complex and processed product as previously referred for other samples. Thus, in the remaining 5 samples declaring lupine flour/protein (#11, #12, #17–#19), it was possible to quantify lupine

in concentrations between $4.12 \pm 0.53\%$ and $22.9 \pm 1.0\%$, which seems to be in good agreement with labelled information (Table 4).

Table 4. Results of analysed commercial samples by qualitative PCR and normalised quantitative real-time PCR targeting the Lup a 4 encoding gene of lupine.

Samples	Relevant label information	Qualitative PCR			Real-time PCR	
		EG-F/ EG-R	La4-F2/ La4-R2	EG-F/EG-R (Ct $\pm$ SD) ^a	La4-F2/La4-R2 (Ct $\pm$ SD) ^a	Estimated lupine (%, w/w) (mean $\pm$ SD) ^b
1 Apricot cookies	Without lupine addition	+	-	NA	NA	
2 Cookies	May contain traces of lupine	+	+	15.83 $\pm$ 0.29	37.12 $\pm$ 0.95	<0.25%
3 Cookies	May contain traces of lupine	+	-	NA	NA	
4 Cookies	Sweet lupine flour	+	+	21.43 $\pm$ 4.40	40.53 $\pm$ 3.64	<0.25%
5 Cookies	Sweet lupine flour	+	-	NA	NA	
6 Cookies	May contain traces of lupine	+	-	NA	NA	
7 Cookies	Without lupine addition	+	-	NA	NA	
8 Chocolate cookies	May contain traces of lupine	+	-	NA	NA	
9 Chocolate cookies	May contain traces of lupine	+	-	NA	NA	
10 Fior di Sole cookies	May contain traces of lupine	+	-	NA	NA	
11 Lupine cookies with lemon flavour	Lupine flour (4.9%)	+	+	17.49 $\pm$ 0.05	27.89 $\pm$ 0.21	7.82 $\pm$ 0.57
12 Lupine biscuits	Lupine flour (5.0%)	+	+	17.42 $\pm$ 0.08	27.74 $\pm$ 0.13	8.59 $\pm$ 0.41
13 Plain biscuit	May contain traces of lupine	+	-	NA	NA	
14 Bread	May contain traces of lupine	+	-	NA	NA	
15 Cereal bread	May contain traces of lupine	+	+	17.15 $\pm$ 0.03	37.76 $\pm$ 0.00	<0.25%
16 Cereal rustic bread	May contain traces of lupine	+	-	NA	NA	
17 Crostini	Lupine protein	+	+	19.34 $\pm$ 0.33	30.64 $\pm$ 0.21	4.12 $\pm$ 0.53
18 Flour for bread	Lupine protein	+	+	17.37 $\pm$ 1.23	26.14 $\pm$ 0.06	22.9 $\pm$ 1.0
19 Pan Carré	Lupine protein	+	+	22.13 $\pm$ 0.20	33.15 $\pm$ 0.11	5.43 $\pm$ 0.27
20 Pan gratí	May contain traces of lupine	+	-	NA	NA	
21 Marble cake	May contain traces of lupine	+	-	NA	NA	
22 Cupcake	May contain traces of lupine	+	-	NA	NA	
23 Panzerottini	Lupine protein	+	-	NA	NA	
24 Wafers	May contain traces of lupine	+	-	NA	NA	
25 Chocolate wafers	Sweet lupine flour	+	+/-	15.53 $\pm$ 0.00	37.14 $\pm$ 0.89	<0.25%
26 Cream wafers	Sweet lupine flour	+	+/-	20.76 $\pm$ 0.54	-	

^a Mean cycle threshold (Ct) values $\pm$ standard deviation (SD) (n=8) of two independent runs; ^b Mean percentage (%) values $\pm$ SD (n=8) of two independent runs; (+) Positive amplification; (-) Negative amplification; +/- Doubtful amplification; NA – not applicable.

CONCLUSIONS

The present work proposes a normalised real-time PCR approach using a hydrolysis probe based on the Δ Ct method to detect a sequence of the allergenic PR-10 protein (Lup a 4) encoding gene of *L. albus* in processed foods. The method allowed detecting and quantifying lupine as a potential allergenic food since both the LOD and LOQ were down to 0.0005% (5 mg/kg) of lupine in rice flour. Food matrix and thermal processing clearly affected the sensitivity of the method, which was 20-fold lower in wheat flour model mixtures

than in rice flour. The baking process had also a negative effect on the sensitivity of lupine detection in bread compared with rice or wheat flour models, decreasing also the PCR efficiency to values slightly out of the acceptable range. The developed method was also successfully applied to 26 processed food samples, revealing that 2 out of 14 declaring that may contain traces of lupine were positive for lupine, suggesting the excessive use of the precautionary labelling. From five samples that declared lupine as an ingredient, its content could be effectively estimated in the range 4.12-22.9%.

This study demonstrated that real-time PCR is an accurate and powerful tool for the detection and quantification of lupine as an allergen, which can contribute to a more effective management of allergens by the food industry and the regulatory agencies, thus helping protecting the health of sensitised/allergic consumers.

Acknowledgements

This work was supported by FCT (Fundação para a Ciência e Tecnologia) through projects AlleRiskAssess – POCI-01-0145-FEDER031720, UID/QUI/50006/2013 – POCI/01/0145/FEDER/007265 with financial support from FCT/MEC through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 and by the project NORTE-01-0145-FEDER-000011. Caterina Villa and Joana Costa are grateful to FCT grants (PD/BD/114576/2016 and SFRH/BPD/ 102404/2014, respectively) financed by POPH-QREN (subsidised by FSE and MCTES).

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3.2.2. Immunoreactivity of lupine and soybean allergens in foods as affected by thermal processing

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ABSTRACT

Lupine and soybean are important technological aids for the food industry. However, they are also capable of inducing severe allergic reactions in food-sensitized/allergic individuals. In this context, this work intended to study the combined effects of thermal processing and food matrix on the immunoreactivity of lupine and soybean proteins used as ingredients in bakery and meat products, respectively. For this purpose, the effects of baking, mild oven cooking, and autoclaving on the protein profiles were evaluated, using model mixtures simulating the production of lupine containing breads and soybean-containing cooked hams/sausages, by native- and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting using specific antibodies. The results showed that lupine gamma-conglutin immunoreactivity was slightly decreased in wheat flour mixtures compared to rice, but it was more pronounced in baked products. In meat mixtures, substantial protein fragmentation was noted after autoclaving, with decreased immunoreactivity of soybean trypsin inhibitor. The analysis of 22 commercial products enabled the identification of lupine gamma-conglutin in four bakery samples and soybean trypsin-inhibitor in five sausages, and further differentiated autoclaved from other milder thermally treated products. Generally, the immunoreactivity of target proteins was reduced by all the tested thermal treatments, though at a higher extent after autoclaving, being slightly altered by the food matrix.

Keywords: *Lupinus albus*; *Glycine max*; proteins; food processing; meat products; bakery products.

INTRODUCTION

Legumes, belonging to the Fabaceae family, are consumed worldwide due to their high contents of proteins and essential elements, including vitamins and lipids. However, they play an important role in the scenario of food allergy, with an increased sensitization to legumes among populations from Mediterranean and Asian countries, as well as from western countries, in the last years [1]. Immunoglobulin E (IgE)-binding proteins have been identified in the majority of legumes, being responsible for causing mild skin reactions to life-threatening anaphylactic shocks in sensitized individuals after their ingestion or inhalation. Lupine and soybean are important legumes capable of inducing severe allergic reactions. Lupine has been ranked ninth in the category of “risk allergens” [2] while soybean is one of the eight groups of foods accountable for about 90% of food-allergic reactions [1,3]. Among sensitized children, 6% were diagnosed with soybean allergy while an overall prevalence of 2.1% was found in a study involving several European countries, the USA, and Australia [1,4,5]. Regarding lupine allergy, the prevalence varies, depending on dietary habits and geographical differences [1,6].

The addition of lupine and soybean protein materials as food ingredients has been increasing due to their important nutritional and technological characteristics, such as emulsifier properties, gelling capability, texture improvement, and water-binding property [7]. These materials are classified as protein isolates (PI, >90% protein content), protein concentrates (PC, 65%–90% protein content), or protein flours (PF, 50%–65% protein content), being frequently added to several food products by food manufacturers [8]. Lupine protein materials can be isolated from four *Lupinus* species, namely *L. albus* (white lupine), *L. angustifolius* (blue or narrow-leafed lupine), *L. luteus* (yellow lupine), and *L. mutabilis* (pink or Andean lupine) [9], while soybean protein materials are normally extracted from *Glycine maxima* species. Lupine flour is a common ingredient used in the production of bakery and pastry products, whereas soybean protein isolate (SPI) and soybean protein concentrate (SPC) are often found in meat products, such as cooked hams and sausages [7]. To protect sensitized consumers, the European regulations about food labelling included soybean and lupine as allergenic foods that must be emphasized in the list of ingredients of pre-packaged foods [10]. The enforcement of labelling legislation helps allergic consumers adopting an elimination diet avoid the risk of having adverse immunological reactions caused by the inadvertent ingestion of the offending food.

Most of the food products containing lupine or soybean are submitted to thermal treatments (baking, boiling, roasting, autoclaving, microwave heating, etc.) during their industrial production or cooking at home. It is reported that heat treatment of food proteins may produce different modifications, such as hydrolysis of peptide bonds, denaturation,

aggregation by disulfide and noncovalent bonds, and reactions with other molecules present in the food matrix, namely lipids, sugars, or carbohydrates [11]. Such modifications might affect the integrity of epitopes recognized by specific IgE, influencing protein allergenicity, by either enhancing (exposure of epitopes or generation of new ones) or reducing it (loss of epitopes), possibly leading to an altered capacity to elicit an allergic reaction [3]. Some studies have been developed in order to find strategies to reduce lupine [12–16] and soybean [17–22] allergenicity by the application of thermal processing technologies. The literature shows that heat treatments can affect lupine and soybean allergenicity differently, depending on a wide range of factors that include the duration of the process, the intensity, and the presence of a food matrix. Besides processing, the effect of the food matrix is also of major importance, in particular with regard to the interactions of allergens with other food components that might alter their properties [23].

In this context, the aim of this work was to study the combined effects of thermal processing and the food matrix on the immunoreactivity of lupine and soybean proteins used as ingredients in bakery and meat products, respectively. For this purpose, the effects of baking, oven cooking, and autoclaving were evaluated after their application to model mixtures simulating the production of lupine-containing bread and cooked hams and sausages containing soybean PI (SPI) and soybean PC (SPC), respectively, using immunochemical assays. Commercial samples that stated the presence of lupine or soybean proteins in their list of ingredients or were suspected of containing them were also tested.

MATERIALS AND METHODS

Sampling

Flour of *L. luteus* was provided by the company Germisem (Coimbra, Portugal) and *L. albus* (Biosagesse, France) was acquired at a local market while the seeds of *L. mutabilis* (accession no. 90-0581D) and *L. angustifolius* (accession no. PI180708) were provided by the US National Plant Germplasm System (NPGS) through the National Genetic Resources Program of the US department of Agriculture (NGRP-USDA) (WA, USA). The soybean materials (SPI and SPC) used in this study were provided by FORMULAB (Maia, Portugal). For antibody specificity testing, several food species ($n = 10$) reported to be cross-reactive with lupine and soybean were evaluated, namely wheat, tree nuts (hazelnut and walnut), legumes (peanut, fava bean, bean, chickpea, pea, and lentil), and milk. Twenty-two commercial foods labelled as containing lupine (cookies, cakes, wafers, and bakery products) or soybean (cooked hams and sausages) were acquired at different Portuguese retail markets and are described in Table 1.

Table 1. List of commercial and model samples tested in this work.

