

Novel Analytical Methods for Allergy-Related Studies

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Abstract

Managing and treating diseases require a deep understanding of the pathologies coupled with accurate diagnostic procedures. The constant development and improvement of bioanalytical methods reflect the need of diagnosis ameliorations with respect to accuracy, sensitivity and efficiency. The field of allergy diagnosis is a good example. Despite affecting almost a third of the population in western countries, allergies still present complex challenges for the physicians in terms of diagnosis and allergen characterization.

This thesis presents novel analytical methods developed for the fast and personalized allergy component-resolved diagnosis (CRD), the allergen identification and quantification in food matrices and the characterization of allergen modifications, notably nitration. Immunomagnetic separation (IMS) was used to perform the allergy diagnosis at the molecular level by extracting the IgE antibodies from allergic patient blood sera and probing them against individual allergens and natural allergenic extracts. The fast and highly selective IMS procedure was coupled to matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry (MS) to identify accurately and with high sensitivity the proteins recognized as sensitizers by the patient antibodies. The developed CRD methods were successful to diagnose cow's milk, egg, peanut and tree nuts allergies, allowing the resolution of several complex clinical cases. Additionally, precious information was collected for the cross-reactivity and cross-sensitization in peanut and tree nuts allergies using blood sera from the *PronNut* clinical study.

Allergen nitration is one of the potential causes for the increase in allergy prevalence observed recently and requires thorough investigation. Herein, various nitrating agents were tested and compared for *in vitro* allergen nitration. After modifying allergens using two of the most efficient nitrating procedures, the nitration sites were identified by bottom-up proteomics (BUP) and compared to the known epitopes, bringing useful information and novel insights to the problem of correlation between protein nitration and allergenicity.

The presence of a minute amount of allergens in a food product is responsible for potential life-threatening reactions in highly allergic patients and sensitive analytical methods are therefore required in food quality assessments. A proteomic-based method was used for the quality control of several food products suspected of causing severe reactions for peanut and hazelnut-allergic patients. Peptide markers were identified in a first screening of the food extracts by BUP using a high-resolution MS instrument. A standard addition method was then used to quantify peanut and hazelnut proteins by BUP using an untargeted “*shotgun*” approach.

Keywords: allergen, allergy, component-resolved diagnosis, immunomagnetic separation, magnetic beads, mass spectrometry, allergen nitration, cross-reactivity, cross-sensitization, proteomics, matrix-assisted laser desorption/ionization, electrospray ionization, food quality control.

Résumé

La gestion et le traitement des maladies nécessitent une compréhension approfondie des pathologies, associée à des procédures de diagnostic précises. Le développement et le perfectionnement continus des méthodes bio-analytiques reflètent le besoin d'amélioration des diagnostics en termes de précision, de sensibilité et d'efficacité. Le domaine du diagnostic des allergies en est un bon exemple. Bien qu'elles touchent près du tiers de la population des pays occidentaux, les allergies posent encore aux médecins des problèmes complexes en termes de diagnostic et de caractérisation des allergènes.

Cette thèse présente de nouvelles méthodes analytiques développées pour le diagnostic rapide et personnalisé par résolution des composants allergéniques (CRD), l'identification et la quantification des allergènes dans les matrices alimentaires et la caractérisation de modifications des protéines allergéniques, notamment la nitration. La séparation immunomagnétique (IMS) a été utilisée pour effectuer le diagnostic des allergies au niveau moléculaire en extrayant les anticorps IgE des sérums sanguins de patients allergiques et en les testant contre des allergènes individuels et des extraits d'allergènes naturels. La procédure IMS, rapide et hautement sélective, a été couplée à la spectrométrie de masse (MS) et à une source de désorption/ionisation laser assistée par matrice (MALDI) et à l'ionisation par électronébuliseur (ESI) afin d'identifier avec précision et avec une sensibilité élevée les protéines reconnues comme sensibilisants par les anticorps du patient. Les méthodes de CRD développées ont permis de diagnostiquer les allergies au lait de vache, aux œufs, aux arachides et aux noix, ainsi que la résolution de plusieurs cas cliniques complexes. De plus, des informations précieuses sur la réactivité croisée et la sensibilisation croisée des allergies aux arachides et aux noix ont été recueillies à l'aide de sérums sanguins issus de l'étude clinique *ProNut*.

La nitration des allergènes est l'une des causes potentielles de l'augmentation de la prévalence des allergies observée récemment et nécessite des investigations approfondies. Ici, divers

agents de nitration ont été testés et comparés pour la nitration d'allergène *in vitro*. Après modification des allergènes en utilisant les deux procédures de nitration les plus efficaces, les sites de nitration ont été identifiés par la protéomique ascendante (BUP) et comparés aux épitopes connus, apportant des informations utiles et de nouvelles perspectives au problème de la corrélation entre la nitration des protéines et l'allergénicité.

La présence d'une quantité minimale d'allergènes dans un produit alimentaire peut être responsable de réactions potentiellement mortelles chez les patients hautement allergiques. Des méthodes d'analyse sensibles sont donc requises dans les évaluations de la qualité des aliments. Une méthode BUP a été utilisée pour le contrôle qualité de plusieurs produits alimentaires suspects de provoquer des réactions graves chez les patients allergiques aux arachides et aux noisettes. Les marqueurs peptidiques ont été identifiés lors d'un premier dépistage des extraits alimentaires par BUP à l'aide d'un instrument MS à haute résolution. Une méthode par ajouts dosés a ensuite été utilisée pour quantifier les protéines d'arachide et de noisette par BUP en utilisant une approche non ciblée.

Mots-clés: allergène, allergie, diagnostic résolu par les composants allergéniques, séparation immunomagnétique, billes magnétiques, spectrométrie de masse, nitration, réactivité croisée, sensibilisation croisée, protéomique, désorption/ionisation laser assistée par matrice, ionisation par électrobuliseur, contrôle qualité des aliments.

List of abbreviations

α -LA	α -Lactalbumin
Abs	Antibodies
ACD	Allergic contact dermatitis
ACN	Acetonitrile
AGC	Automatic gain control
APC	Antigen-presenting cell
AUC	Area under the curve
B-LG	β -lactoglobulin
BAT	Basophil activation test
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BUP	Bottom-Up proteomics
CE	Capillary electrophoresis
CID	Collision induced dissociation
CRD	Component-resolved diagnosis
DCM	Dichloromethane
DMS	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1,4-dithio-DL-threitol
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ExpASy	Expert protein analysis system
FA	Formic acid
HAc	Acetic acid
HCD	Higher-energy collisional dissociation
HDX	Hydrogen-deuterium exchange
HPLC	High-performance liquid chromatography
HR	High-resolution
IAA	Iodoacetamide
IACE	Immunoaffinity capillary electrophoresis
ICP	Inductively coupled plasma
IDT	Intradermal test
IL	Interleukin
IMS	Immunomagnetic separation
LC	Liquid chromatography
LF	Lactoferrin
LOD	Limit of detection
LOQ	Limit of quantification

MALDI	Matrix-assisted laser desorption/ionization
MBs	Magnetic beads
MOSF	Macroporous ordered siliceous foam
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NCE	Normalized collision energy
ND	Nitration degree
NMR	Nuclear magnetic resonance
NTyr	3-nitrotyrosine residue
OFC	Oral food challenge
OD	Oxidation degree
PBS	Phosphate buffer saline
PCM	Polydimethylcyclsiloxane
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail
PMF	Peptide mass fingerprinting
POD	Peroxidase
PR-10	Pathogenesis-related protein group
PrSMs	Proteoforms spectrum matches
PS	PeptideShaker
PTM	Post-translational modification
RAST	Radioallergosorbent test
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SASA	Solvent accessible surface area
SGUI	SearchGUI
SIM	Single ion monitoring
SIT	Specific immunotherapy
S/N	Signal-to-noise ratio
SPE	Solid-phase extraction
SPT	Skin prick test
SRM	Selective reaction monitoring
TDP	Top-Down proteomics
TFA	Trifluoroacetic acid
TG	Transglutaminase
Th	T helper cell
TNM	Tetranitromethane
TOF	Time-of-flight
TPP	Trans-proteomic pipeline
Tyr	Tyrosine residue
UHT	Ultra-high temperature
UV	Ultraviolet
XIC	Extracted-ion chromatogram

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Chapter 1.

Introduction

1.1 Immune system and hypersensitivity reactions

Immunology is the biomedical science whose study field is the immune system. Compared to other medical sciences, immunology is considered relatively new: the terms “*immune system*” and “*immune response*” were firstly used in 1963 and 1950, respectively. (1) Nevertheless, some immunological terms and principles date back to more than 2000 years. For example, the principle of immunity, the capacity of the body to resist to a particular infection, was already a common knowledge as early as 430 BC in Ancient Greece. (1,2) Until the studies of Louis Pasteur on chicken cholera, anthrax and rabies vaccination, as well as Robert Koch’s studies on anthrax and tuberculosis during the 1870s and 1880s, the immunization mechanism was not understood due to the lack of basic immunology knowledge. (3) Antibodies (Abs) were only discovered later by Emil von Behring and Shibasaburo Kitasato in 1890 in the serum of vaccinated people and were described at that time as substances that bind specifically to the corresponding pathogens. (4) The Abs structure determination by Rodney Porter and Gerard Edelman in 1959-1962 was a large breakthrough in immunology and helped understanding the mechanisms of the immune system. (3) They were both rewarded with the Nobel Prize in Physiology and Medicine in 1972 for their respective works.

The immune system is a very complex machinery and any malfunctions of the immune response result potentially in different kinds of illnesses or diseases. Resulting immunopathologies are defined as immunodeficiency, autoimmunity or hypersensitivity reactions. These disorders correspond, respectively, to a missing or ineffective immune response, an immune reaction of organism to its own healthy cells or tissues and an overactive immune response to otherwise innocuous antigens. (5)

Hypersensitivity is described as an excessive or uncontrollable response of the immune system to an antigen. (6) Hypersensitivity disorders have been sorted in four categories in 1963 by Gell and Coombs. (7) Type I hypersensitivity is the most common form of hypersensitivity and is mediated by the presence of antigen-specific IgE-Abs. This class of hypersensitivity is equated with allergy. Type II hypersensitivity is mediated by IgG and IgM-Abs, which may bind to surface antigens of various cells and induce the activation of the complement system. This activation results in opsonisation (marking of the cell, usually resulting in phagocytose), cell lysis and/or the agglutination of blood cells. (5,7) Type III hypersensitivity is also mediated by IgG-Abs, which bind to soluble antigens and deposit in tissues as large complexes. These complexes are recognized by inflammatory cells, such as neutrophils, resulting in the liberation of mediators causing tissue damages and inflammation. (4,5,7) Meanwhile, type IV hypersensitivity is cell-mediated and not Abs-dependent. The symptoms of the type IV hypersensitivity are relatively long to develop, up to several days, and it is therefore referred as delayed hypersensitivity. (5,8) In the past, type IV hypersensitivity has been associated with a *Th1* lymphocyte mechanism. Thanks to the better comprehension of the inflammatory cascades cause by the type IV hypersensitivity, it has been divided in four sub-classes: *IVa*, *IVb*, *IVc* and *IVd*. They are activated, respectively, by *Th1*/monocytes, *Th2*/eosinophils, *T CD8+*, and *T CD4+/T CD8+*/neutrophils. (9)

1.2 Mechanism of allergy

Allergies correspond to the type I hypersensitivities and result from the ability to produce an IgE-response to an environmental allergen. (10) The allergy biochemical mechanism is very complex and is not completely understood at the moment. Moreover, the physiopathology of allergies is multi-factor dependent. The route of exposure, the allergen dose entering into contact with the immune system, and simultaneous exposition to infections or commensal bacteria, affect greatly the response of the immune system and the symptoms of allergy. (11) A general mechanism of the allergic immune response is shown in Figure 1. (12)

Sensitization of the immune system to a specific antigen occurs after the uptake of this molecule by the professional antigen-presenting cells (APCs). Various kinds of cells may act as APCs, but the more common ones are the dendritic cells, present in the skin, the respiratory system and the digestive tract. After this uptake, the APCs have the ability to degrade the absorbed allergens and to present the native or degraded allergens on their surface using the class II major

histocompatibility complex (MHC II). When naïve helper T cells (*Th0*, *CD4+*) enter into contact with APCs presenting a complete or fragmented allergen, they adopt a *Th2* cell phenotype characterized by the production of interleukins (ILs) 4, 5, 9 and 13. (4,10,12)