Sample	Type of product	Relevant label information
<i>Lupine</i>		
a	Cereal bread	May contain traces of lupine
b	Cookies	May contain traces of lupine
c	Lupine biscuits	Lupine flour
d	Pan Carré (bread)	Lupine protein
e	Crostini (mini toasts)	Lupine protein
f	Lupine cookies with lemon flavour	Lupine flour
g	Flour for bread	Lupine protein
h	Cookies	Sweet lupine flour
i	Chocolate wafers	Sweet lupine flour
j	Bread	Model bread (containing 2.5% of lupine flour)
k	Bread	Model bread (containing 0.25% of lupine flour)
l	Flour for bread	Flour mixture (containing 2.5% of lupine flour)
m	Rice flour	Flour mixture (containing 2.0% of lupine flour)
<i>Soybean</i>		
a	Pork cooked-ham (Fiambre da perna extra)	Flavours (milk, gluten, soybean)
b	Pork cooked-ham (Fiambre da perna extra)	Flavours (contains gluten and soybean)
c	Pork cooked-ham (Fiambre da perna extra)	Flavours (contains soybean products)
d	Turkey ham	Model cooked-ham
e	Turkey breast ham	Soybean protein (may contain traces of milk)
f	Chicken breast ham	May contain traces of milk protein and soybean
g	Turkey breast ham	Milk protein (may contain traces of soybean)
h	Pork sausages (canned)	Soybean protein
i	Pork sausages (canned)	Soybean protein
j	Pork sausages (canned)	Soybean protein
k	Turkey sausage	Model sausages
l	Turkey sausages (bottled)	Soybean protein (may contain traces of milk)
m	Chicken sausages (bottled)	Soybean protein (may contain traces of milk)
n	Turkey Frankfurt sausages (vacuum packed)	May contain traces of soy and milk protein
o	Turkey and chicken sausages (vacuum packed)	No information about soybean (Gluten and milk “free”)

Model Mixtures and Sample Preparation

Several model mixtures spiked with known amounts of lupine or soybean materials were prepared. Lupine flour (*L. albus*), SPI, and SPC were used as lupine and soybean materials, respectively. The exact protein contents of each material were obtained by the Kjeldahl method [24,25]. Regarding lupine reference mixtures, two independent sets of binary model mixtures containing 10.0%, 1.0%, 0.1%, 0.01%, and 0.001% (w/w) of lupine flour in rice or in wheat flours were prepared [25]. The first mixture containing 10% of lupine protein was prepared by adding the required amount of lupine flour to 200 g of rice or wheat flours. The

following mixtures were prepared by successive stepwise additions of the respective matrix flour.

For the preparation of model breads, 180 g of water, 4.5 g of salt, 6 g of baker's yeast, and 3 g of bread improver were added to 300 g of each binary mixture of lupine in wheat flour. Model wheat breads containing 10%–0.001% ($n = 5$, w/w) of lupine flour were prepared as a reference. Two additional model wheat breads containing 2.5% and 0.25% of lupine flour were also prepared to simulate commercial samples (Table 1). Doughs were cooked in a bread machine Moulinex OW6101 (Ecully, France) for 3 h with a maximum temperature of 180 °C for 25 min. After cooling, the breads were cut in the middle to remove slices, which were ground in the laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany).

For the preparation of soybean reference mixtures, two recipes were followed, simulating the production of cooked hams and sausages (Frankfurt type). The preparation of raw hams included minced raw pork meat (1.0 kg), salt (8.0 g), and sugar (4.0 g) while raw sausages were prepared with minced raw pork meat (500 g), salt (20 g), crushed ice (250 g), and pork lard (375 g). The first mixture containing 10% of SPI was prepared by adding 13.7 g of soybean material to 106.3 g of raw ham while the one containing 10% of SPC was prepared by adding 18.5 g of soybean material to 101.5 g of raw sausage, both prepared using a laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). To facilitate homogenization, 10 mL of sterile phosphate-buffered saline solution (136 mM NaCl, 1.4 mM KH_2PO_4 , 8.09 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 2.6 mM KCl, pH 7.2) were added to each mixture. The following mixtures were prepared by successive additions to obtain 10-fold dilutions in the concentration range of 10%–0.001%, similarly to lupine mixtures. Each mixture was divided into 2 portions: The first portions were immediately stored at –20 °C (raw ham and sausage mixtures); the second portions containing SPI were submitted to oven cooking at 68 °C for 5 h to simulate the industrial processing of cooked hams while the second portions containing SPC were autoclaved for 15 min, at 121 °C (autoclaved sausages), to mimic the industrial processing of sausages.

All commercial samples were minced and homogenized with the laboratory knife mill (Grindomix GM200, Retsch, Haan, Germany). All materials and different blender containers were previously treated with a decontamination solution in order to avoid contaminations. All samples were immediately stored at –20 °C until further analysis.

Protein Extraction and Quantification

Prior to protein extraction, two buffers (PBS 0.2 M, pH 7.4 and Tris-HCl 100 mM, pH 8.0) were tested for their suitability to extract good quality protein from all model mixtures and commercial samples. Better protein extracts were obtained with Tris-HCl buffer (100 mM,

pH 8.0), being the elected buffer for extracting proteins in this work. Briefly, 150 mg of sample were weighted and 1.5 mL of Tris-HCl buffer were added, followed by an incubation at 60 °C for 2 h, with stirring at 950 rpm and frequent vortexing to increase the protein yield. After incubation, the mixtures were centrifuged twice at room temperature (9000 g, 30 min). Between centrifugations, the supernatant was collected, and the pellet discarded in order to provide clear supernatants. After extraction, the protein concentration was assessed by UV spectrophotometry on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA), using a Take3 micro-volume plate accessory and the protein280 protocol in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Winooski, VT, USA).

SDS-PAGE and Native-PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5–12%) or native-PAGE (5–12%) gels in discontinuous system were homemade, following the protocols described in the Mini-PROTEAN® Tetra Cell Instruction Manual [26]. All model mixtures and samples were run at 150 V in denaturing and native conditions, using a Mini-PROTEAN® Tetra System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), with 1× Tris/Glycine/SDS (Bio-Rad Laboratories, Inc., Hercules, CA, USA) or 1× Tris/Glycine (Bio-Rad Laboratories, Inc., Hercules, CA, USA) electrophoresis buffer, respectively. Electrophoresis under reducing conditions was carried out by adding 2× Laemmli Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing 50 mM β-mercaptoethanol to each sample in a 1:1 ratio, followed by denaturation for 5 min at 95 °C. For native conditions, Native Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was mixed in a 1:1 ratio with each sample, followed by direct application on native-PAGE gels. Protein quantity loaded into gels ranged from 1 to 15 µg of protein per lane. The proteins were visualized by staining the gels with Coomassie Brilliant Blue G-250 solution or blotted into a nitrocellulose membrane (for further immunoblot analysis). A gel image was collected using a white tray and processed with Image Lab 5.2.1 software (Gel Doc™ EZ Imager, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Precision Plus Protein™ Dual Color Standards (10–250 kDa, Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used as a protein molecular weight reference.

Immunoblotting Analysis

After electrophoresis, the gels were blotted into nitrocellulose membranes 0.2 µm transfer pack (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, Laboratories, Inc., Hercules, CA, USA) with an automatic turbo protocol (2.5 A, up to 25 V, 10 min). To verify the efficiency of the transfer,

membranes were colored with Ponceau S 0.1% solution for 10 min. After acquiring an image of Western blot membranes, they were washed with TBST 1×(pH 7.4, 10 mM Tris, 50 mM NaCl, 0.1% Tween 20) at least 3 times, 10 min each, until the red coloration disappeared. Then, the membranes were blocked with TBST 1× containing 2% of gelatin from cold-water fish skin (Sigma-Aldrich, St Louis, MO, USA) for 1 h at room temperature, with constant and gentle agitation. After blocking, the membranes were washed 3 times for 10 min with TBST 1×. The membranes were incubated overnight at 4 °C with a primary antibody specific to soybean (rabbit anti-trypsin inhibitor antibody, Abcam, Netherlands) or to lupine (rabbit anticonglutin gamma globulin antibody, Agrisera, Sweden) diluted 1/40,000 or 1/50,000 in incubation buffer (TBST 1× with 2% fish gelatin), respectively. The anti-rabbit IgG peroxidase antibody produced in goat (Sigma-Aldrich, St Louis, MO, USA), diluted 1/40,000 in incubation buffer, was applied to the membranes for 1 h at room temperature. Between each incubation with antibodies and at the end, the membranes were washed for 10 min (3×) with TBST 1×. The immunoreactive proteins were revealed with Clarity™ Western ECL (Bio-Rad Laboratories, Hercules, CA, USA) for a few minutes and chemiluminescence was acquired on a ChemiDoc system (the membrane was normally instantly revealed).

RESULTS AND DISCUSSION

In this study, different model mixtures were prepared containing lupine or soybean protein materials. In the case of lupine, two different matrix flours (wheat or rice) were used to prepare model mixtures in order to evaluate the matrix effect. A third set of model mixtures was prepared using the lupine flour in the wheat dough, which was baked to simulate the production of bread (to assess the baking effect). The mixtures for the cooked hams and sausages (both raw and processed) with the addition of SPI and SPC, respectively, were also used. The protein profiles of the raw and processed model mixtures were compared and the immunoreactivity of lupine or soybean proteins was assessed by the use of specific polyclonal antibodies targeting the lupine gamma-conglutin or the soybean trypsin-inhibitor, which were critically selected from the available commercial antibodies. Both proteins, the gamma-conglutin (lupine) or the trypsin-inhibitor (soybean), are classified as allergens by the ALLERGOME database [27]. Similarly, commercial samples containing lupine and/or soybean proteins were also analyzed.

Antibody Specificity

Despite their high specificity, antibodies are raised in biological systems (animals), and as such, they can present some unintended reactivity with none target proteins, meaning that it is very important to determine their experimental specificity. Patients allergic to lupine or soybean often suffer from adverse immunological responses upon contact with proteins

from other food sources, particularly legumes [28]. Peanut, lentils, beans, peas, and chickpeas are all legumes of the same botanical family (Fabaceae or Leguminosae), which explains their protein homology and the presence of highly similar epitopes with subsequent IgE recognition [29]. Therefore, in order to evaluate the specificity of both primary commercial antibodies (anti-conglutin gamma-globulin and anti-soybean trypsin inhibitor) and to avoid any false positive results, all the referred species were tested. Moreover, wheat and rice were also included in this evaluation, since they were used as food matrices in model mixtures, as well as three additional species of lupine commonly used as food ingredients (*L. luteus*, *L. mutabilis*, and *L. angustifolius*).

Regarding the specificity of the commercial anti-lupine antibody, for the same protein quantity of each species (2.5 µg), all four species of lupine presented different immunoreactive bands (Figure 1A, lanes 3–6). Gamma-conglutin was already identified as an allergenic protein of lupine [15,30], although it can show unspecific binding properties [31]. Lupine gamma-conglutin is a lectin-like glycoprotein, with a high affinity for galactose, which can bind N-glycosylated proteins, for example, Fc fragments of IgE, but without eliciting clinical symptoms and thus giving, in some cases, false positive results regarding allergy diagnosis [32]. Different patterns of immunoreactive bands among the four species of lupine might be explained by the different degree of protein glycosylation and the presence of distinct subunits according to the target species. Some bands can be identified as corresponding to the allergenic gamma-like large and small subunits of the protein, namely at 29 and 17 kDa, respectively (Figure 1A, lanes 3, 5, and 6), as already reported by Magni et al. [30]. Two bands also appeared at 43 kDa, which might coincide with the unreduced gamma-conglutin of *L. albus* (Figure 1A, lane 3). Moreover, bands with approximately 50 kDa can be observed in *L. luteus*, *L. mutabilis*, and *L. angustifolius*, already identified as the gamma-conglutin precursor [31]. A strong band at approximately 37 kDa in *L. albus* seems to be the most reactive, also observed by Holden et al. [15] in lupine flour using sera from lupine-allergic patients. So far, allergenic gamma-conglutins have been identified in different *Lupinus* species, namely in *L. albus* (Lup a gamma-conglutin) and *L. angustifolius* (Lup an gamma-conglutin) [27], being in good agreement with the immunorecognition of gamma-conglutins in all the tested species. None of the other tested plant species or milk reacted with anti-lupine antibody, except for peanut, where a band of weak intensity was observed at approximately 20 kDa (Figure 1A, lane 11). This anti-gamma conglutin antibody recognized the target allergenic protein in all tested lupine species, thus allowing their unequivocal identification.

In the case of anti-soybean antibody, only soybean materials (isolate and concentrate) presented a band at 21 kDa, corresponding to the allergenic soybean trypsin inhibitor [27],

thus confirming the specificity of this antibody for unequivocal soybean identification (Figure 1B, lanes 1 and 2).

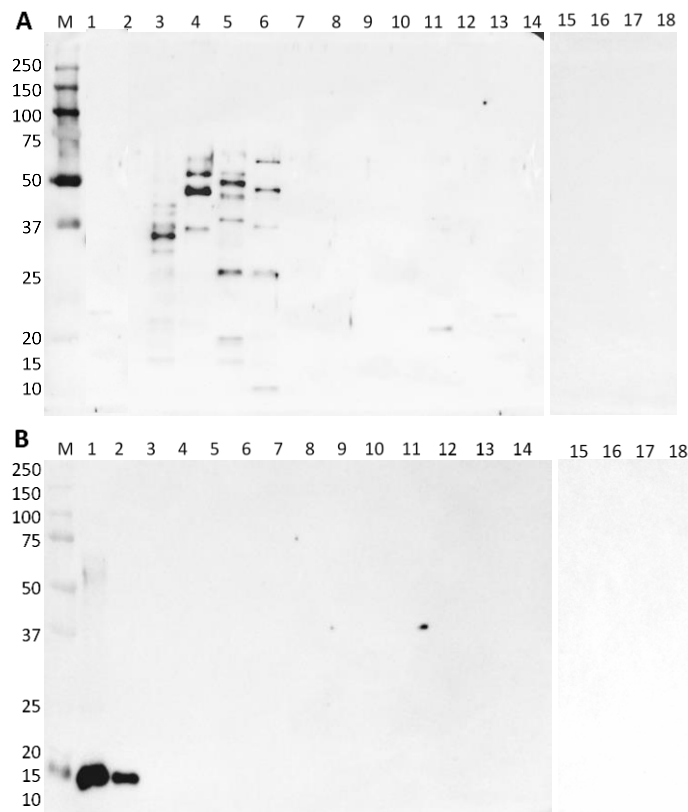


Figure 1. Immunoblot membranes using anti-gamma-conglutinin polyclonal antibody (**A**) and anti-trypsin inhibitor polyclonal antibody (**B**) for specificity test with different plant species. Legend: lane 1, SPI; lane 2, SPC; lane 3, *L. albus*; lane 4, *L. luteus*; lane 5, *L. mutabilis*; lane 6, *L. angustifolius*; lane 7, rice; lane 8, wheat; lane 9, maize; lane 10, milk protein concentrate; lane 11, peanut; lane 12, walnut; lane 13, hazelnut; lane 14, fava bean; lane 15, chickpeas; lane 16, bean; lane 17, lentil; lane 18, pea; lane M, Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Effect of the Food Matrix and Heat Processing

Lupine Proteins

For the evaluation of the effect of the food matrix on lupine proteins, model mixtures containing known amounts of lupine flour in wheat or rice flours were prepared. To simulate the production of bread and evaluate the effect of the heat treatment, dough containing wheat model mixtures were submitted to a baking process for 3 h with a maximum temperature of 180 °C during 25 min. The protein profiles (10 µg loaded in each lane) (Supplementary Material, Figure S1) have different patterns according to the food matrix used in the preparation of model mixtures. SDS-PAGE results show that the protein profile of rice flour presents visible bands only at 75, 45, 37, and 17 kDa (Supplementary Material, Figure S1A, lane 8), while wheat flour exhibits complex band patterns (Supplementary

Material, Figure S1A,B, lane 14). In native conditions, both matrices present bands with high molecular weights (Supplementary Material, Figure S1C,D, lanes 8 and 14). Moreover, the protein profiles of model mixtures are clearly affected by the baking process since most of the protein bands from the breads appear to be almost fully degraded (Supplementary Material, Figure S1B,D).

The immunoreactivity of the lupine material in the model mixtures was then investigated by immunoblotting with the specific anti-conglutin antibody. Membranes contained 0.5 or 2.5 µg of the total protein for raw or processed model mixtures, respectively, are shown in Figure 2. As previously highlighted, the immunoreactive bands of *L. albus* in denaturing conditions are between 30 and 50 kDa, with the most intense at approximately 37 and 43 kDa, with the latter one probably corresponding to the unreduced form of gamma-conglutin (Figure 2A,B, lane 2). When analyzed in native conditions, a single smeared band at 200 kDa can be observed, corresponding to the proteins in their native state (Figure 2C,D, lane 2). Comparing the use of rice and wheat flours as matrices in denaturing conditions, for the same protein quantity in each lane (0.5 µg), it is possible to observe a stronger immunoreactivity in model mixtures prepared with rice than with wheat flour. However, in lupine/wheat flour mixtures, the immunoreactive bands are still visible at 0.1% of lupine (Figure 2A,B, lane 11) while in lupine/rice flour mixtures the signal is visible until 1% of lupine (Figure 2A, lane 4). In native conditions, the band corresponding to lupine gamma-conglutin is visible until 1% or 10% in rice or wheat flours, respectively (Figure 2C, lanes 4 and 9), which might be related with the conformational structure of native protein. Regarding the effect of processing on the immunoreactivity of lupine gamma-conglutin, the results obtained in denaturing conditions show a clear negative effect of heat treatment, mainly at higher lupine proportions, with a reduction of the intensity of target bands, but still with visible signals until 0.1% of lupine in both raw and processed model mixtures (Figure 2B, lanes 11 and 17). In native conditions, the same negative effect is observed, but with immunoreactive bands only in the 10% lupine mixtures (Figure 2D, lanes 9 and 15).

In general, a reduction of lupine immunoreactivity was observed after the baking treatment at 180 °C, with significant effects on the integrity and structure of lupine gamma-conglutin. As already reported by Álvarez-Álvarez et al. [12], autoclaving at 138 °C for at least 20 min induced a reduction in the overall allergenicity of lupine seeds while boiling, microwave, and extrusion cooking did not produce any modification. Holden et al. [15] used *L. albus* seeds in a tofu-like product (Lopino), which was prepared with soaked seeds that were blended, whose filtrate was boiled and pressed. The subsequent IgE-binding capacity of the resultant lupine proteins, assessed using sera from lupine allergic patients, was decreased in heat-treated lupine. In opposition to this, Álvarez-Álvarez et al. [12] verified an important reduction of the IgE-binding capacity of lupine only after prolonged autoclaving.

This fact can be explained by the use of a real food matrix that might modulate the changes caused by the processing, thereby affecting the allergenicity of lupine proteins because the induction of an IgE response depends on their intrinsic properties, as well as the matrix in which they are administered [15,33].

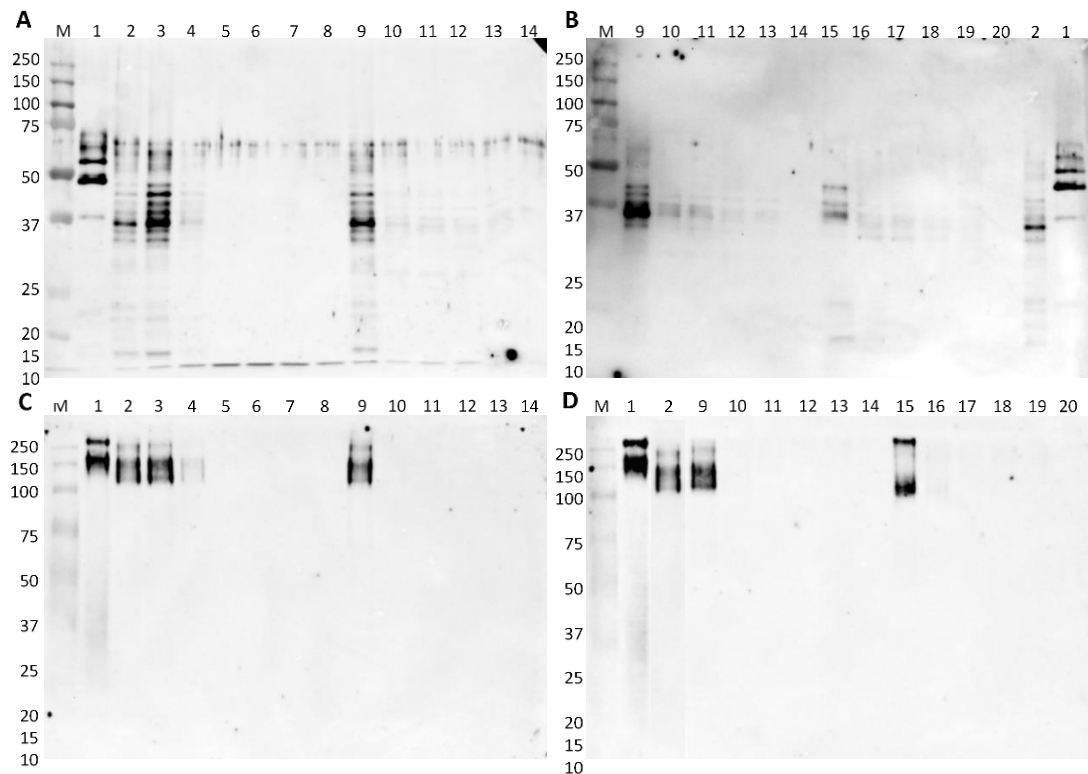


Figure 2. Immunoblot membranes for lupine testing using anti-gamma-conglutinin polyclonal antibody in denaturing (A,B) and native conditions (C,D) comparing rice and wheat flours model mixture (A,C) and wheat flour with model breads (B,D). Legend: lane 1, *L. luteus*; lane 2, *L. albus*; lanes 3–7, 10%, 1%, 0.1%, 0.01%, and 0.001% of *L. albus* in rice flour; lane 8, rice flour (0% lupine flour); lanes 9–13, 10%, 1%, 0.1%, 0.01%, and 0.001% of *L. albus* in wheat flour; lane 14, wheat flour (0% lupine flour) lanes 15–19, 10%, 1%, 0.1%, 0.01%, and 0.001% of *L. albus* in bread; lane 20, wheat bread (0% lupine flour); lane M, Precision Plus Protein Dual Color Standard 10–250 kDa.

Soybean Proteins

The preparation of cooked hams and sausages involves industrial processes that apply distinct heat treatments. In the case of cooked hams, the industrial process includes a slow oven cooking (5 h) using moderate temperature (68 °C) while for sausages the thermal treatment is more severe with the application of high temperatures (121 °C) and pressure for a short period of time (15 min). Model mixtures of pork meat containing known amounts of soybean materials (SPI and SPC) were prepared, simulating both treatments of mild oven cooking and autoclaving. The protein profiles of raw and processed mixtures were then compared by PAGE in denaturing and native conditions. The effect of oven cooking and autoclaving on the protein profiles (15 µg of protein in each lane) of all model mixtures is

clearly shown in Figure S2 (Supplementary Material). In denaturing conditions, most protein bands from processed soybean and pork meat appear to be almost fully degraded, although this effect is more drastic in autoclaved sausages (Supplementary Material, Figure S2B, lanes 21–26) than in cooked hams (Supplementary Material, Figure S2A, lanes 8–13). It is also important to highlight that there are several bands corresponding to high molecular weight proteins (150–250 kDa) in cooked hams, preserving the same profile as in raw hams (Supplementary Material, Figure S2A), suggesting that oven cooking only partially affects the integrity of some proteins. When analyzing the protein profile of processed model mixtures in native conditions, only few bands are visible at the molecular weight around 75 kDa, which might result from the aggregation of smaller proteins (Supplementary Material, Figure S2C,D, lanes 8–13). Like in denaturing conditions, the autoclaving process (Supplementary Material, Figure S2D, lanes 21–26) seems to be more drastic in altering the conformation and size of the proteins.