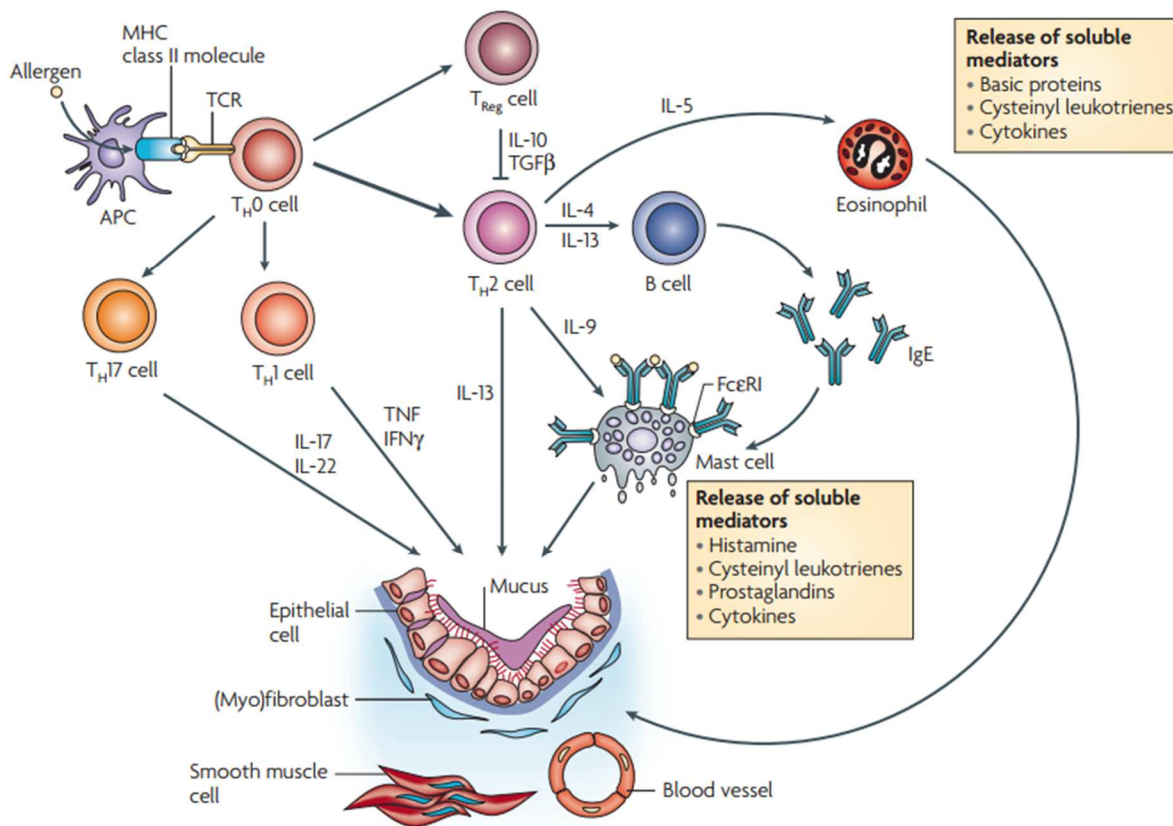


Figure 1: Simplified schematic representation of allergy mechanism. Reprinted from Nat. Rev. Immunol., 8 (3), 218–230, 2008. (12)

IL-4 is responsible for the class switching of B lymphocytes, resulting in the synthesis and liberation of IgE-Abs, specific to the allergen. The other interleukins are also responsible for the recruitment and proliferation of mast cells (IL-4, IL-9 and IL-13), the maturation of eosinophils in the bone marrow (IL-3 and IL-5) and the maturation of basophils (IL-3 and IL-4). (12) Mast cells and basophils have the ability to bind to their surface the produced antigen-specific IgE-Abs using a high-affinity receptor to the fragment crystallisable region of the IgE-Abs (FcεRI). (13)

Upon the second encounter of the host with the allergen, the cross-linking of the allergen with the specific IgE-Abs attached to the mast cells stimulates the liberation of chemical com-

pounds present in the internal vesicles and the production of additional mediators. Released chemicals include histamine, prostaglandins, and proteases, responsible for the early allergic symptoms. Mast cells also secrete cytokines and chemokines, resulting in the recruitment and activation of eosinophils and basophils. Upon activation, these two types of cells produce *inter alia* histamine, proteoglycan 2 (major basic protein), heparin and cytokines. (3,12) The effects of such chemical mediators, proteases and cytotoxic chemicals are diverse and depend on the site where the allergic reaction occurs. They include a vasodilatation, an increase in vascular permeability, a production of mucus, the contraction of smooth muscles and the formation of oedema. (4)

The contact of *Th0* cells with APCs may also result in some cases in their maturation into *Th1* or *Th17* cells and successive *Th1/Th17* responses, having a pro-inflammatory effect. These responses are not considered allergic reactions, as they are not IgE-mediated. (12) Regulatory T cells (*Treg*) act on the allergy mechanism by down-regulating *Th* cells proliferation and inhibiting the *Th1* cytokines production by releasing IL-10 and transforming growth factor- β (TGF- β). (14,15) As the allergic pathways is highly regulated, presence of specific-IgE-Abs does not always reflect the appearance of clinical symptoms upon contact of the immune system with the allergen.

1.3 Class of allergens

Allergens are defined as normally innocuous environmental antigens responsible for an abnormal immune response mediated by specific IgE-Abs. Sources of allergens are multiple, but the most common allergies are caused by molecules originated from plant pollen, animal dander, dust mites, mold, insect venom, latex, drug and food. (4)

Until the 1960s, it was believed that a minimal molecular mass of 5 to 10 kDa was required for a molecule to act as an allergen. (16) Therefore, proteins were considered as the only potential allergens and smaller compounds were mainly acknowledged as non-antigenic. It was later found, that small non-immunologic molecules such as drugs and therapeutic compounds have the ability to produce an IgE-mediated immune response when combined with a carrier protein, forming a hapten-carrier complex. (17) Nowadays, it is also known that IgE-Abs may be specific to carbohydrates attached to proteins, such as asparagine-linked glycans. (18) Moreover, nickel (Ni^{2+}) is commonly referred as the most common contact allergen responsible for allergic contact dermatitis (ACD). (19) Despite its name, ACD is not an allergic reaction as previously defined, because it involves a T lymphocytes response without IgE-Abs production. ACD is therefore

a delayed hypersensitivity reaction (type IV). (19–21) Despite these few examples, nearly all allergens are proteins.

The World Health Organization and the International Union of Immunological Societies developed in 1986 an official nomenclature for the allergens, based on the Linnaean binomial nomenclature of genus and species. (22) As an example, in this nomenclature α -lactalbumin, an allergenic protein found in cow's milk, is noted *Bos d 4*, as domestic cattle is part of the *Bos domesticus* specie. A database of the allergen names is available on www.allergen.org and regroups more than 800 common allergens. (22) In the present work, both the biochemical names of the proteins and the official allergen nomenclature are used interchangeably.

1.4 Allergy symptoms

Immediate reactions are caused by the exposure of the allergic individual to allergens. The resulting effects and symptoms vary according to the site of the allergen entry: the skin, the eyes, the digestive tract, the respiratory system or the bloodstream. (23) The apparition of the symptoms is fast, usually less than 10 minutes, and is caused by the activation of mast cells and their degranulation, as described above. The liberation of chemical mediators from mast cells results mainly in an increase of local vascular permeability, an increased blood flow, the contraction of smooth muscle, mucus production and itching. These effects are limited to the vicinity of the contact point between the allergen and the immune system. (3,4,23) In many cases, the immediate reactions are followed by a late-phase reaction occurring within 6 to 48 hours after the onset of the allergic reaction. This late reaction is due to the recruitment of *Th2* lymphocytes, mast cells, eosinophils and basophils to the allergic site resulting in the prolongation of the inflammatory response and possible oedema formation. (3)

If the allergen contact is elicited in the skin, the reactions are generally characterized by redness, swelling and itching, due to the action of histamine on cutaneous sensory nerves. In the nasal and eye mucosa, allergens cause nasal discharge, itching, watering and sneezing. When an airborne allergen is absorbed in the respiratory system, the main symptoms induce a rapid swelling of the affected tissues, called an angioedema. The possible airway obstruction caused by angioedema is a severe life-threatening condition. After ingestion of an allergen, the immune response may cause direct symptoms such as diarrhea, colic and vomiting.

Rapid absorption of the allergen by the guts or direct injection in the bloodstream may cause a systemic anaphylaxis caused by the activation of mast cells in the whole body of the allergic patient. This reaction leads to dramatic decrease in blood pressure, constriction of the airways, swelling in the epiglottis and cardiovascular collapse. (4,23) Breathing problems (bronchoconstriction or angioedema) and anaphylaxis reactions make allergy a serious life-threatening condition.

1.5 Allergy treatments

The management of allergies is mainly based on allergen avoidance measures and environmental control precautions, as a primary prophylaxis. (12,23) In case of food allergies, the patient needs to follow a strict diet to avoid culprit allergens. If an allergic reaction affects a patient, despite the measures taken to avoid contacts with the allergen, pharmacotherapy is used to relieve and control the allergic symptoms. (12,23) Different chemical compounds including antihistamine, corticosteroids, broncodilators, leukotriene antagonists and anti-inflammatory drugs are used to decrease or block the symptoms. (12,23,24)

Allergen-specific immunotherapy (SIT) is the only allergy treatment acting on the causes and not the symptoms. (24) SITs are recommended for allergic rhinitis, venom allergies, drug allergies and allergic asthma, like hay fever. By producing repeated expositions to an increasing amount of allergen, SIT results in an immunological tolerance by increasing the allergen-specific IgA and IgG4-Abs levels and decreasing the level of IgE-Abs. (12,25) SITs are usually performed by the oral, sublingual or epicutaneous routes using incremental regimes of native or modified allergens. (12,26) Induction of the tolerance requires between several days to several months of treatment. (12) This kind of treatment is not completely safe for the patient: deaths caused by SIT have been reported (24,27) and approximately 0.1 to 5 % of patients show anaphylactic side-effects. (12) Different approaches have been used to decrease the risks and improve the efficiency of the immunotherapies (28) such as including peptides modifying the activity of regulatory T cells, (29) blocking the allergens in biodegradable nanoparticles (30) or using modified allergens called allergoids. (31,32) Immunotherapies that are non-allergen specific are very promising and include humanized monoclonal anti-human IgE-Abs (Omalizumab), (33) peptide immunotherapies, (29,34) and traditional Chinese medicine. (26)

1.6 Analytical tools for allergy diagnosis and allergen identification

Having reliable and fast allergy diagnostic tests is necessary as this kind of immune disorder can be life threatening and touches a large portion of the population. It is currently estimated that approximately 20 % of the population of developed countries are affected by allergies. (35) In Switzerland, the *aha! Swiss Allergy Centre* estimates that 35 % of the Swiss population are sensitive to one or several allergens and that 20 to 25 % have suffered from allergic symptoms after the contact with an allergen. (36)

The ideal allergy diagnostic tool should be fast, reliable and non-expensive, while presenting no unpleasantness or dangerousness for the patient. As for other diseases, the first step in the diagnosis is the careful study of the medical history of the patient and preliminary physical examination. If the physician suspects an allergy, various tests exist to confirm or infirm this hypothesis. The existing allergy diagnostic techniques are separated into two categories: the *in vivo* and the *in vitro* tests. Performing the *in vitro* diagnosis at the molecular level, called the component-resolved diagnosis (CRD), is used to define the exact allergen(s) responsible for the patient sensitivity. (26,37,38) Knowing the precise sensitizer, responsible for the allergy, allows its avoidance by the patient, decreases the probabilities of an allergic or anaphylaxis reactions, and helps defining a possible immunotherapy. The available CRD methods have the potential to be modified and coupled to mass spectrometry (MS) for the identification and structural characterization of allergens.

1.6.1 *In vivo* allergy diagnoses

The *in vivo* diagnostic tools, also called provocation tests, are based on the monitoring of the immune response of a patient after direct contact with a specific allergen source. The skin tests are widely used and are usually the first tests performed on the patient. They are separated into skin prick test (SPT), intradermal test (IDT) and patch test. (39) SPT and IDT are the most common *in vivo* tests, whereas patch test is limited to very specific clinical cases.