The immunoblotting results of model hams and sausages with soybean materials, using membranes containing 1.5 or 15 µg of total protein for raw or processed model mixtures, respectively, incubated with a polyclonal antibody against soybean trypsin-inhibitor, are presented in Figure 3. In denaturing conditions, intense bands of 21 kDa identifying the soybean trypsin inhibitor (target protein) are recognized in SPI (Figure 3A, lane 1), and in the model mixtures of 10% of soybean material in raw (Figure 3A, lane 7) and cooked ham (Figure 3A, lane 13). A faint band is also identified in the 1% SPI mixture in cooked ham run in denaturing conditions (Figure 3A, lane 12). In the model sausages (Figure 3B), the results are similar to raw hams, with the identification of strong immunoreactivity in the SPC and 10% SPC mixtures of both raw and autoclaved sausages (Figure 3B, lanes 14, 20, and 26). A very weak signal is identified in the autoclaved 1% SPC mixture (Figure 3B, lane 25), which is due to the 10-fold higher concentration of this sample compared to its raw counterpart. The results in native conditions are in good agreement with the denaturing conditions, although the pattern of the soybean trypsin inhibitor seems slightly different, probably due to the protein total charge (Figure 3C,D). The bands at 17–18 kDa of the soybean trypsin inhibitor in cooked hams and autoclaved sausages (Figure 3C,D, lane 26) might result from partial degradation during processing. There are also some very weak bands at higher molecular weights (25 and 40 kDa) in processed mixtures (Figure 3C,D, lanes 13 and 26), a fact that might be justified by the formation of reactive aggregates during heat treatments. In native conditions, trypsin inhibitor may undergo conformational alterations at mild temperatures, leading to a molten globule structure (structure that preserves partial spatial conformation, native-like secondary structure) [34]. The formation of such globular structures during thermal processing can expose some hidden epitopes or create new allergenic determinants, increasing protein immunoreactivity.

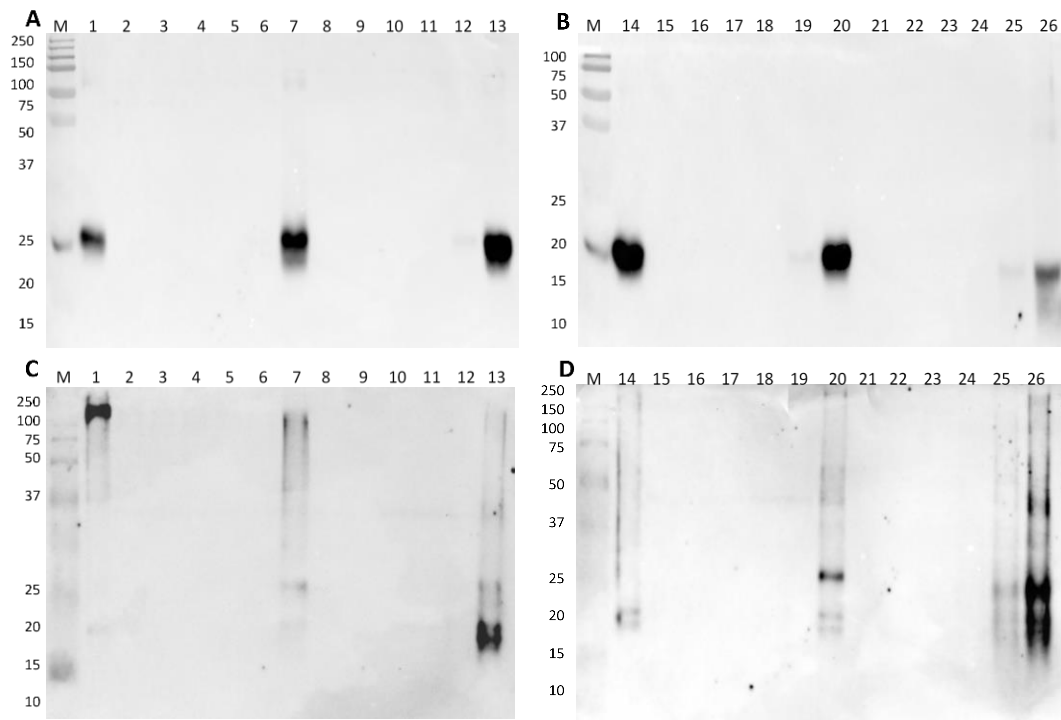


Figure 3. Immunoblot membranes for soybean testing using anti-trypsin inhibitor polyclonal antibody in denaturing (**A,B**) and native conditions (**C,D**) with model mixtures simulating raw and cooked hams (**A** and **C**) and raw and autoclaved sausages (**B** and **D**). Legend: lane 1, SPI; lane 2, raw pork ham (0% SPI); lanes 3–7, 0.001%, 0.01%, 0.1%, 1%, and 10% of SPI in raw pork ham; lane 8, cooked-pork ham (0% SPI); lanes 9–13, 0.001%, 0.01%, 0.1%, 1%, and 10% of SPI in cooked pork ham; lane 14, SPC; lane 15, raw pork sausage (0% SPC); lanes 16–20, 0.001%, 0.01%, 0.1%, 1%, and 10% of SPC in raw pork sausage; lane 21, autoclaved pork sausage 0% SPC; lanes 22–26, 0.001%, 0.01%, 0.1%, 1%, and 10% of SPC in autoclaved pork sausage; lane M, Precision Plus Protein Dual Color Standard 10–250 kDa.

Analysis of Commercial Samples

Lupine-Containing Products

A set of commercial samples containing lupine as an ingredient, such as bakery and pastry products, were analyzed by SDS-PAGE in denaturing conditions, native-PAGE, and immunoblotting to assess the molecular structure and immunoreactivity of lupine proteins. The protein profiles of the analyzed food samples are presented in Figure S3 (Supplementary Material), together with the four main *Lupinus* species and relevant flours frequently used as ingredients of those foods for comparative purposes. Using the same amount of proteins (10 µg per lane), the highly processed samples like biscuits, cookies, and breads exhibit fragmented protein patterns, including mostly faint/smeared bands because of being submitted to harsh processing conditions (Supplementary Material, Figure S3A,B, lanes a–e, h–k). Contrarily, the flour samples for making bread present more complex protein patterns (samples #g, #i, and #m). Cookies and breads showed generally

similar patterns due to the presence of wheat flour proteins, with more intense bands at 50 and 70 kDa (samples #c, #f, #h, #j, and #k). In native conditions, most of the samples present smeared bands or no bands, pointing out to protein fragmentation owing to severe processing, while the few bands at high molecular weight might be aggregates formed during baking (Supplementary Material, Figure S3C,D).

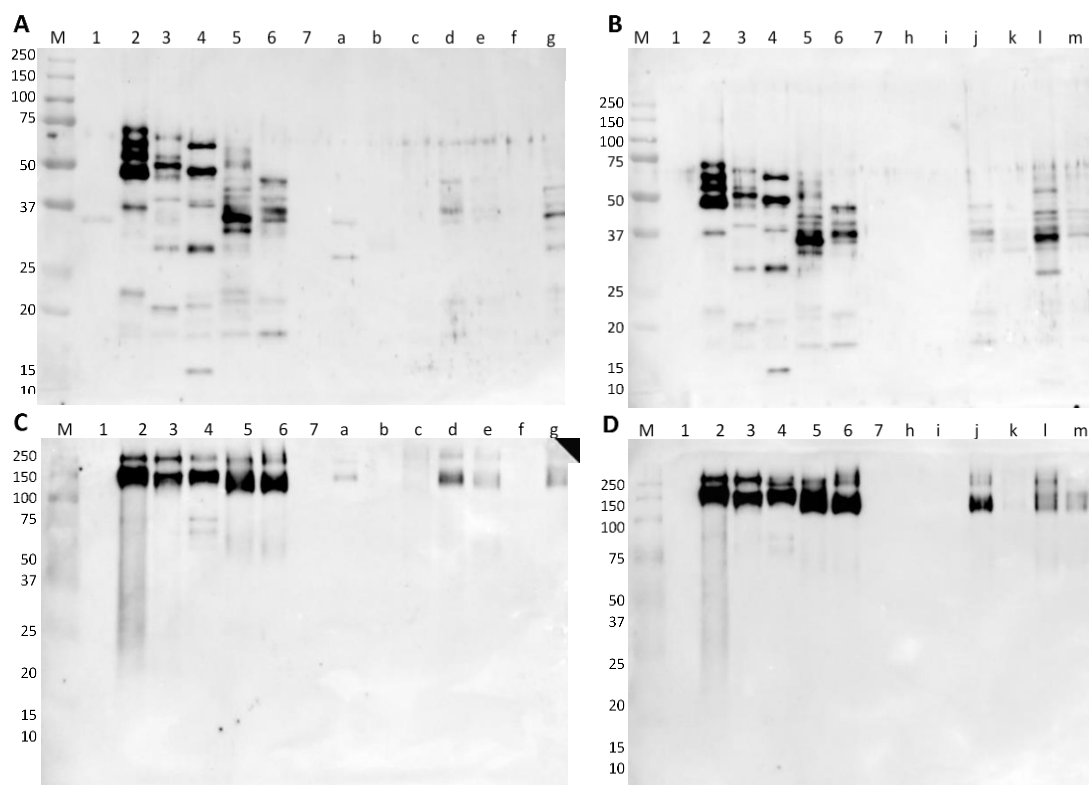


Figure 4. Immunoblot membranes for lupine testing using anti-gamma-conglutin polyclonal antibody in denaturing (A,B) and native conditions (C,D) with commercial and in-house-made samples. Legend: lane 1, SPI; lane 2, *L. luteus*; lane 3, *L. mutabilis*; lane 4, *L. angustifolius*; lane 5, *L. albus*; lane 6, 10% of *L. albus* in bread; lane 7, wheat bread (0% lupine flour); lane a, cereal bread; lane b, cookies; lane c, lupine biscuits; lane d, Pan Carré; lane e, “crostini”; lane f, lupine cookies with lemon flavor; lane g, flour for bread; lane h, cookies; lane i, chocolate wafers; lane j, model bread containing 2.5% of lupine flour; model bread containing 0.25% of lupine flour; lane l, flour mixture containing 2.5% of lupine in wheat; lane m, flour mixture containing 2.0% of lupine in rice; lane M, Precision Plus Protein Dual Color Standard 10–250 kDa.

From the immunoblotting results (5 µg of protein per lane), it was possible to identify immunoreactive bands of gamma-conglutin in four commercial samples, namely in a cereal bread, Pan Carré, “crostini”, and flour for bread, both in native and denaturing conditions (Figure 4A,C, samples #a, #d, #e, and #g). The two bands, at approximately 37 and 27 kDa in sample #a, seem to be different from the bands of samples #d, #e, and #g, which exhibit patterns close to *L. albus*. These bands could correspond to *L. mutabilis* or *L. angustifolius*, highlighting the potential use of these two lupine species in processed foods. Sample #a stated “may contain traces of lupine” in its label while the other three mentioned lupine

protein, which is in good agreement with the obtained results. Samples that declared lupine flour as an ingredient, but without exhibiting any reactivity, were probably submitted to treatments that were more aggressive or have undetectable lupine proteins by the antibody. The model wheat breads containing 2.5% and 0.25% of lupine, as well as the flour mixtures with lupine, presented immunoreactive bands corresponding to the protein pattern of *L. albus*, with the raw wheat flour mixture being the most reactive in denaturing conditions (Figure 4B, sample #l). Contrarily, in native conditions, the most reactive bands are observed in bread (Figure 3D, sample #j). In these conditions, the proteins might form aggregates with increased immunoreactivity, probably as result of the formation of new conformational epitopes [35].