SPT is mainly used to determine the IgE-sensitization to pollens, foods, insect venoms, some pharmacological compounds, (39–41) molds, house dust mites and animal dander. (23) These tests are based on the immune response analysis after exposing the patient to a small quantity of diluted allergen solution by the percutaneous route. In principle, the physician deposes on the patient skin, usually on the arm or the back, a small quantity of allergen solution and pricks it into the skin surface. (40–42) About 15 to 20 minutes after the exposition, the sites where aller-

gens were deposited are examined for a wheal and flare reaction. (40) The wheal size is compared to a positive control (histamine) and negative control (saline solution) to determine the sensibility of the patient to the tested allergen. (23,42) SPT is very easy to perform, cheap and provide results in minutes. (39) Moreover, SPT is relatively safe, only a very small number of tested patients exhibit a severe reaction: it is estimated that the overall risk of SPT-induced anaphylaxis reaction is less than 0.02 %. (43) Nevertheless, fatal reactions to SPT have been reported. (42) It is usually recommended to consider patients with anaphylaxis history, pregnant women, infants and uncontrolled asthma patients as higher risks. (23)

IDT is usually used to test the patient against insect venom and drugs such as penicillin (42) or when SPT are negative or inconclusive. (40) In these tests, allergen solutions are injected in the dermal layer of the patient. IDTs are responsible for more anaphylaxis reactions than SPT. As IDTs induce more risks and are less specific, they are recommended as complementary procedures and not as first-choice tests. (39)

Several problems arise from the skin tests carried out as the allergy diagnosis. The results are visually evaluated and are therefore dependent on the proficiencies of the physician performing the test. (41) For example, the evaluation of wheal size with irregular shapes or pseudopods is problematic. (44) Nevertheless, if the physician is correctly trained, results obtained from skin tests are reproducible. (40) SPT and IDT are not pertinent when the patient suffers from skin disorders such as dermatitis, eczema, dermatographic urticaria. (23,40) Moreover, the uptake of antihistamine or other medications may influence greatly the results. (23,40) Young patients, children and infants may reveal themselves uncooperative, complicating the skin tests. In those cases, patch tests are possible substitutes. (45)

In case of food allergies, skin tests are sometimes limited by an apparent variability in specificity and sensitivity to food allergens. (44) For this reason, oral food challenge (OFC), performed in double-blind and placebo-controlled, is considered as the golden standard for the diagnosis of food allergies. (44,46) OFC is based on the observation of the allergic symptoms after ingestion of the suspected food or allergen. (47) OFCs are time consuming, expensive and troublesome for physicians and patients. (44,48) Moreover, it is recommended to perform OFCs only in places suitable for the handling of side effects and possible anaphylaxis. (26,48)

1.6.2 *In vitro* allergy diagnoses

As *in vivo* tests have limitations, different kinds of *in vitro* methods have been developed to assist in the allergy diagnosis. The more common and used *in vitro* tests are based on the quantification of total and/or allergen-specific IgE-Abs in the blood serum of the patient, as IgE-Abs are biomarkers for type I hypersensitivity. For a non-allergic person, the total concentration of IgE-Abs in blood serum varies according to the life period: it increases during childhood until the age of 15-20, stabilizes until approximately age 60 and then slowly decreases. In adults, the normal concentration is around $240 \text{ ng}\cdot\text{mL}^{-1}$ ($100 \text{ IU}\cdot\text{mL}^{-1}$). (49) Total IgE-Abs concentrations above this value may indicate the sensitivity of the patient to one or more allergens. The concentration of each kind of allergen-specific IgE-Abs vary greatly for every patient depending on the type of allergen. It is important to emphasize that these tests quantify the IgE-Abs in circulation and not the ones bound to mast cells. (50) Performing the *in vitro* diagnosis at the allergen level, often referred to component-resolved diagnosis (CRD), is also useful in estimating the risk associated with particular allergen classes and allergy persistence. (51,52)

Radioallergosorbent test (RAST) was the first *in vitro* test developed to diagnose allergies. (41) In this test, allergens are bound to an insoluble polymer and added to the blood serum of the patient. The specific IgE-Abs of the patient then bind to the allergens and are later detected and quantified by adding radio-labelled anti-human IgE-Abs. (53) Nowadays, the use of RAST is not recommended anymore for the diagnosis of allergies (54) and enzyme-linked immunosorbent assay (ELISA)-based tests are preferred.

Immunoassays, mainly based on ELISA, are now the standard *in vivo* tests thanks to their accuracy, sensitivity and partial automation. Such tests are now commercially available and, currently, three tests share the majority of the global market of *in vitro* allergy diagnosis: ImmunoCAP for total (55) or specific-IgE-Abs (56) quantification (Phadia, ThermoScientific, Sweden), Immulite 2000 (57) (Siemens, Germany) and Hytec-288 (Hycor, USA). (58) The general principles of mentioned tests are very similar. First, a certain volume of patient blood serum is added to allergens, bound to a well surface or to a particle, in order to capture the patient IgE-Abs (Figure 2.1). Then, enzyme-conjugated anti-human IgE-Abs are added to the system and they bind to the constant domain of the patient IgE-Abs (Figure 2.2). The obtained immunocomplexes are then detected and quantified by colorimetry or fluorescence in the presence of corresponding substrate (Figure 2.3-4). A comparative study of these three tests has been recently published. (58) ELISA-based tests

provide a minimum limit of quantification (LOQ) down to $0.24 \text{ ng}\cdot\text{mL}^{-1}$ (ImmunoCAP for specific-IgE-Abs). Phadia (ThermoScientific) has also developed a biochip for semi-quantitative measurement of specific IgE-Abs called ImmunoCAP ISAC. (59) Described commercial tests require less than $100 \mu\text{L}$ of patient blood serum but are relatively long to perform (3-5 hours).

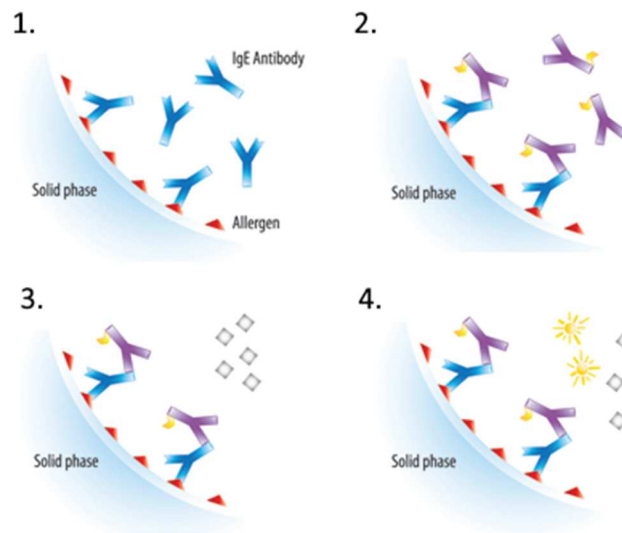


Figure 2. Representation of the ELISA-based design of ImmunoCAP for specific-IgE-Abs quantification. (56)

Other immunoassays have been developed in different research laboratories in the past years, but none of them is currently commercially available. They are based, for example, on microarray using diamond-like carbon-coated chip, (60) microarray with proteins immobilized in a 3D-hydrogel, (61) surface plasmon resonance detection, (62–64) photoimmobilized microarray (65), magnetophoretic immunoassay (66) or aptamer-based electrosensor. (49) Their limit of quantification, limit of detection, costs, sensibility and analysis duration vary greatly. Point-of-care diagnosis based on lateral flow assay have also been developed (67,68) and are commercially available. (69,70) Recently, our group developed two CRD procedures using mass spectrometry (MS) as the detection method. In the first one, immunoaffinity capillary electrophoresis (IACE) coupled with matrix-assisted laser desorption/ionization MS (MALDI-MS) was used to capture the patient IgE-Abs for the total IgE-Abs quantification and the cow's milk CRD using only $2 \mu\text{L}$ of blood serum. (71) Meanwhile, immunomagnetic separation using magnetic beads coupled to mass-barcode gold nanoparticles was used to perform the multiplex allergy CRD using MALDI-MS for

the detection of the mass-barcodes. (72) Both methods display the power of MS-based procedure for the accurate and sensitive allergy CRD.

A subclass of *in vitro* tests is sometimes referred as *ex vivo* assays. They are based on the isolation of immune cells from the blood serum of the patient and their analyses in presence of allergens or allergoids. The most popular *ex vivo* assay is the basophil activation test (BAT), monitoring by flow cytometry the expression of the marker molecules after addition of the allergen. These surface proteins are only expressed after activation of basophils and BAT reflects, therefore, the allergenicity level of a given allergen. (73) Other methods are based on the detection of inflammatory mediators release from immune cells. Potential diagnostic markers include histamine released from basophils (74) and tryptase released from mast cells. (75,76) Nevertheless, these *ex vivo* methods are less popular than BAT due to their insufficient sensitivity and specificity. (73,76)

In most cases, *in vitro* tests are an important complement to SPT or OFC for the diagnosis of type I hypersensitivity, (40) but are not enough to confirm alone the allergy diagnosis. *In vitro* tests are, moreover, dependent on the quality and purity of the allergen used. Allergens either are extracted from natural sources or produced using recombinant technologies. (23,40) The tests may also be complicated by the presence of isoallergens in the allergen extracts. Isoallergens are defined as allergen isoforms with similar molecular size and identical biological function. For example, the major white birch allergen, *Bet v 1*, has 31 different isoallergens. (77) Overall, *in vitro* CRD gives highly specific results with no risk of anaphylaxis for the patient.

1.6.3 Mass spectrometry-based method for allergy diagnosis and allergen characterization

The recent developments and improvements of MS instruments, coupled to efficient separation techniques such as immunomagnetic separation, 2D gel electrophoresis or liquid chromatography, opened the possibility of using MS for the sensitive and accurate allergy CRD (71,72) and for the identification, characterization and quantification of allergens in food products or immunotherapy drug products. (78) Proteomics, defined as the large-scale quantitative and qualitative study of proteins to characterize biological processes, (79) has an extremely wide potential and is considered the base of a new era in allergy studies. (80,81) When applied to allergen identification and characterization, proteomics strategies are sometimes referred to "*allergenomics*". (82,83)

Bottom-up (BUP) and top-down proteomics (TDP) are the two main MS-based proteomic approaches used in allergenomics (Figure 3). BUP is the most common approach, in which a protein sample is digested using a protease prior to mono- or multi-dimensional peptide separation by liquid chromatography and analysis by MS/MS. The recording of the peptides in MS¹ scans and their fragment products in MS² scans allows the protein quantification and characterization, based on the peptides matched to known proteins by the bioinformatics tools used for the data processing. (84) BUP procedures are ideal for the fast and high-throughput protein identification at the proteome levels. However, BUP is limited by the lack of specificity of small peptides and the potential loss of information (post-translational modifications, proteoform variations, and amino acids sequence differences) as not all peptides are identified. (85) On the other hand, TDP procedures are based on the analysis of intact proteins and their fragment ions in the MS instrument after their separation using liquid chromatography, without an enzymatic digestion as shown in Figure 3. (84) TDP analyses allow identifying easily post-translational modifications (PTMs) and detecting proteoforms or degradation products, while avoiding protein inference problems and modifications induced during the enzymatic proteolysis. (85,86) Additionally, as no protein digestion is required, TDP procedures result usually in a full protein coverage without the loss of PTM sites. (85) Due to the extremely high complexity of MS scans collected in TDP analyses, MS instrument with high mass accuracy and resolution are required to separate spectrally the peaks and to identify precisely small mass-shifts between proteoforms. (87) TDP methods are complementary to BUP procedures and have been recently proposed for food authentication and allergen identification in food products. (88–90)

Hundreds of allergens are currently known. They are classified as minor or major allergens based on their prevalence, while they are identified on the basis of their reactivity towards IgE-Abs. (91) A deep understanding of allergy and allergen reactivity passes through the complete sequencing of allergenic proteins. For years, Edman degradation was used to sequence allergens (92–95) but this tedious process was replaced by MS-based proteomics. (96–98) In addition to allergen sequencing, MS-based techniques are helpful to identify and characterize the binding interactions between the allergens and the IgE-Abs. The determination of the IgE epitopes is crucial for the development of immunoassays and the production of allergoids for immunotherapies. (12,26,99) Proteomic approaches offer the perfect alternative to NMR and X-ray diffraction for the

complete identification of IgE epitopes (*i.e.* epitope mapping) following epitope excision and extraction procedures in BUP analyses (100) or hydrogen deuterium exchange (HDX) MS. (78)

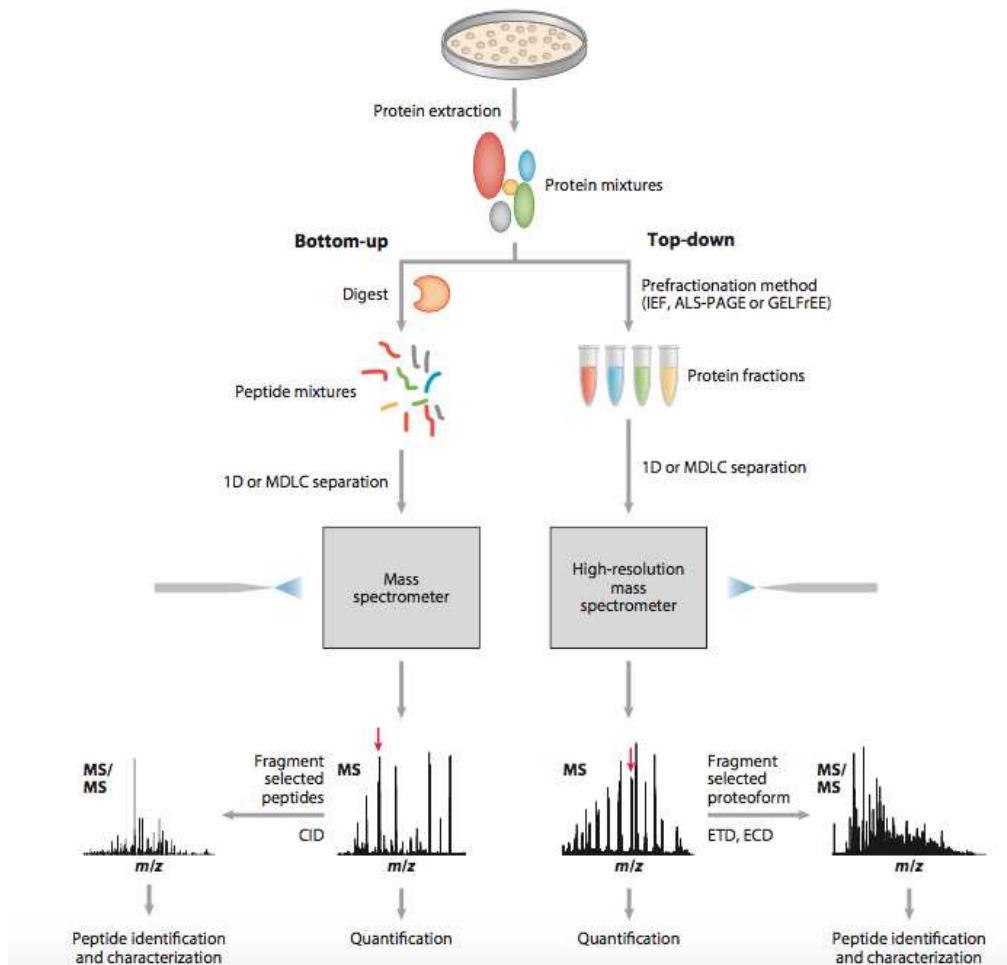


Figure 3. General workflow for typical MS-based bottom-up and top-down proteomics. Reprinted from Annu. Rev. Anal. Chem. 2014, 7, 427-454. (84)

1.7 Discovery of previously unknown allergens

The identification of previously unknown allergens is extremely important for the understanding of allergic diseases, their reliable diagnosis and for potential immunotherapies. Various analytical methods are used to discover allergenic proteins, to confirm their IgE-binding properties and to measure their ability to trigger an immune response. (101,102) The initial discovery of new allergens is based on the screening of a protein extract using the IgE-Abs present in human blood sera. The most common procedures used for this screening are using isoelectric focusing or polyacrylamide gel electrophoresis (1D, 2D) to separate the proteins based on their charge or mass. (101,103) Then, western blot and related procedures are used to detect the binding of IgE-Abs to the previously separated proteins by using a secondary labeled Abs. The detection is usually car-

ried out by staining, radioactivity or immunofluorescence and the procedure is often referred to as IgE immunoblotting. (104–106)

The proteins for which an IgE-binding was detected are extracted and purified in order to identify them formally and to characterize their allergenic properties. The purification of native proteins from a complex natural extract is performed by combining various chromatographic techniques, whereas recombinant proteins may be produced using various expression systems. The allergens activity is estimated *in vitro* by techniques such as ELISA and ImmunoCAP, (106) whereas a full identification and structural characterization may be performed using MS-based proteomics techniques. (107) Finally, the ability of the newly discovered allergens to trigger an immune response is tested using for example SPT, BAT or histamine release tests.

The procedures currently used for the detection and identification of previously unknown allergens are tedious and suffer from several limitations. (101) As the initial screening of an extract is carried out usually by pooling together the blood sera of a given number of patients, the ability of detecting a new allergen is closely related to the quality of the serum pool. Moreover, demographic factors are known to have a large impact on allergic diseases; an allergenic protein for a tested group of patients may be innocuous for people from other geographic regions. Additionally, false-positive hits may arise during the screening of protein extracts from cross-reactivity, caused by similarity in protein structures resulting in the binding of IgE-Abs to innocuous proteins. Lastly, the separation methods, such as IEF or SDS-PAGE, result potentially in protein modification, denaturation and/or IgE-epitope loss or creation.

1.8 Modifications of allergens

Nowadays, modified allergens are typically used for hypoallergenic food production and vaccines creation for allergen-specific immunotherapies. Allergens are also referred to allergoids when intentionally modified with the goal to reduce their allergenicity. In this case, the purpose of allergen modifications is to decrease the IgE-binding capacity while retaining its immunogenicity, by preserving structural features necessary for recognition by the T lymphocytes. (108) These modifications are also widely applied in food processing in order to decrease the allergenic potential of food products. (109) Modifications are mainly performed by physical, chemical, enzymatic or genetic engineering pathways. (108)

Different methods exist to assess the allergenicity of native and modified allergens. Typically, they are based on the same techniques used for the allergy diagnosis. The ability of the allergens to trigger an immune response is linked to its ability to bind to IgE-Abs and its ability to activate the effector cells (mast cells, basophils and eosinophils). *In vitro* immunoassays such as RAST, ELISA, and immunoblotting are used to test the IgE-Abs against allergens and allergoids, allowing the determination of the IgE-binding potential of the tested molecules. A reduced IgE-binding ability of the allergoids compared to the native allergens indicates a potential decrease in the allergenicity, but is not always correlated with reduced symptoms. On the other hand, the allergenicity may truly be assessed by *in vivo/ex vivo* tests as they depend on the IgE-binding capacity of the allergens and the monitoring of immune cells activation.

Animal models are also used to determine the allergic pathway, the sensitizing potential of proteins and to test allergoids before their use in SIT. These models allow an ethical study of the complete immune response to an allergen, and not only the IgE-binding potential or chemical effectors liberation. (110) The most often exploited animals are rodents, (109) but the murine models tend to develop tolerance to orally ingested allergens, limiting their application to food allergy or oral SIT. (111,112) Larger animals such as pigs, dogs and sheep have also been used as animal models in the study of allergies. These animals are anatomically, histologically and physiologically closer to human than rats and mice, (113) allowing a better allergenicity prediction. In general, the animal model should be chosen wisely depending especially on the administration route, the mode of action of the allergoids, the adjuvants used and the dose range of allergens. (24,114) Animal models are a powerful tool to study the allergenicity of proteins but differences between these models and the human immune system limit their application.

In vivo tests (SPT and OFC) are usually expensive, time consuming and not applicable to patient susceptible to suffer an anaphylaxis reaction. However, they are the final proof to assess the allergenicity of allergens or allergoids and to prove the safety of new compounds developed for SIT. (109)

1.8.1 Physical and structural changes of allergens during food processing

The processing of food products during their production result in potential physical and structural changes. The most frequently studied industrial process is the heat treatments of food.

In the food industry, the main purposes of thermal processing are the building or enhancement of properties, textures and flavor, the increase of digestibility, and microbiologic safety. (115)

Depending on their origins and properties, food allergens are affected differently by cooking or high temperature treatments. In some cases, protein allergenicity is not modified by this common technological food process. The main peach allergen *Pru p 1*, a lipid transfer protein, has been shown to be extremely stable and to conserve its allergenicity even after a 30 minutes heat treatment at 121°C. (116) One example of a known modification decreasing the allergenicity of the protein is the structural changes of β -lactoglobulin (β -LG), one of the main cow's milk allergens. At high temperature, β -LG unfolds and aggregates in structures of approximately 50 nm in diameter. (117) Unfolding reveals a free cysteine, normally hidden in the tertiary structure of the protein, allowing the formation of non-native disulfide bonds. (118) The creation of disulfide cross-links with other β -LG molecules or other proteins results in a stable gel like structure. (119) Heat processing of β -LG has been shown to reduce significantly, but not totally, its IgE-binding ability, and therefore its allergenic potential. (120) Meanwhile, another study showed the formation of a new epitope, not found in the native state of the protein, after heat-treatment of β -LG, corresponding to an increase of its allergenicity. (115)

Other industrial process such as gamma irradiation for food safety, fermentation, and high pressure treatment have been less studied and display different allergenicity modulations when applied to food allergens. (121) In all cases, protein degradation and/or agglomeration are responsible for the physical and allergenic modifications. Additionally, partial unfolding and aggregation of proteins have also been reported to be induced by mixing and shearing (122,123) or by absorption of proteins at interfaces in food emulsions. (124–126)

1.8.2 Enzymatic modifications

Different enzymes are currently used in the food industry. The most common process is the enzymatic hydrolysis of food products for hypoallergenic preparation of milk and baby food formula. (121,127) The goal of this process is to degrade extensively the proteins in short peptides for better assimilation with a reduced allergenicity, by destroying the IgE epitopes. (121) The quality of the hydrolysate and the average length of the peptides are easily tailored by choosing correctly the proteolytic enzymes. (128)

Instead of degrading the proteins, cross-linking them is the other enzymatic strategy to hinder the IgE epitopes and obtain hypoallergenic products. Additionally, the protein cross-linking is used to tailor the food structure and texture by modifying the rheological properties, creating and improving gel formation and foaming properties. (129,130) As an example, transglutaminases (TG) belong to the acyltransferase proteins group and are able to catalyze the reaction between a glutamyl residue and the primary amino group of lysine to form an isopeptide bond, resulting in polymerization of proteins and liberation of ammonia, as shown in Figure 3. (129,131) The capacity of TG to polymerize proteins varies according to the nature of the protein substrates. For example, caseins, which are non-globular milk proteins with flexible tertiary structure, are good substrate for TG resulting in a high level of polymerization. (132–134) Globular whey proteins, such as β -LG, are poor substrates for TG, compared to caseins. (134,135) Enzymatic cross-linking with TGs has been used to reduce the allergenicity of β -casein, (136) β -LG, (137) natural rubber latex, (138) wheat flour proteins, (139,140) and soy proteins. (141) TGs are recognized as safe according to the GRAS Notice Inventory of the Food and Drug Administration (FDA, USA), with usage level below 200 ppm.

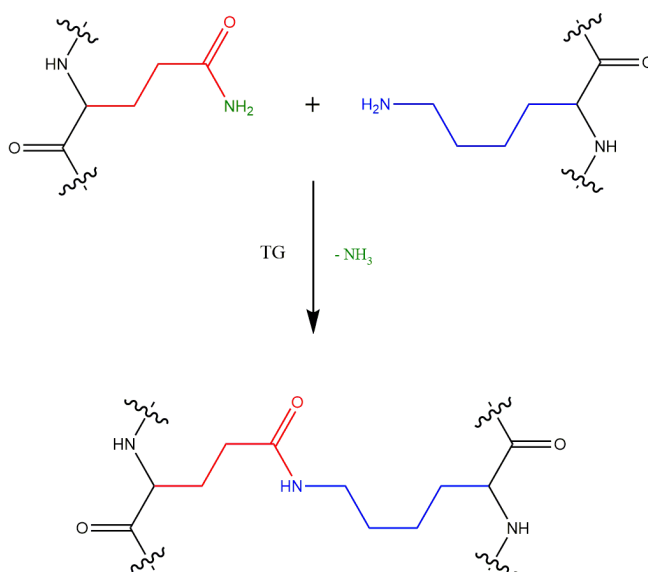


Figure 3. Polymerization of proteins by the reaction between their lysine and glutamine residues triggered with TG.

Another class of enzymes having the ability of proteins cross-linking is the peroxidases (PODs). These heme-containing proteins oxidize phenols, aromatic phenols, indoles, amines and thiols using hydrogen peroxide as an electron donor. Oxidation results in the formation of a radi-

cal, which reacts easily with other substrates resulting in a cross-linking. (109,129) It has been shown that treatments of allergens with PODs may reduce their allergenicity. (142,143) Nevertheless, it was also found that PODs are possible allergens themselves. (144,145) Laccases and phenoloxidases are other enzyme classes able to oxidize phenolic compounds. (146,147) β -LG has been cross-linked by laccase, resulting in a decrease of the β -LG allergenicity with an increased digestibility. (148) Similar results were obtained for β -casein cross-linking using laccase. (136) Phenoloxidases, such as tyrosinase, have been also used to mitigate the IgE-binding capacity and allergenicity of different allergens. (136,149–152)

1.8.3 Genetic modifications

The aim of inducing genetic modifications in the DNA sequence coding for the allergens is mainly to decrease the IgE-binding ability of the newly produced allergens, while retaining their general features and structures, primary for immunotherapy purposes. This method requires the isolation of the gene corresponding to the allergen and the use of common expression vector such as *E. coli* or yeast cells. (108)

One simple genetic method of gene/protein modifications, based on the gene-shuffling strategy, is called the directed molecular evolution. It is based on random mutations of the gene occurring during the replication protocol, resulting in chimeric gene and proteins having potentially new functional properties or structures. (108,153) DNA-shuffling has been successfully used to create and find proteins with low allergenicity but high immunogenicity from tree pollen allergens (154) and dust mite allergens. (155,156)

Site-directed mutagenesis is a powerful method to make intentional modification in the DNA sequence of the gene coding the allergen, in order to modify its allergenicity. It is based on the rational design of genetic modifications by replacement, insertion or deletion of a codon. (157) Such genetic modifications require a good knowledge of the allergen structure, the IgE-binding epitopes positions, sequence and conformations. (109) This technique has been successfully used to produce different chimeric allergoids originated, for example, from latex, (158) grass pollen, (159) birch pollen, (160) and fish. (161)

Linking of genes coding for different allergens and expression of the resulting chimeric protein, called a hybrid allergen, has been applied for the production of allergoids. Hybridization of allergens results in a completely new protein, for which structure and IgE-binding ability are hard

or impossible to predict. (108) This method has been successfully applied to form hybrid allergens of Fagales pollen, (162) bee venom (163) and grass pollen. (164) Other genetic engineering approaches, such as synthesis of T-cell epitope peptides (24,165–167) or folding variant of allergens, (168,169) have been carried out and the resulting allergoids were used with success in immunotherapies.

1.8.4 Chemical modifications

Chemical modifications are by far the most common and prevalent alterations, whether naturally occurring or purposely designed, that are found in proteins. The goal of chemical modifications of proteins is mainly the tuning of desired properties such as function, structure, reactivity, solubility or allergenicity. (109) Induced modifications, when altering the protein allergenicity, are useful for the development of allergoids for immunotherapies or for the production of hypoallergenic foods. In both cases, if toxic compounds are used to perform the protein alterations, complex and time-consuming procedures are required to remove the reagents from the allergen matrix. (109) Hereafter, a selection of the most common chemical modifications applied to allergens are discussed. Carbamoylation, (170–172) conjugation with polysaccharides, (173–177) and non-covalent modification using phytic acid, polysaccharides, and oxidized lipids (178–181) are some of the other modification processes used to modulate the protein allergenicity but are not discussed herein.