Soybean-Containing Products

Different commercial samples of cooked hams and sausages made of pork or poultry meats were analyzed by PAGE, both in denaturing and native conditions (Supplementary Material, Figure S4). In general, the protein profiles of cooked hams are clearer and with well-defined molecular weight bands (Supplementary Material, Figure S4A,C) than the ones of autoclaved sausages (Supplementary Material, Figure S4B,D), which might be due to the differences in the thermal treatment. In autoclaved sausages (canned), the high temperature in combination with high pressure might have contributed to form aggregated macromolecules with very high molecular weight that can explain the intense smeared bands at 150–250 kDa in the pork sausage samples (Supplementary Material, Figure S4B,D, lanes 13, h, i, and j). In the bottled poultry sausages (autoclaved), the intense smeared bands are also visible, though at lower molecular weights (Supplementary Material, Figure S4B,D, lanes k–l). The vacuum-packed sausages (Supplementary Material, Figure S4B,D, lanes n and o) have been subjected to a boiling treatment, much softer than autoclaving, presenting protein profiles that are close and consistent with the profiles of poultry cooked hams (Supplementary Material, Figure S4B,D, lanes e–g). Like in the case of autoclaved model mixtures, the protein profiles of commercial autoclaved samples in native conditions (Supplementary Material, Figure S4C,D) are smeared bands, denoting protein fragmentation. Generally, these results highlight that the protein structure was more significantly affected by the severe thermal processing of autoclaving (Supplementary Material, Figure S4D) than by soft cooking/boiling (Supplementary Material, Figure S4C).

The commercial samples of cooked hams and sausages were also tested by immunoblotting, targeting the soybean allergen trypsin inhibitor (Gly m TI). In both denaturing and native conditions, no immunoreactivity to soybean can be observed for all

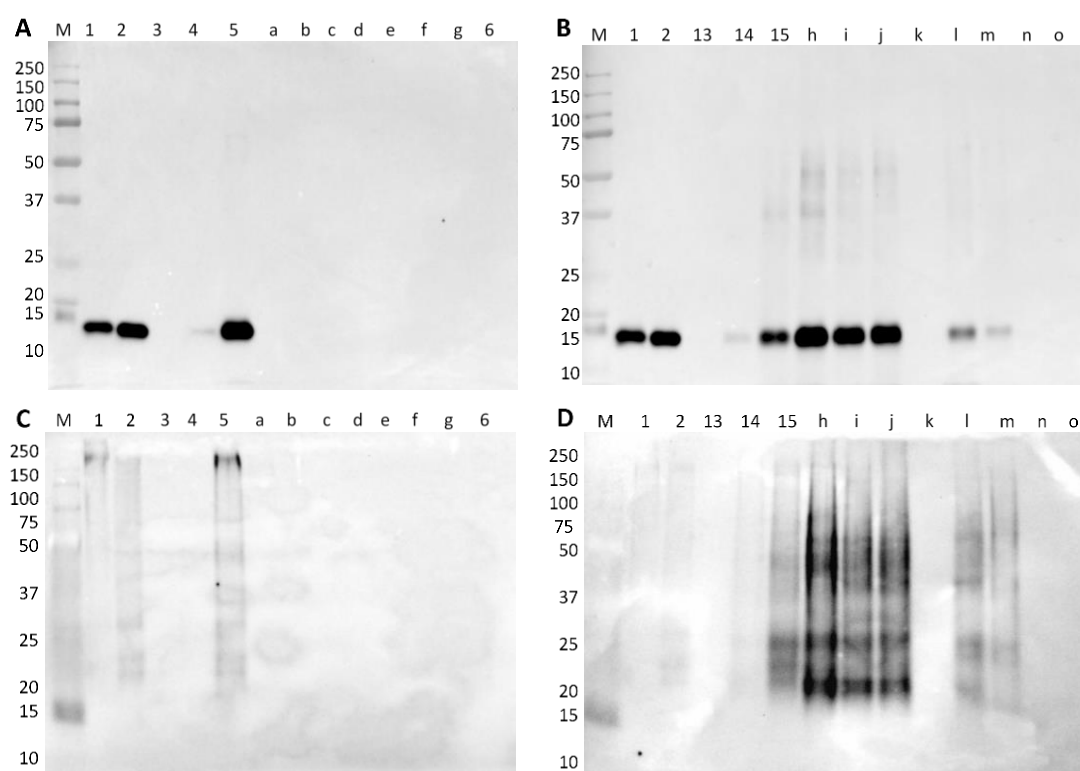


Figure 5. Immunoblot membranes for soybean testing using anti-trypsin inhibitor polyclonal antibody in denaturing (**A,B**) and native conditions (**C,D**) with hams (**A,C**) and sausages (**B,D**) commercial and in-house-made samples. Legend: lane 1, SPI; lane 2, SPC; lane 3, model pork cooked ham (0% of SPI) (negative control); lanes 4,5, pork cooked ham with 1% and 10% of SPI (positive control); lanes a–c, commercial pork cooked hams; lane d, model turkey cooked ham (0% of SPI); lane e, commercial turkey cooked ham; lane f, commercial chicken cooked ham; lane g, commercial turkey cooked ham; lane 6, milk protein concentrate; lane 13, model pork sausage (0% of SPC); lanes 14,15 model autoclaved pork sausage with 1% and 10% of SPC; lanes h–j, commercial pork sausages; lane k, model turkey sausage (0% of SPC); lane l, commercial turkey sausages; lane m, commercial chicken sausages; lane n, commercial turkey sausages; lane o, commercial turkey and chicken sausages; lane M, Precision Plus Protein Dual Color Standard 10–250 kDa.

cooked-ham samples (pork and turkey/chicken) (Figure 5A,C). All these samples were labelled as “fiambre da perna extra”, which according to the Portuguese Standard [36] are products that cannot contain proteins from vegetable origin. Thus, these samples were not expected to contain soybean proteins (at least considering the qualitative result in this type of immunoassay). With respect to Regulation (EU) No 1169/2011 [10] that establishes the mandatory labelling of soybean and products thereof, among other potentially allergenic foods (including soybean), the statement of “may contain or contain soybean” (Table 1) suggests the practice of precautionary labelling since soybean was not detected. From the seven samples of commercial sausages, five of them presented strong immunoreactivity to soybean trypsin inhibitor protein (around 21 kDa) (Figure 5B). In the canned samples (#h, #i, and #j), the amount of soybean protein seems to be higher than 10%, considering the band intensities when compared with the respective model mixture of 10% SPC in

autoclaved pork sausage (Figure 5B, lane 15). The two bottled samples, #l and #m (Figure 5B, lanes l and m), present bands with low intensities, though higher than the 1% SPC model mixture (Figure 5B, lane 14), while in vacuum-packed samples soybean was not detected. These findings are consistent with the estimated higher amounts of soybean material in the canned sausage samples than in bottled ones, suggesting that the lower cost canned products contain more soybean probably for cost reduction [37]. According to Portuguese Standards defined for the characteristics of Frankfurt-type [38] and raw sausages [39], a maximum addition of 5% of vegetable proteins is recommended. Therefore, to verify if sausage samples are according to that recommendation, a quantitative analysis should be performed to determine the amount of soybean protein [37]. Nevertheless, they are all in good agreement with Regulation (EU) No 1169/2011 [10], regarding the labelling of soybean ingredients (Table 1). The immunoblot results obtained in native conditions (Figure 5C,D) confirmed all data obtained in denaturing conditions (Figure 5A,B).

CONCLUSIONS

In summary, with this work, it was possible to evaluate the effect of food processing on the immunoreactivity of lupine and soybean proteins used as technological ingredients in food products. All the tested thermal treatments, namely baking, mild oven cooking, and autoclaving, were able to reduce the immunoreactivity of target proteins, although at greater extension in the case of autoclaved sausages compared to cooked hams. The food matrix was also proven to affect the immunoreactivity of allergenic proteins since lupine/rice flour mixtures presented more intense bands compared to bands of lupine in wheat, suggesting the interaction of lupine proteins with wheat molecules, which can decrease IgE binding. Therefore, the importance of the use of model mixtures to simulate, as much as possible, the processing and matrix of a real food was clearly demonstrated. More studies about the effect of processing in food allergens are needed, mainly towards the development of strategies able to reduce protein allergenicity.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, SDS- and native-PAGE containing protein profiles of model mixtures prepared with soybean or lupine technological materials and of commercial foods are presented as in the supplementary material section (Figures S1–S4). Figure S1: SDS-PAGE run in denaturing (A, B) and PAGE native conditions (C, D) comparing model flour mixtures of lupine in rice and lupine in wheat flours (A, C) and model breads with lupine in wheat flour (B, D), Figure S2: SDS-PAGE gels in denaturing (A and B) and PAGE native (C and D) conditions comparing model mixtures simulating pork hams (A and C) and pork sausages (B and D),

Figure S3: SDS-PAGE run in denaturing (A, B) and PAGE in native conditions (C, D), Figure S4: SDS-PAGE gels run in denaturing (A and B) and PAGE native (C and D) conditions with commercial samples of cooked-hams (A and C) and sausages (B and D).

Author Contributions: Conceptualization, I.M. and J.C.; methodology, C.V. and M.B.M.V.M.; validation, C.V. and M.B.M.V.M.; formal analysis, C.V., M.B.M.V.M. and J.C.; investigation, J.C. and I.M.; writing - original draft preparation, C.V.; writing - review and editing, I.M. and J.C.; supervision, J.C. and I.M.; project administration, I.M.; funding acquisition, I.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Fundação para a Ciência e Tecnologia under the Partnership Agreement UIDB 50006/2020 and by the projects AlleRiskAssess—PTDC/BAA-AGR/31720/2017 and NORTE-01-0145- FEDER-00001.

Acknowledgments: Caterina Villa is grateful to FCT grant (PD/BD/114576/2016) financed by POPH-QREN (subsidized by FSE and MCTES). The authors also acknowledge the US National Plant Germplasm System (NPGS) through the National Genetic Resources Program of the US department of Agriculture (NGRP-USDA) (WA, USA) for gently providing vouches seeds of *L. mutabilis* (accession no. 90-0581D) and *L. angustifolius* (accession no. PI180708) and FORMULAB (Maia, Portugal) for providing the soybean protein materials (SPI and SPC).

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Materials

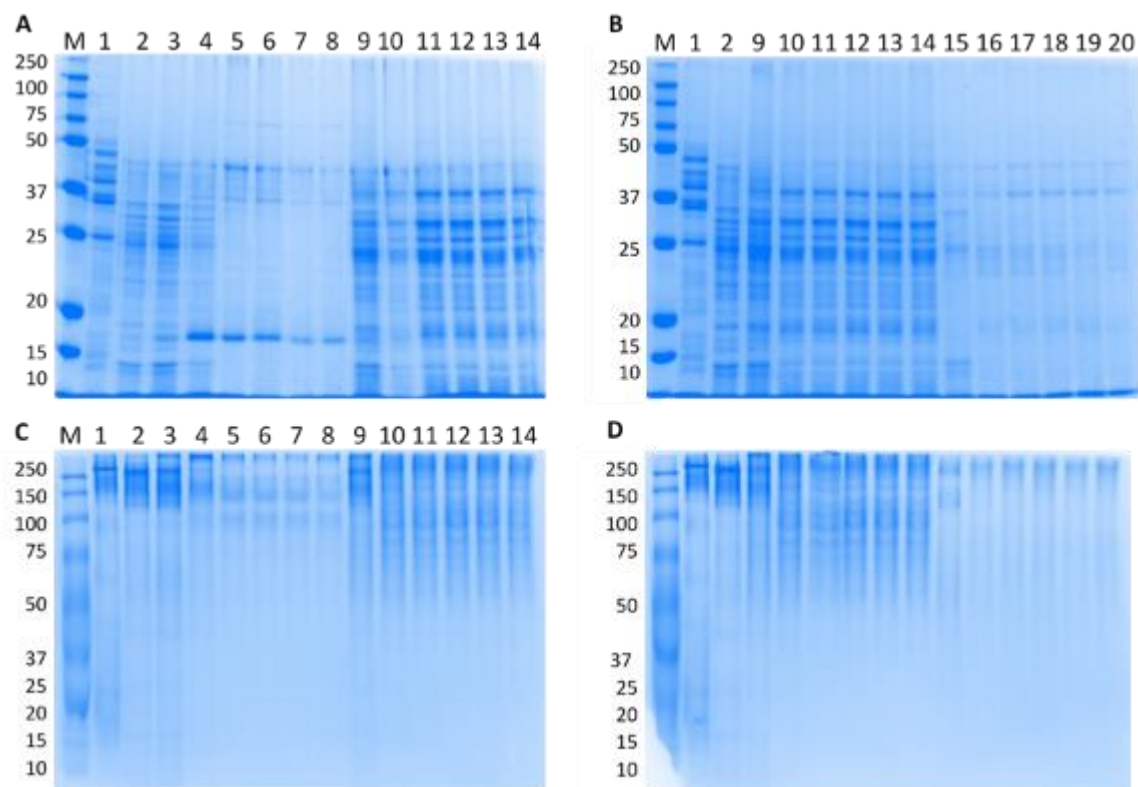


Figure S1. SDS-PAGE run in denaturing (A, B) and PAGE native conditions (C, D) comparing model flour mixtures of lupine in rice and lupine in wheat flours (A, C) and model breads with lupine in wheat flour (B, D). Legend: lane 1, *L. luteus*; lane 2, *L. albus*; lane 3-7, 10%, 1%, 0.1%, 0.01% and 0.001% of *L. albus* in rice flour; lane 8, rice flour; lanes 9-13, 10%, 1%, 0.1%, 0.01% and 0.001% of *L. albus* in wheat flour; lane 14, wheat flour; lanes 15-19, 10%, 1%, 0.1%, 0.01% and 0.001% of *L. albus* in wheat bread; lane 20, wheat bread; lane M, Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