1.8.4.1 Polymerized allergoids

Historically, one of the first chemical modifications applied to allergens was their polymerization using aldehydes, mainly formaldehyde and glutaraldehyde, in order to produce allergoids for subcutaneous specific immunotherapy. (109) The goal of this method is the aggregation of allergens to decrease or block the IgE-binding potential, while retaining the immunogenicity and producing a normal immune response. The aldehyde groups react easily with the free amino groups of the lysine residues, resulting in bridge formations and random cross-linking of allergens. (109)

Glutaraldehyde has been firstly used to aggregate crude timothy grass pollen extracts, decreasing their reactivity towards IgE-Abs and inducing an IgG-Abs response. (181) Formaldehyde and glutaraldehyde treatments have been successfully applied to ragweed allergens, (182,183) ovalbumin, (184,185) birch allergens (186) and timothy pollen extracts. (187) Marsh *et al.* com-

pared formaldehyde and glutaraldehyde for the polymerization of ragweed allergens. It was found that using formaldehyde instead of glutaraldehyde is more efficient in term of allergenicity decrease, with similar or increased immunogenicity in comparison to native allergens. (182) Meanwhile, glutaraldehyde-modified pollen allergens were recently shown to have a lower IgE-binding ability compared to allergens polymerized with formaldehyde. Nevertheless, allergens modified with glutaraldehyde showed a lower dendritic cell uptake and subsequent T cell response, resulting in a partial loss of immunogenicity. (188) Those contrasted results show the efficiency of polymerized allergoids for reduction of allergenicity but a controversial change in immunogenicity.

1.8.4.2 Acylation of allergens

Chemical modifications of allergens have been performed with acetic, maleic or succinic anhydride. The treatments of allergens with acylation agents result in the acylation of the positively charged amino groups of the lysine residues. (109) The acetic anhydride treatment results in a neutralization of the normally positive amino group of lysine residues, whereas a negatively charged group appears after the protein modification using succinic or maleic anhydrides. Acylation therefore produces highly acidic proteins with a decreased total charge, changing their physicochemical and immunological properties in comparison to the native protein. (108,189) Modification of the allergenicity for highly negatively charged allergoids is not completely understood. Scavenger receptors are present on immune cells, such as dendritic cells, and have a high affinity for negatively charged ligands. The acylation treatments of allergens are believed to enhance their uptake by dendritic cell, resulting in increased immunogenicity and promotion of a non-allergic *Th1* immune response. (190–192)

It has been shown that allergoids of grass pollen obtained by maleic and succinic anhydride treatment yield a reduced allergenicity, while preserving immunogenicity, without oligomerization or aggregation of the allergoids. (189) The major mugwort pollen allergen was treated with acetic anhydride producing a highly acidic allergoid with a retained monomeric structure and reduced IgE-binding affinity. This allergoid was tested in SPT and BAT, yielding a decreased reactivity and retaining its immunogenicity. (190) Other acylation agents such as citraconic anhydride or 2,3-dimethylmaleic anhydride have been successfully used to produce allergoids with reduced allergenicity. (193)

1.8.4.3 Allergen reduction

The disulfide bonds (S—S) play an important role in the structure, stability and, therefore, allergenicity of the proteins. (194) After reduction of the disulfide bonds to free thiols (-SH), the tertiary structure of the allergens is modified and their allergenicity may decrease, when the IgE-epitopes are dependent on the disulfide bonds. (109,195) A large range of reducing agents is available to perform this modification, including small molecules such as 2-mercaptoethanol or dithiothreitol and proteins such as glutaredoxin and thioredoxin. (196)

To mitigate the allergenicity of food allergens during food processing or vaccine production for immunotherapy, the use of a non-toxic reducing agent is compulsory. Among the listed above molecules, proteins are, therefore, the most interesting potential reducing agents. Thioredoxin is a small (12 kDa) ubiquitous protein, present in almost all organisms, and is used for its disulfide bond reduction capacity. (197) Reduction of S—S bonds using thioredoxin has been applied to different food allergens and demonstrated a significant decrease in the allergenicity of wheat allergens, (197) β -LG, (198) and 2S albumins (seed proteins). (199) The decreased allergenicity may be explained by the change in conformation of the epitopes or by an increased proteolytic digestibility of the allergens. (197) On the other hand, some allergens, such as the major peanut allergen *Ara h 1*, do not contain disulfide bonds, thus their structures and allergenicity are not affected by thioredoxin treatment. (200) However, Glaser *et al.* showed that thioredoxins are a novel family of cross-reactive allergens in atopic eczema and allergic asthma, limiting their application in processing of hypoallergenic food products. (201,202)

Despite the need of supplementary purification steps to remove toxic compounds, standard reduction and subsequent alkylation of allergens using dithiothreitol and iodoacetamide has been performed with peanut allergens, resulting in a decreased allergenicity. (203,204)

1.8.4.4 Nitration of allergens

Under certain circumstances, proteins might undergo nitration reaction. In the organism, nitration and other oxidation reactions are caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). These molecules and the products of oxidation reactions play an important role as they are acting as chemical messengers. (205) ROS include mainly the hydroxyl radical (\cdot OH), superoxide anion ($O_2^{\cdot-}$), hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2), whereas RNS include nitric oxide (NO^{\cdot}) and its derivative. At high concentrations, listed compounds

induce the oxidation of DNA, proteins and other molecules, resulting, for example, in the nitration of tyrosine residues of proteins. The oxidative products were detected in several conditions and are used as biomarker for inflammatory response, (206) nitrate stress (207) or degenerative diseases. (208,209)

Tyrosine (Tyr) residue nitration is induced by its scavenging of radicals, resulting in the formation of Tyr radicals. The subsequent reaction of Tyr radicals with nitrogen dioxide (NO_2^\bullet) produces a 3-nitrotyrosine residue (NTyr), (210) as shown in Figure 4. Production of nitrogen dioxide is performed by several pathways, as shown in Figure 5, including direct oxidation of nitric oxide by oxygen. (211) The formation of peroxynitrite (ONOO^-) and its transformations in peroxynitrous acid (ONOOH) or nitrosoperoxy carbonate (ONOOCO_2^-) are also responsible for the liberation of nitrogen dioxide, as shown in Figure 5. (210,212)

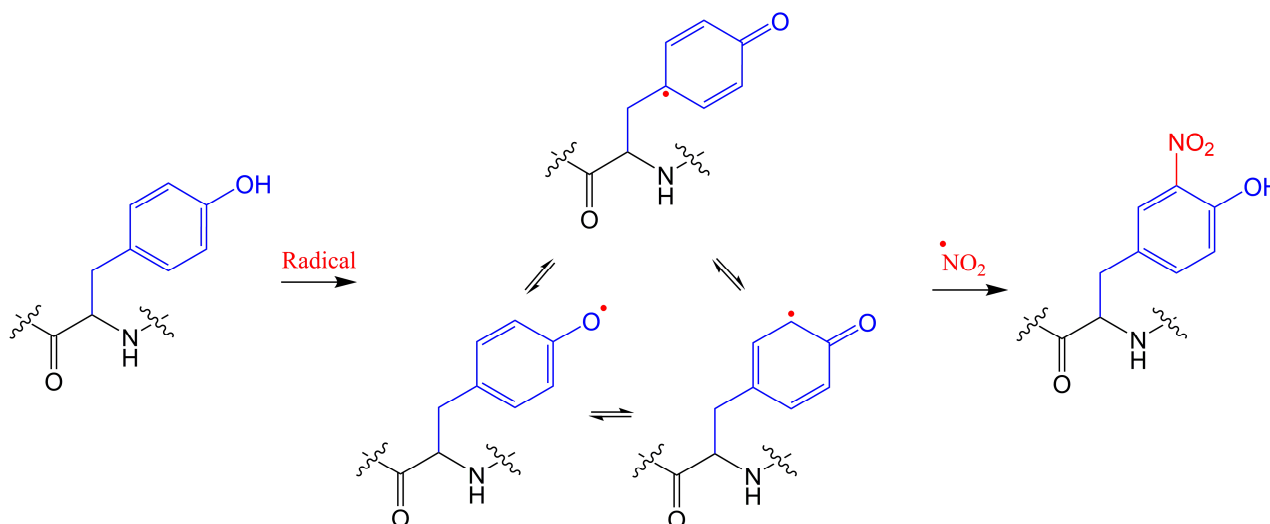


Figure 4. Radical nitration of Tyr residue yielding NTyr. The tyrosyl radical intermediate is stabilized by the delocalization of the radical.

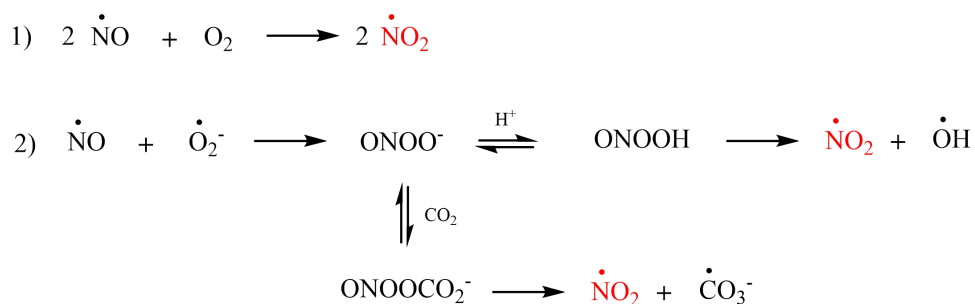


Figure 5. Mechanisms of nitrogen dioxide production by (1) oxidation of nitrogen monoxide and (2) decomposition of peroxynitrite and its derivatives.

Additionally, Tyr nitration is also induced enzymatically by peroxidases or superoxide dismutases in the presence of nitrite and hydrogen peroxide. (212) these enzymes are metalloproteins or heme-binding proteins able to catalyze the hydroxyl radical liberation from hydrogen peroxide, called a Fenton reaction. Hydroxyl radicals react with nitrite anions and Tyr residues to form nitrogen dioxide and tyrosine radicals, respectively, resulting in NTyr formation. (212) Recently, cupric ions have been shown to act as a better Fenton catalyst than Fe (III) or heme for the Tyr nitration following a chemical Fenton-like mechanism. (205)

It has been hypothesized that the urban air pollution may be responsible for the increase in allergic diseases in western countries. (213) High concentrations of ozone and nitrogen dioxide in urban smog have been shown to be responsible for the nitration of airborne proteins, like the major birch allergen *Bet v 1*. Moreover, nitrated proteins were detected in several urban samples such as urban road dust, window dust and fine air particulate matter. (214) An aerosol flow tube technique has been used to study the nitration kinetics of aerosolized bovine serum albumin (BSA) by O₃ and NO₂. (215) The results obtained with this technique showed that proteins on the surface of aerosolized particles are readily nitrated by ozone and nitrogen dioxide. Tyr oxidation and nitration have been recently extensively studied by different computational methods. (216) Nitration of ovalbumin and *Bet v 1* has been demonstrated to increase their allergenicity compared to the native allergen forms, using mouse models. (217) Ackaert *et al.* have demonstrated recently that the nitration of birch allergen *Bet v 1* modifies its immunogenicity toward an increase in allergenicity, due to the change in the allergen structure. (218) A complete study of *Bet v 1* structure and nitration degree upon nitration has been performed by LC-MS/MS under various nitration conditions. (219) In this study, peroxyxynitrite and O₃/NO₂ have been used as nitrating agents corresponding to physiological conditions and environmental pollution, respectively. Performed reactions yielded various nitration degree. Moreover, Tyr nitration occurred at different positions of allergen sequence depending on the nitrating agents. In addition to the increased allergenicity upon protein nitration, it is known that air pollutants, such as hydrocarbons and exhaust gases, can cause an oxidative stress of the respiratory system and drive pro-allergic inflammation. (220)

Food allergens may undergo nitration as well, either naturally or during food processing. It was shown that nitration of ovalbumin and β-LG enhances their allergenicity in mouse models. (206,221) Nevertheless, nitration of ovalbumin resulted in an increased digestibility and,

therefore, a decreased *de novo* sensitizing capability by oral route. (206) Meanwhile, nitration of ovomucoid, another egg allergen, did not affect its allergenicity. (221)

Based on these results, it is impossible to rationalize the effects of allergens nitration on their allergenicity and biological properties compared to the natural unmodified allergens. More efficient nitration techniques and CRD compatible with nitrated allergen are required to have a better understanding of allergen nitration impact.