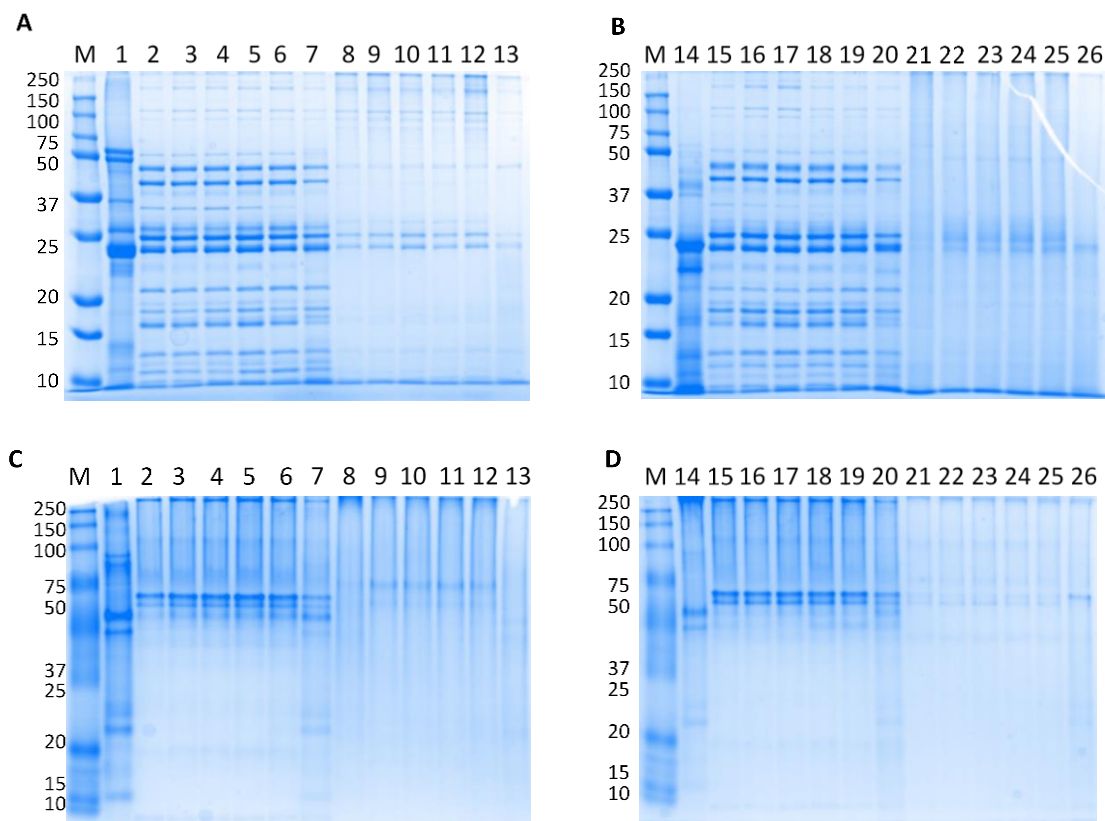


Figure S2. SDS-PAGE gels in denaturing (A and B) and PAGE native (C and D) conditions comparing model mixtures simulating pork hams (A and C) and pork sausage (B and D). Legend: lane 1, SPI; lane 2, raw pork ham; lanes 3-7, 0.001%, 0.01%, 0.1% 1% and 10% of SPI in raw pork ham; lane 8, cooked-pork ham; lanes 9-13, 0.001% 0.01%, 0.1%, 1% and 10% of SPI in cooked-pork ham; lane 14, SPC; lane 15, raw por sausage; lanes 16-19, 0.001%, 0.01%, 0.1%, 1% and 10% of SPC in raw pork sausage lane 20, autoclaved pork sausage; lanes 21-26, 0.001%, 0.01%, 0.1%, 1% and 10% o SPC in pork sausage (autoclaved); M, Precision Plus Protein Dual Color Standard 10-25 kDa (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

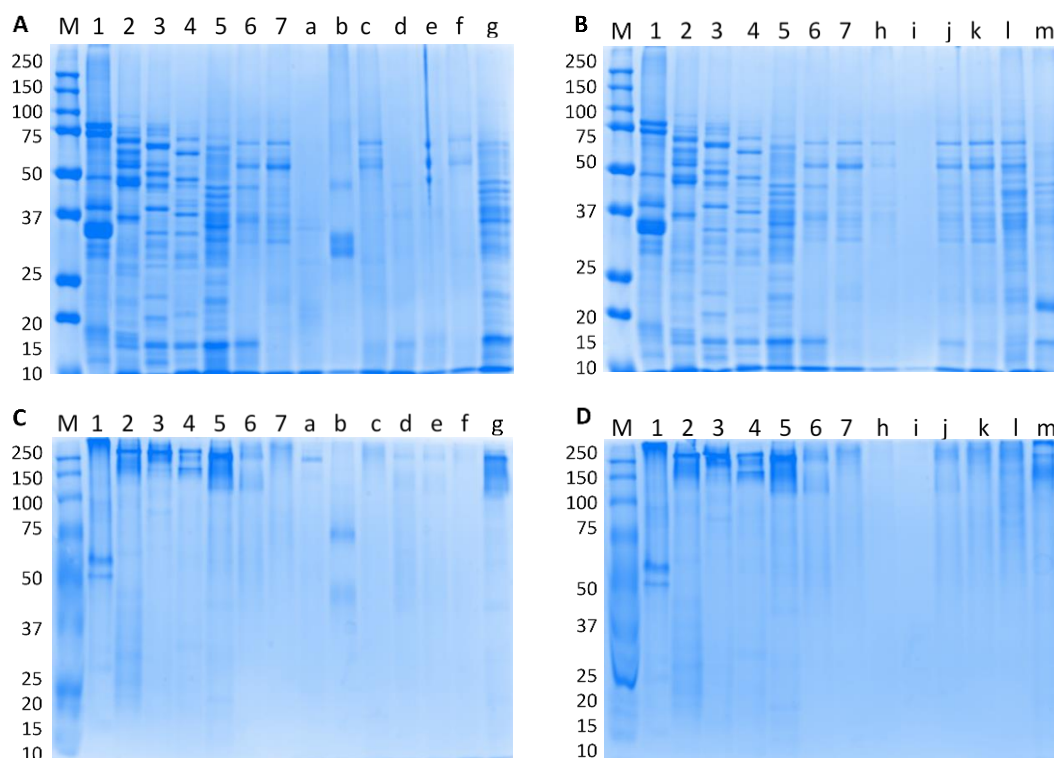


Figure S3. SDS-PAGE run in denaturing (A, B) and PAGE in native conditions (C, D) with commercial samples. Legend: lane 1, SPI; lane 2, *L. luteus*; lane 3, *L. mutabilis*; lane 4, *L. angustifolius*; lane 5, *L. albus*; lane 6, 10% of *L. albus* in bread; lane 7, wheat bread; lane a, cereal bread; lane b, cookies; lane c, lupine biscuits; lane d, Pan Carré; lane e, “crostini”; lane f, lupine cookies with lemon flavour; lane g, flour for bread; lane h, cookies; lane i, chocolate wafers; lane j, model bread containing 2.5% of lupine flour; model bread containing 0.25% of lupine flour; lane l, flour mixture containing 2.5% of lupine in wheat; lane m, flour mixture containing 2.0% of lupine in rice; lane M, Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

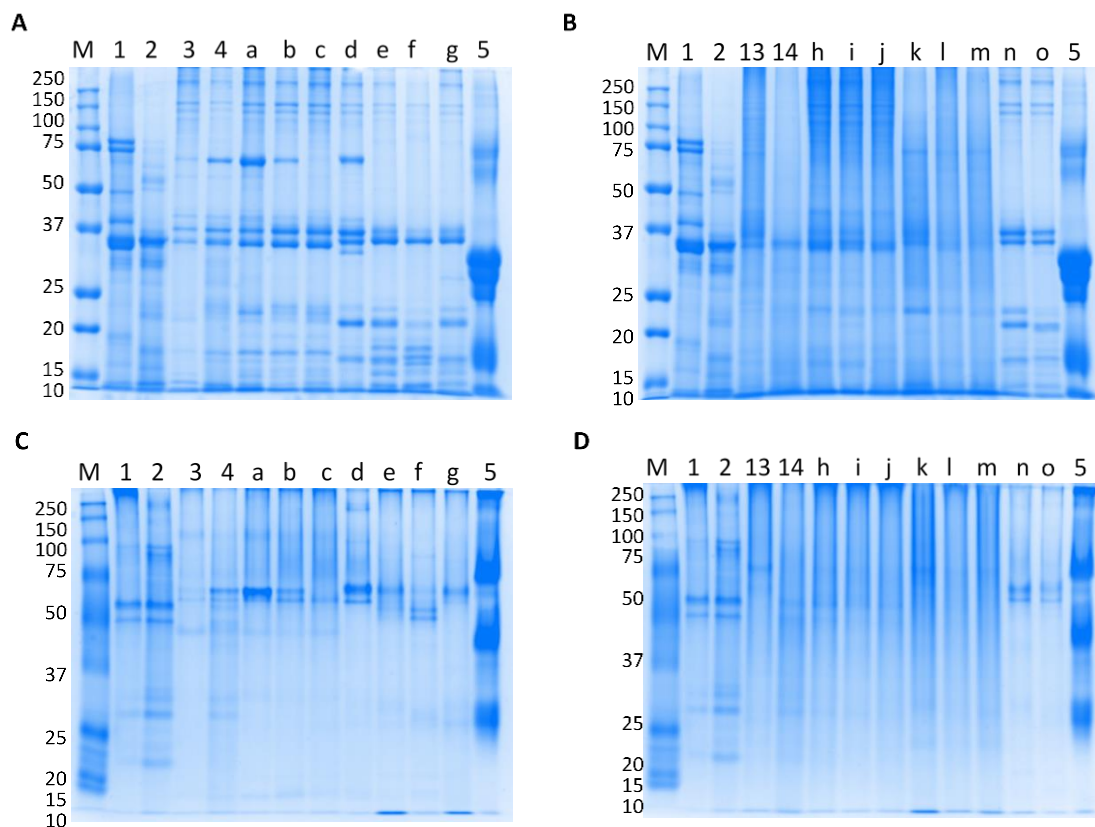


Figure S4. SDS-PAGE gels run in denaturing (A and B) and PAGE native (C and D) conditions with commercial samples of cooked-hams (A and C) and sausages (B and D). Legend: lane 1, SPI; lane 2, SPC; lane 3, cooked-pork ham; lane 4, cooked-pork ham with 10% of SPI; lanes a-c, commercial cooked-pork hams; lane d, cooked-turkey ham; lane e, commercial cooked-turkey ham; lane f, commercial cooked-chicken ham; lane g, commercial cooked-turkey ham; lane 5, milk protein concentrate; lane 13, autoclaved pork sausage; lane 14, 10% of SPC in autoclaved pork sausage; lanes h-j, commercial pork sausages; lane k, autoclaved turkey sausage; lane l, commercial turkey sausages; lane m, commercial chicken sausages; lane n, commercial turkey sausages; lane o, commercial turkey and chicken sausages; lane M, Precision Plus Protein Dual Color Standard 10-250 kDa (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

CHAPTER 4. Final discussion

FINAL DISCUSSION

Currently, there is a global and constant concern about food allergies. The mechanisms of allergic sensitisation are not fully understood and an effective treatment for food allergy is still far to be found. The food industry, the regulatory agencies, the policy makers, the caretakers, the clinicians and the researchers are making efforts in order to find strategies to protect sensitised individuals and to improve their quality of life. At a global scale, the food legislation within the European Union is one of the most restrictive, demanding the mandatory labelling of the largest number of potentially allergenic foods. Since unintended cross-contamination during food production is possible to occur, the use of precautionary labelling is widely applied, thus restricting/diminishing the options of sensitised/allergic consumers, when selecting prepackaged processed foods. Moreover, there is a generalised lack of official methods and reference materials, which are both fundamental to harmonise food allergen control. Besides the prevention of food allergies through labelling, at the clinical level, there are currently some desensitisation treatments, but not enough to provide total tolerance to the offending food. At the food processing level, some strategies, including the use of specific food treatments, seem to have promising results in the reduction of the allergenicity of certain foods. Nevertheless, more studies combining the effects of conventional and novel food processing technologies are still required, as well as knowledge regarding the influence of food matrix and digestion on the allergenic potential of foods.

In this context, the present work was focused on two main research studies: (i) the development of highly sensitive and specific analytical methods to detect and quantify milk and lupine as hidden allergens in processed foodstuffs and (ii) the evaluation of the impact of different heat treatments and *in vitro* digestion on the structural and immunogenic properties of selected allergenic proteins.

4.1. Development of real-time PCR methodologies for food allergen detection

4.1.1. The importance of using model mixtures in allergen analysis

Presently, DNA-based methods relying on PCR amplification are considered suitable tools for food allergen analysis, presenting wide application to most of the allergenic foods. Additionally, PCR-based methods are now being implemented, by institutional/governmental laboratories (e.g. Germany, Japan) for food control, as standard methods for food allergen quantification, thus reinforcing their importance for routine analysis [1]. Different PCR-based methods have been recently proposed for lupine detection, among other food allergens. However, their application to milk detection is still limited. Although

most of them rely on real-time PCR, they are all based on rough estimations of the food allergens, such as the lupine content as an ingredient, lacking suitable quantitative models. To fill this gap, in this work, model mixtures were prepared and used as standards for the development of calibration models. Considering the lack of reference materials for milk and lupine detection, the preparation of model mixtures simulating the addition of the target allergenic food in common and highly consumed food matrices, namely meat and bakery products for milk and lupine detection, respectively, was essential. In the case of meat products such as cooked-hams and sausages, they both contain several ingredients, namely salt, sugar, fat and water, which can greatly affect the yield and purity of DNA extracts and, consequently, decrease the PCR efficiency due to the eventual presence of inhibitor compounds. This fact can lead to unaccurate quantitative analysis. Similarly, the presence of components such as fats, carbohydrates or other plant metabolites in wheat and rice flours might influence the results. Therefore, the use of model mixtures was proposed in the present work to account the influence of food matrix on the detection of the target allergenic food ingredients. Accordingly, the results obtained in this thesis showed that the correlation of the method for milk detection was slightly worsened in the raw ham model mixtures, probably due to the instability of the raw matrix compared with cooked matrix and with the raw sausage matrix (Section 2.2.2.). Similarly, the sensitivity of lupine detection was 20-fold lower using wheat matrix compared to rice flour, demonstrating that the effect of food matrix cannot be neglected in the development of this type of analysis (Section 3.2.1.).

Guaranteeing the absence of any potential cross-contamination during the preparation of the referred model mixtures was a difficult task to succeed in both works, due to the highly finely ground flours (lupine) and protein concentrates (milk) used, which could easily spread in the working area. From the point of view of a large-scale preparation, such as in food industry, this fact can be very problematic. Lupine flours, as well as milk protein concentrates and isolates, are frequently used in a wide variety of processed foods due to their technological and nutritional value. The use of such powdered ingredients may lead to a high probability of unintended cross-contaminations during product manufacturing. Thus, foods that are not supposed to contain these allergenic ingredients can pose a great risk to the allergic consumers.

4.1.2. DNA extraction method and target sequence selection

DNA extraction is a critical step in the development of a real-time PCR method since it is fundamental for the elimination of potential inhibitors that could negatively affect the activity of DNA polymerase. Currently, there are several options of commercial DNA extraction kits that are optimised according to the applied biological system. Besides, the

classical CTAB (cetyltrimethylammonium bromide)-based method is still widely used, especially for plant DNA extraction, despite being time-consuming and difficult to perform. Therefore, the choice and optimisation of the DNA extraction method needs to be critically evaluated, considering the food matrix to be analysed. The effective elimination of potential PCR inhibitory components and interfering substances, such as food additives, polysaccharides, proteins, phenolic compounds, and other secondary metabolites should be assured in the extracted DNA. Additionally, the absence of some chemicals used in the DNA extraction, namely detergents and alcohols, should be attested since they might also inhibit or interfere the PCR performance. Even using previously optimised DNA extraction protocols as the commercial kits, the optimisation of some steps during the extraction process are important and has already demonstrated their usefulness in obtaining improved DNA purity and yields. For example, the cell lysis step is optimal at 65 °C during 60 min, while all centrifugations should be performed at 4 °C in order to avoid DNA degradation. Other factor leading to the improvement of the quality of the extracts is the controlled addition of RNase after the cell lysis step to degrade all the present RNA, which can decrease PCR efficiency. Accordingly, the RNase concentration and incubation time must be carefully previously optimised for each food matrix since the suggested conditions according to kit manufactures normally contribute to low yield DNA extracts due to unintended DNA degradation. In the presented studies, two methods were evaluated and used for DNA extraction, namely NucleoSpin Food kit (Macherey-Nagel, Düren, Germany) and Wizard-based method. The later combines the use of in-house prepared lysis buffer with the resin suspension and the silica-based columns of a commercial kit (Wizard Plus Minipreps DNA Purification System, Promega, Madison, WI, USA), which is normally very efficient for extracting DNA from animal matrices, such as meat products [2-4]. However, in the present meat-based model mixtures (cooked-hams and sausages), the NucleoSpin Food kit evidenced a better performance than the Wizard method, particularly in terms of real-time PCR efficiency. The optimised use of RNase with the NucleoSpin Food kit clearly improved real-time PCR amplification. Equally, in the case of lupine model mixtures, the NucleoSpin Food kit combined with the use of RNase provided the best compromise of DNA yield and PCR amplification.

The choice of a proper target sequence is also essential for the success of a real-time PCR assay. Normally, for better sensitivities, multi-copy genes that are present in several organelles of the cell, such as chloroplasts or mitochondria, are the most suitable. However, multi-copy genes are not ideal for accurate quantitative analysis and are more prone to the occurrence of cross-reactivity between closely related species, since they are normally conserved genes, like *rbcL* or *matK*. Therefore, the use of uni-copy genes is advisable when analysing and quantifying closely related species. The allergen-encoding genes are suitable

targets because they are nuclear uni-copy genes, allowing the identification of a sequence that, after its translation process in the cytosol, will originate an allergenic protein [1]. In this work, a sequence of the uni-copy gene encoding the allergenic protein Lup a 4 was considered a suitable target for lupine detection and quantification, providing high sensitivity and specificity. However, the search for an optimal DNA marker to detect bovine milk revealed to be a harsh task. Firstly, the amount of DNA obtained from milk products is much lower than from other products such as lupine flours. However, this drawback was overcome by taking advantage of the high sensitivity of real-time PCR technique. The major problem with the development of a milk detection PCR method was related with the choice of the marker gene, mostly because of their low inter-species variability particularly between cow and other species (turkey, pork or goat) commonly used in meat products. This finding demanded huge efforts in searching for a molecular marker that could provide an adequate compromise of specificity and sensitivity for milk detection.

The NCBI database was used to perform an initial search of all the deposited nucleotidic sequences from *Bos* spp. ($n=480,874$), from which 229,014 sequences were identified as belonging to *Bos taurus*. An extensive literature review was performed regarding the application of specific molecular markers used for milk authentication and/or for milk allergens analysis [5,6]. The most common genes found on the literature were used for refining the initial *in silico* search and to reduce the number of the obtained sequences. From each selected gene, the obtained sequences were evaluated by BLAST, eliminating all sequences with high homology rates ($>90\%$) towards other mammalian species. At the end, 10 promising genes were selected and used for the design of specific primers for cow. Several primers were designed taking into account their specificity as assessed by primer-BLAST software from NCBI. At the end, 21 candidate primer pairs were obtained and analysed in order to find the best combination of specificity/sensitivity by qualitative PCR, including 2 nuclear genes encoding the allergenic proteins β -casein (Bos d 11) and β -lactoglobulin (Bos d 5) and 19 sequences from the mitochondrial genome of cow (multi-copy genes). Despite the expected higher specificity of the nuclear genes as analysed by *in silico* and experimentally, real-time PCR results were not satisfactory, without being able to provide acceptable Ct values to construct a reliable calibration curve. The search for alternative markers demanded an enormous bioinformatic work to identify regions with enough inter-species nucleotide differences among closely related species to cow, such as pork or goat. Despite the encouraging *in silico* data of the majority of the selected sequences from multi-copy genes, the experimental PCR results were often contradictory, revealing high level of cross-reactivity among sequences of common animal species (cow, goat, pork, turkey, chicken). Most of the deposited sequences in NCBI databases that were studied in this work, namely in the 12S rRNA (7 sequences), *cytb* (5 sequences), NADH (3

sequences), *tRNA* (3 sequences) and COI (1 sequence) genes, were specifically chosen for their known sufficient inter-species variability with other species [7]. However, when the designed primers were tested by qualitative PCR, similar amplification levels were obtained with many species, even with those not closely related to cow, namely turkey and chicken. This fact can be due to the lack of corresponding sequences in NCBI, disabling a proper *in silico* analysis of sequence homology and primer specificity. It is important to highlight that the low specificity results of the target regions were reached at high sensitivity testing levels (above 30 cycles of amplification), contrarily to other reports in the literature mainly focusing on the detection of cow species for authentication purposes [8-12].

4.1.3. Achievements from real-time PCR method development

After the evaluation of 21 target sequences (mitochondrial vs nuclear) by PCR, the 12S rRNA region from *Bos domesticus* revealed to be the most specific (only minor reactivity with turkey and goat above 38 cycles of amplification), providing a linear dynamic range of 10–0.05% of MPC in raw and cooked-hams, with acceptable real-time PCR performance parameters (Section 2.2.1.). Using the selected target sequence, the following step was the development of 4 normalised calibration approaches (ΔC_t method) using the two types of model meat mixtures (hams and sausages), raw and processed, to evaluate the effects of food matrix and processing (oven-cooking or autoclaving) in the PCR performance. Sensitivities down to 0.01% (w/w) of MPC in model ham mixtures (raw and cooked) and autoclaved sausages were achieved, while in raw sausage mixtures an even lower value was reached (0.005%, w/w). The normalisation of the method with the use of a reference endogenous gene had a positive effect on the real-time PCR performance parameters, leading to a 5-fold increase in the sensitivity, from 0.05% to 0.01% (100 mg/kg, w/w) in both ham model mixtures, even considering the 38-cycle limit above which the amplification is unreliable due to cross-reactivity with other species. Additionally, it was possible to infer that autoclaving, as being the most severe treatment, had a minor effect on the detection of MPC by real-time PCR, still maintaining an optimal PCR efficiency. The influence of food matrix was demonstrated from the use of the two different meat models since a clear delay in the amplification of DNA from ham mixtures was noted in relation to sausages. Lastly, the developed systems were successfully validated with blind samples, applied to commercial samples, and further compared with an immunochemical assay (commercially available ELISA), obtaining well corroborated results (Section 2.2.2.).