1.9 Allergen cross-reactivity

In allergy, the cross-reactivity is defined as the ability of an allergen to be recognized by IgE-Abs normally specific to another allergenic protein, resulting in the appearance of clinical symptoms. (222) Cross-reactivity is caused by the presence of shared IgE epitopes in both allergens, resulting from similarities in sequences and structures. (222) On the other hand, cross-sensitization refers to the detection of specific-IgE-Abs raised against two similar allergens with the absence of clinical allergy upon contact with the cross-sensitizers. (223) As both cases demonstrate positive results in standard *in vitro* determination of IgE-Abs, *ex vivo* and *in vivo* tests are required to distinguish formally cross-reactivity from cross-sensitization. As a consequence of cross-reactivity, patients may experience allergic reactions from certain allergenic proteins even without prior exposure. (214)

The allergens causing cross-reactivity are usually originating from related species. A good example is the allergies to peanut and tree nuts, for which cross-reactive allergens are relatively common as they are part of the seed storage proteins. (225) For example, the major peanut allergen *Ara h 1*, member of the vicilin (7s globulin) family demonstrates cross-reactivity with other vicilins from walnut, hazelnut and cashew nut. (226) However, seemingly unrelated allergens might display cross-reactivity caused by high levels of similarity due to the conservation of structures and roles during the evolution process. (227) For example, the prevalence of fruit allergy is relatively low in Northern Europe. It was found that apple allergy is rarely present without the presence of specific-IgE against the major birch pollen allergen *Bet v 1*. The major apple allergen *Mal d 1* is considered inefficient to induce the production of IgE-Abs and it is therefore rare for a patient to be primary allergic to apple. (228) However, between 47 and 80 % of birch-allergic patient reported allergic reaction to apple (229,230) and the *Mal d 1* epitopes are also identified on

Bet v 1. (231) Birch is therefore considered as the primary allergen in most of the cases and sensitivity to apple in birch-allergic patients arise because of cross-reactivity.

As cross-reactivity may induce severe and potentially life-threatening allergic reactions, the clinicians often advise the avoidance of whole group of food based on the positive results of allergy tests. Recognizing cross-reactivity and distinguishing it from cross-sensitization is therefore important to adapt accordingly the patient's diets and avoid unnecessary eviction, resulting in increased quality of life and better nutrition.

1.10 Traces of allergens in food

The allergen amount required to induce the first allergic symptoms varies greatly for each patient and for each allergy. For example, peanut and sesame seed display similarities in their allergenic properties and allergists suggest to test peanut-allergic children for sesame allergy. (232) However, the eliciting doses of the two allergenic sources are extremely different. In sesame seed allergy, 30 mg have been reported as the lowest reactive threshold, (233) whereas only 100 µg of peanut proteins may be enough to identify the first allergic symptoms. (234) As even small allergen traces in food are potentially life-threatening, highly allergic patients are required to be correctly diagnosed and they need to be extremely attentive to the safety labels on food products. Additionally, the manufacturers should identify accurately the composition of their products and label them accordingly. Presence of hidden allergens is still possible due to the adventitious contamination during the food production and errors in labelling.

Despite all the precautionary measures taken by the manufacturers and the patients themselves, fatalities are still reported after the ingestion of allergens hidden in normally innocuous food. (235,236) The accurate and sensitive allergen quantification in food products is therefore of paramount importance for the safety of allergic consumers. High-throughput procedures have been developed for the quality control of industrial products. ELISA-based procedures are amongst the more popular method as they offer reliable, sensitive and highly specific results thanks to the use of specially designed Abs, making them highly interesting for routine analyses. However, the ELISA limitations include the tedious process of method development, the relatively long experiment time and the possible non-detection of allergens caused by physical or chemical modifications at the epitope sites during the food processing. (237–239) Furthermore, large quantitative differences are observed when testing identical food products on various ELISA kits. (240–

242) Those variations are caused by differences in sample preparation procedure, antibody composition, targeted allergens and calibration procedures. (243) The LODs obtained with ELISA for the detection of allergens are commonly in the ppm or sub-ppm range. (244–246) At the same time, the amplification and detection of genes, coding for known allergens, by polymerase chain reaction (PCR) is extensively used in food quality control. LODs relatively similar as ELISA-based tests were obtained for the quantification of quantify milk, peanut, almond and soy allergens. (247) PCR-based methods have the advantages of being easily automated and multiplexed. However, these tests are indirect methods of allergen detection and the detection of a DNA strand does not always correlate with the corresponding allergen presence in the sample. (248)

The improvements and development of MS in the last decades opened the possibility of using it for the accurate and sensitive identification of allergens in food following BUP approaches. In BUP, the proteins are first extracted from the sample and submitted to a reduction and alkylation process before performing an enzymatic digestion. The obtained peptides are separated by liquid-chromatography before analyzing them with the MS instrument. Standard MS techniques are not adapted for the direct protein quantification, nevertheless, various quantification methods based on protein labelling with stable isotopes have been developed. (249,250) Label-free methods also exist and are usually preferred for food allergens. (251) Label-free quantification methods are based on spectral counting or on the measurements of the area under the curve (AUC) for the peptide to quantify and/or the signal intensity of the precursor ions. Spectral counting is based on counting the number of times a specific peptide is selected and fragmented by the MS instrument. Peptide present with higher abundance in the sample are assumed to be selected more often than low abundant peptides, allowing a relative quantification. (252,253) However, suppression of less abundant peptides, differences in peptide ionization efficiencies, and detector saturation effects are not considered, (254) making this method delicate to apply to traces quantification. On the other hand, the quantification procedures relying on peptide ions heights and AUC are based on the notion that the peptide intensity is correlating with its concentration in the sample. (252) Differences in sample complexity is known to affect the intensities of the peptides ions and these methods are therefore limited to the comparison of samples having the same composition and comparable levels of complexity. (253) Additionally, special care is required during the sample preparations and MS analyses to ensure a high level of consistency. (253,255) In general, AUC is preferred compared to other methods; however, AUC is limited by the computational method to

process the data and by peptide co-elution. (256) Quantification based on signal heights is therefore relatively well adapted for the label-free quantification of allergen traces in food and easier to implement than AUC procedures.

Different scan modes are available on modern MS instrument for quantitative analyses. The simplest one is based on the untargeted data collection in full MS scan mode and the creation of extracted ion chromatograms (XIC) in a post-acquisition step for the ions of interest. The peak heights or areas are then used to determine the relative abundance of the peptides. Other techniques such as selected ion monitoring (SIM), selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) are more cumbersome to implement, but demonstrate higher sensitivity. (257) In SIM procedure, the data are acquired in scanning mode with a restriction on the m/z range, creating a window of allowed ions around a chosen m/z value. The narrower the mass range, the more specific the SIM assay. The quantification is then based, as for XIC procedures, on the obtained ion chromatograms. SIM demonstrates in general a better sensitivity than full scan experiments as the MS instrument dwell time is longer. (257) On the other hand, SRM and MRM are based on monitoring the presence and intensity of pairs of parent ion and one of its fragment ions for specific fragmentation events identified in preliminary experiments, called transitions. (258)

For the different methods of label-free quantification described above, LODs were found to be in the same range as ELISA-based procedures for food quality control. (259–261) In addition to their straightforward multiplexing, the main advantage of MS techniques over ELSIA or PCR procedures is the direct identification of proteins by their proteotypic peptides with indifference towards the presence of epitopes in their native or potentially modified states. Label-free MS methods have been successfully applied to quantify egg, (262–265) milk, (259,262,266–268) and nuts (264,269–271) allergens in various food matrices.

TDP approaches were also used for the quantification of milk allergens in various matrices (272,273) but their uses remain marginal compared to BUP.

1.11 Immunomagnetic separation

Analysis of complex samples, such as the blood serum of an allergic patient or the allergen extracts obtained from natural sources, is a serious challenge. In most cases, such samples are

pre-treated to isolate and concentrate the target molecules from the complex matrix allowing easier analyses. The common separation methods used in the laboratory such as gas chromatography, liquid chromatography and capillary electrophoresis are crucial for the analysis of complex analytes. Additionally, compounds present in low concentration may be hard or impossible to detect depending on the dynamic range of the chosen analytical method. Therefore, a pre-concentration step is sometimes required. Evaporation of the solvent, liquid-liquid (micro-) extraction (LLE or LLME) (274,275) and solid-phase extraction (SPE) (276) are common pre-concentration techniques.

SPE was extensively studied for the extraction of biological macromolecules, because it shortens the extraction time, reduces the solvent consumption and has a high recovery rate. (277) Conventional SPE is based on the injection of the solution into an SPE column, capturing the target molecules as they pass through the adsorbent material, whereas in batch SPE the adsorbent particles are directly added to the solution. (278) Functionalized magnetic materials are widely used in SPE as sorbent for their interesting properties. The most used functionalized magnetic materials are the magnetic beads (MBs) with an iron-oxide core. The coating of their surfaces and the functionalization with specific reactive groups is based on well-known chemistry. Moreover, they have high aqueous dispersibility, large surface-to-volume ratio and their use is relatively straightforward. (278)

Immunomagnetic separation (IMS) is a special case of magnetic-based SPE. IMS uses MBs, functionalized with appropriate Abs, to extract, purify and isolate various analytes from a wide range of matrices including biological fluids like blood plasma and serum. For instance, MBs were successfully used to isolate free nucleic acids from blood plasma, (279) remove white and red blood cells from blood sample to obtain blood plasma, (280) desorb peptides from abundant blood proteins (281) or separate and detect cancer cells from a cell mixture. (282) Moreover, MBs as an immunosorbent are cheap and they offer unique properties of high loading capacity due to large surface-to-volume ratio, a large variety of functional coatings together with facilitated sorbent manipulation, renewal and good reusability. (283) MBs coated with the desired Abs have been immobilized in a capillary electrophoresis system to perform the extraction, separation and on-line detection of target molecules. (284) Recently, such technique, called immunoaffinity capillary electrophoresis (IACE), has been successfully used for the development of a CRD of cow's milk allergy. (71)

1.12 Thesis outlines

This thesis presents novel analytical methods developed for the allergy CRD, the allergen identification and quantification in food matrices and the characterization of allergen modifications, notably nitration. In Chapter 2, a fast and personalized CRD procedure based on IMS-MALDI-MS is presented for the diagnosis of cow's milk and egg allergies and for the resolution of complex clinical cases. Chapter 3 is devoted to the adaptation of the IMS-MALDI-MS procedure and its application to peanut and tree nut allergies using the blood sera of patients taking part in the European clinical study *ProNut*. In addition to the diagnosis outcomes obtained with the developed IMS-nanoESI-HRMS procedure, this chapter yields precious information for the cross-reactivity and cross-sensitization of peanut and tree nuts allergens. Chapter 4 was focused on the allergen nitration using various nitrating agents. Two of the most efficient nitrating procedures in terms of nitration and oxidation degrees were used to nitrate individual allergens or natural extracts and the nitration sites were identified by BUP. In Chapter 5, the presence of minute amount of allergens were identified and quantified in various food products, causing allergenic reaction in peanut and hazelnut patients, using a standard protein extraction procedure followed by BUP analysis. The general conclusions of this thesis and future perspectives of the developed techniques are compiled in Chapter 6.

1.13 References

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Chapter 2.

Component-resolved diagnosis for personalized and rapid test of food-related allergy

Based on M. Frossard et al., Journal of Allergy and Clinical Immunology, 2018, 141 (6), 2297

2.1 Introduction

IgE-mediated allergies are abnormal responses of the immune system, defined as type I hypersensitivity reactions, caused by the production of IgE antibodies (IgE-Abs) against specific antigens, i.e. allergens. It is estimated that 2 % of adults and 2-8 % of the young infants are allergic to one or more food proteins. (1)

Cow's milk allergy and hen's egg allergy were selected as case studies in the present work, as they are the two most common food allergies during childhood. Milk allergy affects 2-3 % of infants in their first year of life and is usually outgrown. Nevertheless, 15 % of these children remain allergic to milk during their entire life. (2) Egg allergy is the second most common food allergy in young children; it is estimated that 0.5 to 2.5 % of them suffer from it. (3–7) As for milk allergy, it is usually outgrown. (8) The major allergens for milk and egg are listed below in Table 1. Less common allergens may be found on online database such as the WHO/IUIS Allergen nomenclature and the Allergome platform. (9,10)

Table 1. Major milk and egg allergens with their standard nomenclature and UniProt accession numbers.

Organism	Protein name	Allergen nomenclature	UniprotKB
Bos taurus	α -Lactalbumin	Bos d 4	P00711
Bos taurus	β -Lactoglobulin	Bos d 5	P02754
Bos taurus	Bovine serum albumin	Bos d 6	P02769

Bos taurus	Immunoglobulin	Bos d 7	--
Bos taurus	Caseins	Bos d 8	--
Bos taurus	α S1-casein	Bos d 9	P02662
Bos taurus	α S2-casein	Bos d 10	P02663
Bos taurus	β -Casein	Bos d 11	P02666
Bos taurus	κ -Casein	Bos d 12	P02668
Bos taurus	Lactoferrin	Bos d LF	P24627
Gallus domesticus	Ovomucoid	Gal d 1	P01005
Gallus domesticus	Ovalbumin	Gal d 2	P01012
Gallus domesticus	Ovotransferrin	Gal d 3	P02789
Gallus domesticus	Lysozyme C	Gal d 4	P00698
Gallus domesticus	Serum albumin	Gal d 5	P19121

The early diagnosis of milk allergy, as well as other food allergies, is crucial for the allergen avoidance by the patient and allergy treatment. The precise history of the allergic reactions is the cornerstone of the correct diagnoses and allows listing all suspected proteins for further tests. A wide panel of diagnostic techniques exists nowadays. (11) Skin prick tests and oral food challenges are the golden standards for *in vivo* food allergy diagnoses. (12) Most of the time, *in vitro* specific IgE-Abs determinations are performed to confirm the *in vivo* diagnosis. These tests, often referred to component-resolved diagnosis (CRD), are also useful in estimating the risk associated with particular allergen classes and allergy persistence. (13,14)

Typically, *in vitro* specific IgE-Abs determination is performed by classical enzyme-linked immunosorbent assay (ELISA) and its microarray variations. Both versions are currently commercially available, (15–18) require 25-40 μ L of patient's blood serum and provide limit of detection (LOD) for IgE-Abs of 0.24 $\text{ng}\cdot\text{mL}^{-1}$. They employ purified recombinant allergens and take 3-4 h. These techniques are able to detect IgE-Abs only against known allergens, thus limiting the clinicians in the choice of potential sensitizers. Utilization of whole food extracts could tackle this issue. This approach could also enable the recognition of culprit proteins, hidden in complex food matrices (e.g. spices).

In this chapter, a new personalized allergy test with whole food extract application was developed. Immunomagnetic separation was chosen, as it brings important features for high throughput diagnosis like a robust equipment, simple design, high specificity and low sample volume required. (19) This method uses magnetic beads (MBs), functionalized with appropriate anti-

bodies to be personalized by the patient's antibodies, to extract, purify and isolate various analytes from a wide range of matrices. (20–23) A common detection method employed after immunomagnetic separation is mass spectrometry (MS), currently becoming a promising tool for allergen detection in food. (24,25) However, for allergy diagnosis MS application is still very limited. (11) Herein, the great potential of MS for IMS-based allergy diagnostic technique was demonstrated using complex components like whole food extracts.

Immunomagnetic separation (IMS) was firstly used to extract the IgE-Abs from the blood serum of allergic patients using MBs functionalized with anti-human IgE-Abs, as illustrated in Figure 1. Then, the CRD of milk allergy was performed by probing the binding of the obtained anti-human IgE/IgE-Abs immunocomplexes with individual protein solutions or whole extracts. After elution of the captured allergens and IgE-Abs from the MBs surface, the extracted proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) or by peptide mass fingerprinting (PMF) after performing the proteolysis with trypsin immobilized into macroporous ordered siliceous foam (MOSF). The whole procedure is conducted just in Eppendorf tube without complicated setups or reagents. It can directly screen food extracts and takes less than 45 min, or 100 min with trypsin digestion, keeping high diagnostic accuracy due to the MS detection. After tests with commercial blood serum samples, we have successfully demonstrated the advantage of the developed diagnostic procedure on several clinical cases.

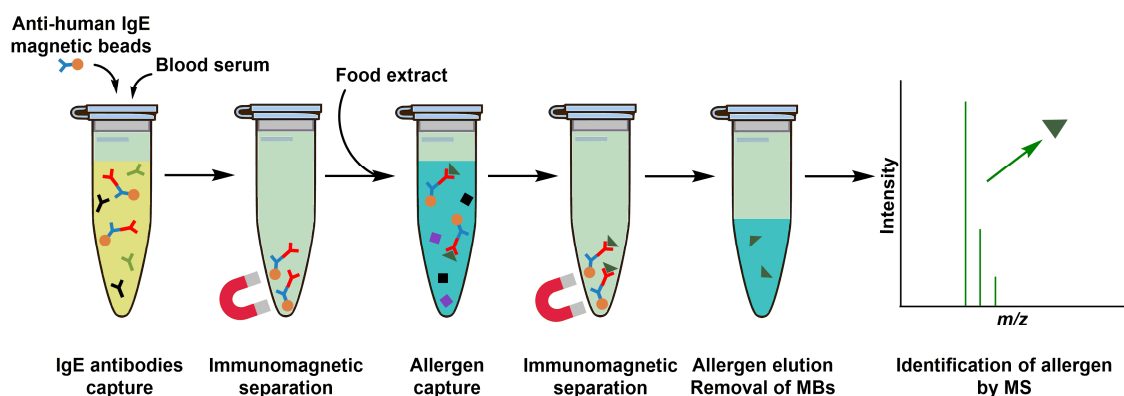


Figure 1. Allergy CRD procedure: MBs coated with anti-human IgE-Abs are used to carry out the immunomagnetic separation of total IgE-Abs, followed by allergenic proteins capture from individual solutions or food extracts. After elution, the captured proteins are identified by MALDI-MS.

2.2 Materials and methods

2.2.1 Chemical and materials

Estapor tosyl-activated superparamagnetic polystyrene beads with a diameter of 1.29 μm were kindly offered by Merck Chimie SAS (Fontenay-sous-Bois, France). Mouse monoclonal anti-human IgE-antibodies (0100-0414) were obtained from AbD Serotec (Oxford, United Kingdom). Dimethyl suberimidate (DMS) and control human IgE were purchased from Pierce Biotechnology (Rockford, IL, USA). Commercial blood serum of a patient with milk allergy was obtained from Bio-reclamation LLC (Westbury, NY, USA). All the milk and egg proteins used, polysorbate surfactant Tween 20, acetone, sodium phosphate mono/dibasic, iodoacetamide (IAA), DL-dithiothreitol (DTT) and 2,5-dihydrobenzoic acid were purchased from Sigma-Aldrich (Buchs, Switzerland). Angiotensin I (trifluoroacetate salt) was obtained from Bachem (Dübendorf, Switzerland). MOSF was kindly offered by Prof. Baohong Liu, Fudan University (Shanghai, China).

Sodium borate, ammonium acetate and methanol were purchased from Merck (Darmstadt, Germany) and sinapinic acid, ammonium bicarbonate, trifluoroacetic acid (TFA), acetic acid (HAc) from Fluka (Buchs, Switzerland) and urea from Acros Organics (Switzerland). Trypsin (from bovine pancreas) was obtained from AppliChem (Darmstadt, Germany). Acetonitrile was obtained from ABCR (Karlsruhe, Germany). Deionized water used for solutions and buffers was produced by an alpha Q-Millipore System (Zug, Switzerland). Whole ultra-high-temperature (UHT) processed cow's milk (3.5 % fat), skimmed milk powder (35 % of protein content by mass), soymilk, UHT goat milk (3 % fat), pasteurized sheep milk (6.3 % fat) and fresh eggs were purchased from a local store. The blood sera of patients with proven egg and milk allergy and the blood sera of clinical patients with potential allergy were kindly provided by the Institut Central des Hôpitaux (Sion, Switzerland) after approbation by the relevant ethics committee (*Commission cantonale valaisanne d'éthique médicale*, CCVEM, example of an approbation letter in *Appendix I*) and with the patient consents.

2.2.2 Fonctionnalization of MBs

The functionalization of MBs was performed according to the manufacturer protocol. (26) First, 20 μL of MBs ($100 \text{ mg}\cdot\text{mL}^{-1}$) were washed three times with a coating buffer (0.1 M sodium borate buffer, pH 9.5) and resuspended in 20 μL of the same buffer. 80 μL of anti-human IgE-antibodies ($1 \text{ mg}\cdot\text{mL}^{-1}$), 260 μL of coating buffer and 166 μL of 3 M ammonium sulphate in coating buffer were added to the MBs suspension. The obtained solution was incubated overnight at room

temperature under continuous moderate stirring to avoid MBs sedimentation. Afterwards, the supernatant was removed and 500 μL of the blocking buffer (10 mM PBS solution, 0.1 % Tween 20, pH 7.4) were added to the MBs for 1 h of incubation at room temperature. Then, the MBs were washed three times with washing buffer (10 mM PBS solution, 0.05 % Tween 20, pH 7.4) and re-suspended in the storage buffer (10 mM PBS solution, 0.025% Tween 20, 0.02% NaN_3 , pH 7.4) to obtain a final concentration of 5 $\text{mg}\cdot\text{mL}^{-1}$. All the MBs were stored at 4°C and used within a period of 3 weeks after their coating.

2.2.3 Chemical cross-linking of IgE Abs to MBs

The procedure of IgE cross-linking to the MBs was adapted from the DMS manufacturer protocol (27) to obtain an efficient cross-linking with minimal loss of antibodies activity. After the IgE-Abs extraction step, 150 μg of the resulting MBs were dispersed in 200 μL of 0.2 M triethanolamine (pH 9.0), then, mixed with 1 mL of 2 mM DMS (for milk and bolete allergies) or 20 μM DMS (for egg white allergy) in 0.2 M triethanolamine and incubated for 30 min at room temperature. To stop the reaction, 200 μL of 50 mM Tris-HCl buffer (pH 7.5) were added to the mixture and incubated for 15 min. After rinsing with washing buffer, the MBs with cross-linked IgE were either directly used for the CRD or stored in the storage buffer at 4°C.

2.2.4 Milk fraction and egg white protein solution preparation

A 20 $\text{mg}\cdot\text{mL}^{-1}$ solution of skimmed milk powder was prepared in deionized water. 1 mL of obtained milk solution was acidified to pH 4.5 by addition of an ammonium acetate buffer (50 mM, pH 4.5) and stirred for 2 min. Then, the milk sample was centrifuged for 2 minutes at 13000 g to accumulate the precipitated casein fraction and separate it from the supernatant. The obtained casein pellet was washed three times with the ammonium acetate buffer and separated from it by centrifugation in order to remove the whey proteins trapped in the casein precipitate. The washed pellet was dissolved in 1 mL of ammonium bicarbonate buffer (25 mM, pH 8.5) for further use. The supernatant containing the whey proteins was mixed with additional amount of ammonium acetate buffer and was incubated under medium stirring during 10 minutes to ensure complete precipitation of the caseins. After centrifugation and precipitate removal, the whey fraction was ready to use. The whole UHT milks originated from cow, sheep and goat were firstly defatted by centrifugation for 5 min at 13000 g and, then, separated into casein and whey fractions using the same procedure as for the skimmed milk powder solution.

The egg white was separated from the yolk of a fresh egg and 1 g of egg white was diluted in 100 mL of 10 mM PBS solution (pH 7.4). As the protein content in egg white is approximately 10 %, the final protein content corresponded to 1 mg·mL⁻¹. The obtained solution was used for the CRD procedure without further processing.

2.2.5 Preparation of *Boletus edulis* protein extract

The protein extraction of *Boletus edulis* was carried out by agitating 0.5 g of freshly ground king bolete at room temperature in 40 mL of PBS buffer (100 mM, pH 7.4). The solution was centrifuged and the supernatant was collected and filtered through a PVDF syringe filter (CHROMAFIL Xtra, PVDF-45/25, Macherey-Nagel AG, Switzerland). A standard cold acetone precipitation, with a 1:7 v/v ratio of sample to acetone, was performed overnight to purify the proteins. After protein pellet washing and resolubilisation in ammonium bicarbonate buffer (25 mM, pH 8.5), the protein solution was lyophilized and stored at -20°C until further use.

2.2.6 Optimized immunomagnetic separation procedure for the milk allergy CRD

150 µg of anti-human IgE-Abs-functionalized MBs were transferred into an Eppendorf tube and washed three times with the washing buffer. 100 µL of the blood serum from an allergic patient were added to the MBs pellet and incubated for 5 min at room temperature under medium stirring. After the incubation, MBs were separated from the supernatant, washed three times with a washing buffer and once with deionized water. An additional step of cross-linking could be carried out to covalently link the patient IgE-Abs to MBs. The cross-linking procedure was adapted from the protocol of the chemical linker manufacturer (27) and is detailed above.

The resulting MBs with the immunocomplexes, formed on their surface by anti-human IgE-Abs and the patient IgE-Abs, were incubated with 200 µL of individual protein solutions (200 µg·mL⁻¹ concentration) or natural extracts at room temperature for 5 or 20 min, respectively. Milk extracts obtained from skimmed milk powder were used as prepared, whereas UHT milk fractions and egg white were diluted three and five times, respectively, before extraction. Then, MBs were separated from the allergen solution, rinsed three times with washing buffer and once with deionized water. The captured proteins were eluted by adding 10 µL of elution solution (5 % acetic acid (HAc) or 1:3 (v/v) methanol/water solutions) to the MBs pellet and 1 µL of the eluate was submitted to the MALDI-MS analysis.

2.2.7 Preparation of trypsin-loaded MOSF

1 mg of MOSF was added to 100 µg of trypsin dissolved in 1 mL of 25 mM ammonium bicarbonate buffer (pH 7.8) and incubated at room temperature for 30 min. After the incubation, the suspension was centrifuged, the supernatant was discarded and the MOSF particles were washed two times with ammonium bicarbonate buffer. The obtained trypsin loaded MOSF was stored at 4°C in 200 µL of ammonium bicarbonate buffer, at a final working MOSF concentration of 5 mg·mL⁻¹.

2.2.8 Allergen digestion using trypsin-loaded MOSF

Prior to the digestion, MBs bearing all immunocomplexes, formed during the allergy CRD, were added into 10 µL of 25 mM ammonium bicarbonate buffer (pH 7.8), and directly denatured at 95°C for 5 min without an elution step. After the denaturation, MBs were removed from the reaction mixture. 1 µL of the trypsin-loaded MOSF was added to the protein solution and incubated at 37°C for 20 or 60 min during CRD with individual milk protein solutions or milk fractions, respectively. After the incubation, 1 µL of the digested solution was analysed by MALDI-MS.