A novel real-time PCR method for the detection/quantification of lupine as hidden allergen in processed foods was also developed in the course of this PhD (Section 3.2.1.). Targeting the Lup a 4 (PR-10 protein) allergen-encoding gene of *Lupinus albus*, this approach was able to reach sensitivities down to 0.0005%, 0.01% and 0.05% (w/w) of lupine

in rice flour, wheat flour and bread, respectively, with adequate real-time PCR performance parameters using the ΔC_t method. Again, it was possible to demonstrate that the normalisation improved the efficiency of the method. Additionally, the effects of food matrix and processing (baking) were also investigated in this work, evidencing a decrease of method sensitivity in wheat flour model mixtures compared to the ones in rice flour (20-fold lower) and in baked breads, and a loss of method performance from the reduction of the PCR efficiency to values slightly out of the acceptable range (90-110%). The application of the proposed real-time PCR method to quantify lupine in commercial samples revealed once more the excessive use of precautionary labelling as also reported in Section 2.2.3. for milk ingredients.

4.1.4. DNA-based methods vs protein-based methods

The majority of the reported works for the detection of food allergens apply protein-based techniques, such as ELISA and MS spectrometry, for the direct identification of target proteins (either allergenic or species marker) [13]. Recently, the use of DNA molecules as indirect markers to detect allergens have increased due to their high thermal stability, an important feature to analyse highly processed foods, but also due to the high reliability associated to the real-time PCR technique.

All the advantages and disadvantages of applying DNA-based techniques as alternatives to protein-based methodologies were already extensively enumerated along this thesis, particularly in the state-of-the-art sections (2.1.1. and 3.1.1.). However, the use of DNA-based methods for food allergen analysis is still a controversial issue. This is mainly due to the indirect identification of the allergenic food without directly identifying the offending protein(s) responsible for the adverse immunological reactions in sensitised individuals. It should be referred that the expression of an allergen is affected by several physiological states of the plant species that are influence by edaphoclimate conditions. Besides, the allergenicity of proteins might be altered by food processing and food matrix, from which other compounds might interact with allergens, changing their physical/chemical structure. These factors may lead to an incorrect correlation between DNA and the allergenic protein [1]. Even so, during the last years, interesting and promising results have been obtained using quantitative real-time PCR methodologies for allergen analysis, fact that is widely demonstrated in this thesis. The developed real-time PCR approaches showed acceptable analytical performance, providing high specificity and sensitivity for lupine detection and even for milk derived products. It is known that milk is predominantly composed of proteins, fat and carbohydrates, having DNA from somatic cells at minute amounts. Moreover, the comparative study of real-time PCR results with data from an ELISA kit proved that both techniques are in good agreement. As far as we know, herein, it is presented the first

successful method able to detect and quantify milk as an allergenic ingredient in processed foods at traces levels (Section 2.2.1.).

This study contributed to fill the gap of required harmonisation regarding the best method for allergen detection, as well as the absence of reference materials, as crucial pieces for method development. It is, therefore, necessary to reach a compromise by food allergen management authorities in order to conciliate the proper strategy to be adopted in food allergen analysis. Undoubtedly, DNA-methods do not intend to substitute or eliminate the use of protein-based methods. On the contrary, they can provide complementary methodologies that should be used as confirmatory tools to improve the reliability of the results.

4.2. Immunoreactivity as affected by food processing

4.2.1. Animal-derived antibodies vs sera from allergic patients

The immunoreactivity evaluation of allergenic proteins using two distinct immunochemical strategies, animal-derived antibodies and sera from allergic patients, was another developed research carried out throughout this thesis. This study was firstly established for the cases of lupine and soybean allergens, based on commercial animal derived-antibodies targeting the lupine γ -conglutin and the soybean-trypsin inhibitor, respectively (Section 3.2.2.). It consisted of assessing the structural modifications of the target proteins caused by food processing (baking for lupine or autoclaving for soybean) and by the interaction with other compounds of the food matrix (rice flour vs wheat flour or hams vs sausages). These alterations can affect the binding between the allergen and its specific antibody, which can possibly lead to a decrease of its immunoreactivity. Accordingly, the first strategy revealed that the evaluation on the behaviour of the referred target proteins (lupine γ -conglutin and the soybean-trypsin inhibitor) after the applied thermal treatments evidenced a clear reduction of their immunoreactivity, especially upon autoclaving.

Sera from allergic patients are very difficult to obtain because many ethical issues are requested by hospitals to protect the privacy of patients. There are several methods using the sera from allergic patients to assess the sensitising and elicitation capacities of different food allergens. However, the use of human sera may also be problematic due to their high variability according to age, gender or the environment surrounding the patient. Different sera from distinct allergic patients can demonstrate variable responses, in particular as diverse patients react to distinct epitopes of each allergen or to different allergens within each specific food. Moreover, the availability of well-defined patients' sera, with a well-documented clinical history is a limiting factor in this type of tests. As the allergens are

always part of different food matrices, the serum IgE may or may not react with the proteins, depending on the epitope recognition, which might lead to false positive or negative results. This can also happen if specific IgE binds to a homologous protein with a common epitope, having implications on *in vitro* cross-reactivity and incorrect interpretation of the results. For these reasons, pooled sera from allergic patients are often used to obviate the referred problems [14,15].

Despite these limitations, sera from allergic patients are very useful in the estimation of the IgE-binding capacity of food allergens, providing rapid and straightforward information on the possible occurrence of clinical manifestations upon ingestion of the offending food. In this thesis, sera from allergic patients to bovine milk were obtained from a hospital in Italy, which, in combination with MS analysis, allowed assessing the influence of autoclaving and digestibility on the IgE-binding capacity of MPC (Section 3.2.2.). Thus, it was possible to demonstrate a significant reduction, or even elimination, of the IgE-binding capacity of milk proteins after severe heat treatment (autoclaving) and at the end of the digestion process, respectively.

4.2.2. Considerations on the immunoreactivity evaluation of food allergens

The study of the effects of *in vitro* digestibility and/or food processing on the immunoreactivity of selected allergens were one of the main goals of this thesis (Sections 2.2.3. and 3.2.2.). This work was carried out using real food matrices, in which the allergenic target proteins were included as part of a food ingredient. It must be pointed out that most reported studies use purified forms of the proteins to evaluate the effects of food processing and/or digestibility directly, without accounting for the influence of food matrix. The interactions among different components of the food matrix (proteins, lipids, sugars) should be considered because they might greatly alter protein allergenicity. Accordingly, here it was proved that lupine incorporated in rice or wheat flour has a distinct immunoreactivity, considering that both matrices have different proportions of other components, especially carbohydrates and proteins. Similarly, the use of model sausages for the study of MPC immunoreactivity after digestion provided a more complete picture on the behaviour of milk allergens within a complex food matrix.

Digestibility studies are important to establish the impact of digestion on the structural features of food allergens, with special focus on immunoreactivity. Different protocols for protein digestion are available, reflecting a certain lack of harmonisation of the most suitable procedure for food allergen analysis. The diversified conditions do not allow inferring any kind of correlation between digestibility and allergenicity and, consequently, hampering the comparison of results among researchers. For example, the use of a large variety of enzymes along the process, different pH, salt concentrations and digestion time might lead

to different results. In this context, the harmonised digestion protocol developed by the COST Action Infogest network was the preferable choice in this work, being considered the one that best simulates the physiological conditions of human complete digestion process [16]. As a general standardised and practical static digestion method for food, it aims at contributing with the production of comparable data among researchers, which was the major reason for its application in section 2.2.3

4.2.3. Achievements from immunoreactivity evaluation

An important achievement of this work regarded the evaluation of the effect of autoclaving applied to model sausages in combination with the effect of simulated gastro-duodenal digestion on the final immunogenicity of milk proteins (Section 2.2.3.). Autoclaving followed by digestion seems to promote a high degree of protein fragmentation with the production of low molecular weight peptides (below 10 kDa). MS analysis showed that these peptides corresponded to the casein fraction, while the whey proteins were completely degraded by proteolytic enzymes. The IgE-binding capacity of milk proteins was tested by immunoblotting with sera from cow's milk allergic patients and the results demonstrated that the IgE-reactivity of whey proteins was abolished after autoclaving, contrarily to caseins that still maintained a residual IgE-binding capacity. From these findings, it can be inferred that whey proteins are more affected by heat due to their complex structure, being more susceptible to this kind of treatment. In opposition, caseins are noncompact and flexible proteins that are not significantly affected by heat treatment, but they are extensively degraded by proteolytic enzymes during digestion, causing the loss of their IgE-binding capacity as confirmed by IEDB (Immune Epitope Database) results.

The application of the flour/breads model mixtures allowed assessing of the effects of food matrix and processing (baking) on the immunoreactivity of lupine allergens, specifically lupine gamma-conglutin (Section 3.2.2.). At the same time, meat model mixtures spiked with soybean protein concentrate/isolate, also considered as an important allergenic food, enabled evaluating the impact of autoclaving and oven-cooking, used for their preparation, on the immunoreactivity of soybean trypsin inhibitor. Generally, all the applied thermal treatments, namely baking, mild oven-cooking and autoclaving, reduced the immunoreactivity of target proteins, although at a higher extension in the case of autoclaved sausages compared to cooked-hams (soybean proteins). The food matrix proved to slightly affect the immunoreactivity of allergenic proteins since lupine/rice flour mixtures presented more intense bands compared to bands of lupine in wheat, suggesting the interaction of lupine proteins with wheat molecules and consequent decrease of their IgE binding capacity. Independently on the biological recognition element (animal derived antibodies vs sera from food-allergic patients), the major impact on the immunoreactivity of selected

allergens (soybean and milk) was undoubtedly achieved with harsh processing conditions, i.e. autoclaving, highlighting that the combination of high pressure with high thermal treatments seems to be determinant for a more pronounced reduction or even elimination the immunoreactivity of soybean or milk allergens.

4.3. Conclusions

With this work, several significant aspects of food allergen management were studied and highlighted. Real-time PCR revealed to be an effective and robust technique for the detection and quantification of milk and lupine as allergenic foods. Despite the difficulties of working with MPC as an allergenic food, it was possible to develop the first successful real-time PCR method for the detection of MPC in meat products with adequate specificity and high sensitivity. Additionally, important findings were achieved by the application of thermal treatments as a strategy to reduce protein allergenicity and possibly, to produce hypoallergenic formulas of different foods. The importance of using model mixtures in any allergen analysis to simulate, as much as possible, the processing and matrix of a real food was clearly demonstrated. The need for harmonisation of the existing methods for allergen detection as well as for allergen risk assessment studies is imperative. Regarding the analytical methods for allergen analysis in foods, there is a generalised lack of consensus on how data is presented in the literature, considering the use and type of calibrants, the reported units, among other pertinent issues. The studies focused on the evaluation of the effects of food processing and matrix face similar lack of consensus, especially considering the wide diversity of *ex vivo*, *in vitro* or *in vivo* assays and the huge variability associated with biological tissues/serum/plasma, making it difficult the comparison and evaluation of the results from different studies and distinct laboratories.

In the last years, many advances have been made in the field of food allergy management, in which the results of this thesis can certainly contribute. However, more efforts are still needed, always considering the well-being and the quality of life of the allergic patients as a final duty.

4.4. Bibliography

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