Before the digestion of egg allergens, the eluted proteins were denatured in 25 mM ammonium bicarbonate buffer containing 6 M urea and reduced by adding DTT to a final concentration of 5 mM. The solution was then incubated at 56°C for one hour, cooled down to room temperature and IAA was added to a final concentration of 15 mM. The alkylation reaction was performed at room temperature in the dark for 30 minutes. Afterwards, the captured allergens were precipitated using a standard chloroform/methanol procedure to remove urea and solubilized in 25 mM ammonium bicarbonate buffer. The digestion was then performed with trypsin-loaded MOSF for 60 min as described above.

2.2.9 MALDI-MS analyses

A MALDI-TOF Microflex mass spectrometer (Bruker Daltonics, Bremen, Germany) was used for all MALDI-MS experiments. The matrix for the protein analysis contained 10 mg·mL⁻¹ of sinapinic acid in 70:29.9:0.1 (v/v) acetonitrile/water/trifluoroacetic acid (TFA) mixture. During the proteolytic peptide analysis, 10 mg·mL⁻¹ of 2,5-dihydrobenzoic acid in 70:29.9:0.1 (v/v) acetonitrile/water/TFA mixture were used as a matrix. For each MALDI-MS analysis, 1 µL of sample was premixed with 1 µL of the corresponding matrix solution, then, was spotted onto the MALDI plate and dried under ambient conditions prior to analysis.

The MALDI-MS spectra of intact proteins were recorded in positive linear mode. Each spectrum was obtained by averaging 800 laser shots at different positions on the sample spot, with a laser frequency of 20 Hz and variable intensity. The sample rate was set to 0.5 or 1 GS/s depending on the desired resolution. All the proteins MS spectra have been smoothed by the Savitzky-Golay algorithm using Origin Pro 9 (OriginLab Corporation, Northampton, USA). A quadratic polynomial order was selected with a window size of 20 to 300 points for each spectrum in a way to decrease the spectral noise without losing peak information. Afterwards, all peaks were fitted with a Gaussian using Origin Pro 9 fitting tool and the protein masses were reported as the centroid of the obtained Gaussian function.

The mass accuracy of the calibrated instrument in the linear mode was determined using bovine α -lactalbumin (α -LA). The accurate mass of α -LA was determined to be 14168.76 Da by deconvolution (Protein Deconvolution 4.0, Thermo Scientific, San Jose, USA) of the MS spectrum recorded with a high-resolution instrument, Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, San Jose, USA). The mass of α -LA obtained by MALDI-TOF-MS was 14186.38 Da, corresponding to a mass accuracy of approximately 1172 ppm.

For the analyses of peptides, the reflectron mode was used and each spectrum was acquired as an average of 500 laser shots. The mass accuracy of the calibrated instrument in this mode was determined using Angiotensin I (protonated monoisotopic mass 1296.6853 Da) and was found to be approximately 400 ppm. The obtained peptides mass spectra were processed using the FindMod tool from ExPASy (Expert Protein Analysis System) of the Swiss Institute of Bioinformatics. (28) The mass tolerance for the similarity analyses was chosen as 0.5 Da with 3 allowed missed cleavages.

2.3 Results and discussion

2.3.1 Optimization of immunomagnetic separation parameters for IgE-Abs extraction from control solution

For the effective CRD development, a crucial step is the extraction of IgE-Abs from the patient blood serum. The human blood serum is a very complex matrix containing a wide range of proteins, in concentrations typically between 60 and 80 g·L⁻¹. Due to such blood serum complexity, we firstly used 2 μ g·mL⁻¹ solutions of control human IgE-Abs to define the starting experimental conditions for immunomagnetic separation.

In the first tests, 50 μg of MBs were used to extract IgE-Abs from 100 μL of 2 $\mu\text{g mL}^{-1}$ IgE-Abs control solutions with a 15 minutes incubation time. The elution was performed using 10 μL of the acidic elution buffer (pH 2.4). The MALDI-MS spectrum obtained from the eluate showed a peak at m/z 151890, (Figure 2.a) though the peak of the control IgE-Abs used should correspond to m/z 194546 (Figure 2.b). These MALDI-MS spectra display very broad peaks, which is typical for such large biomolecules, as the resolution of MALDI-MS decreases at high ratio of mass to charge. (29) The signal-to-noise ratios are low due to the poor sensitivity of typical MALDI-MS instrument towards such large molecules as antibodies. (30,31) The peak at m/z 151890 could either correspond to IgG-Abs (approximate molecular weight: 150 kDa) released from the MBs functional coating or a potential degradation of IgE-Abs under acidic pH used for elution step during immunomagnetic separation. To test the first hypothesis, immunomagnetic separation procedure was performed using pure water as a blank IgE-Abs sample, and no IgG Abs released from the MBs were detected in the respective m/z range (Figure 2.c). To the best of our knowledge, no studies have shown an acidic degradation of human IgE-Abs so far. Therefore, the stability of IgE-Abs at acidic pH was tested by incubating 1 μL of IgE-Abs solution ($100 \mu\text{g}\cdot\text{mL}^{-1}$) with 9 μL of elution buffer (5 % HAc, pH 2.4) during 10 min. The resulting MALDI-MS spectrum (Figure 2.d) revealed no evident degradation of IgE-Abs.

Based on the obtained results, it was assumed that the IgE-Abs were degraded during the elution procedure of the immunomagnetic separation, but this degradation was mediated by the combination of the acidic conditions with the IgE-Abs binding conformation on the MBs. The intensity of the IgE-Abs peak was further used to evaluate the extraction parameters during the immunomagnetic separation conditions optimization.

The quantity of MBs is a major parameter of immunomagnetic separation and represents a large portion of the analysis costs due to the price of MBs and their anti-human IgE-Abs coating. Initially, the amount of MBs used to perform the immunomagnetic separation from control solution was set to 50 μg (Figure 3.a). Increasing this amount to 100 μg resulted in a slight increase in the intensity of the IgE-Abs peak obtained in the MALDI-MS spectrum (Figure 3.b), whereas using 25 μg of MBs gave no peak (data not shown). When adapting this method to blood serum, a larger quantity of MBs may be required to compensate for the crowding of the solution.

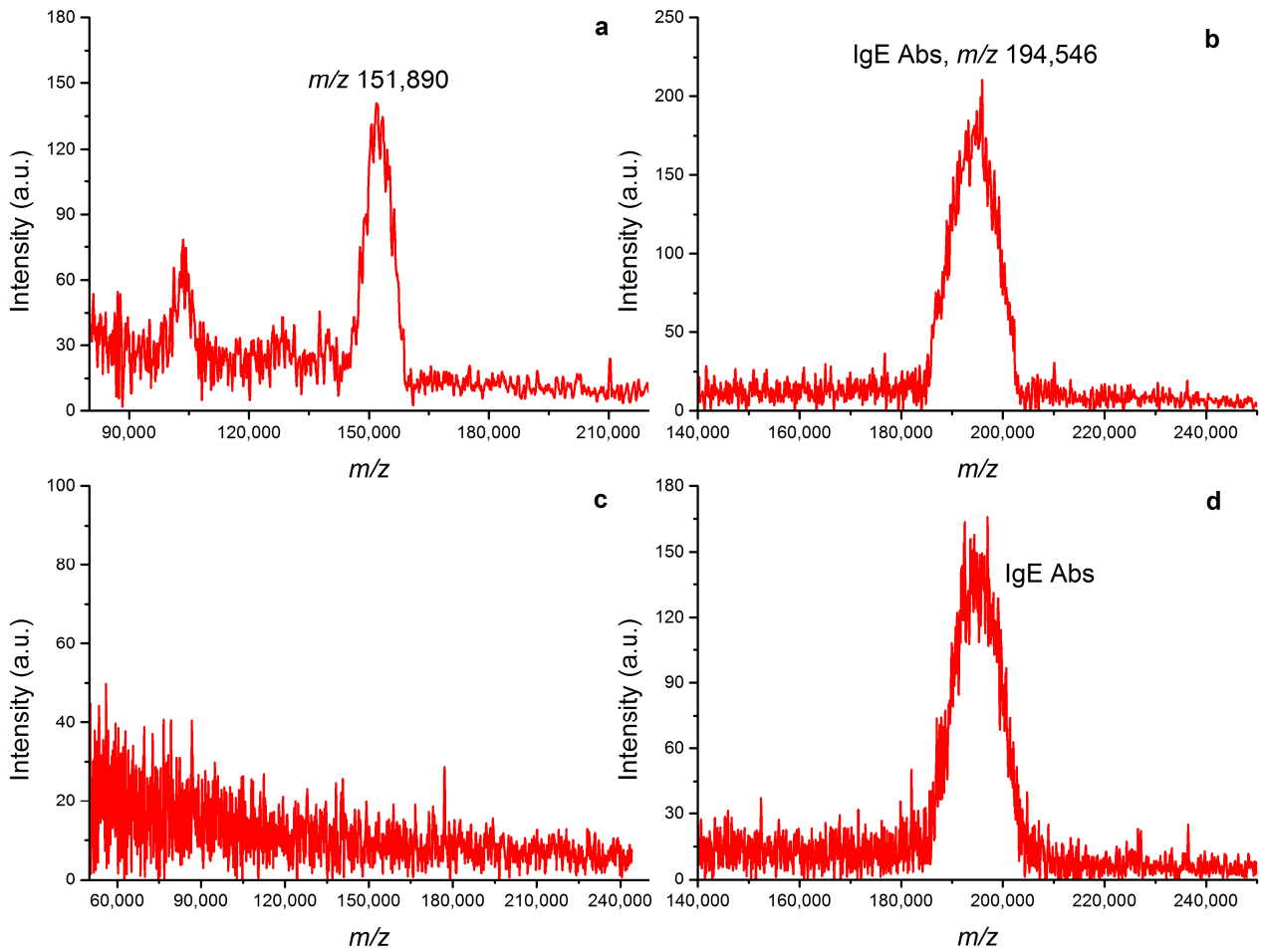


Figure 2. (a) MALDI-MS spectrum obtained during the IgE-Abs extraction by immunomagnetic separation from an IgE-Abs control solution ($2 \mu\text{g}\cdot\text{mL}^{-1}$). (b) MALDI-MS spectrum of the control IgE-Abs at $75 \mu\text{g}\cdot\text{mL}^{-1}$. (c) MALDI-MS spectrum obtained from immunomagnetic separation of a blank solution. (d) MALDI-MS spectrum obtained after a 10 min incubation of control IgE-Abs solution ($100 \mu\text{g}\cdot\text{mL}^{-1}$) with 5 % HAc. Immunomagnetic separation parameters: 50 μg of MBs, 15 min incubation, 10 μL of elution solution (pH 2.4).

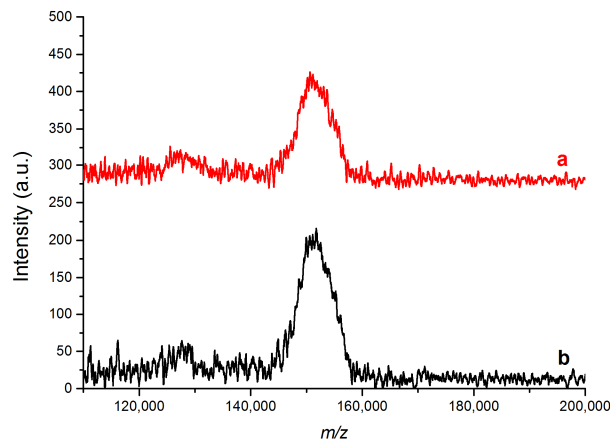


Figure 3. MALDI-MS spectra obtained during IgE-Abs extraction by immunomagnetic separation from a control solution using (a) 50 μg and (b) 100 μg of MBs. Parameters: 100 μL of $2 \mu\text{g mL}^{-1}$ IgE-Abs control solution, 15 min incubation, 10 μL of elution buffer, pH 2.4.

MBs manufacturers advise 5 min as a minimal incubation time for typical immunomagnetic separation of proteins. (32,33) It was thus assessed that 15 min incubation were enough to have a maximal capture of IgE-Abs and this incubation time was not modified. The incubation temperature plays an important role in the binding kinetics and thermodynamics of the antibodies to their targets. Many antibody-antigen interactions have a higher affinity at low temperature due to the formation of hydrogen bonds and polar interactions. (34) To predict the effect of the temperature on the intermolecular interactions, it is necessary to determine the amino acid sequence of the epitopes and paratopes. In order to keep the CRD procedure as straightforward as possible and with minimal equipment, the incubation of all immunomagnetic separation experiments was performed at room temperature.

As described before, the elution of IgE-Abs from the MBs was carried out using 10 μL of elution buffer (5 % HAc, pH 2.4) and resulted in their partial degradation (Figure 2.a). To check the effect of more acidic elution buffer on the efficiency of IgE-Abs elution, two immunomagnetic separation procedures were performed in parallel applying either 10 μL of a strong acidic elution buffer (10 % HAc and 0.1 % TFA, pH 1.5) or 10 μL of the previously used elution buffer (5 % HAc, pH 2.4). The respective MALDI-MS spectra of the eluates (Figure 4.a and 4.b) displayed similar elution efficiency and the same degradation of IgE-Abs.

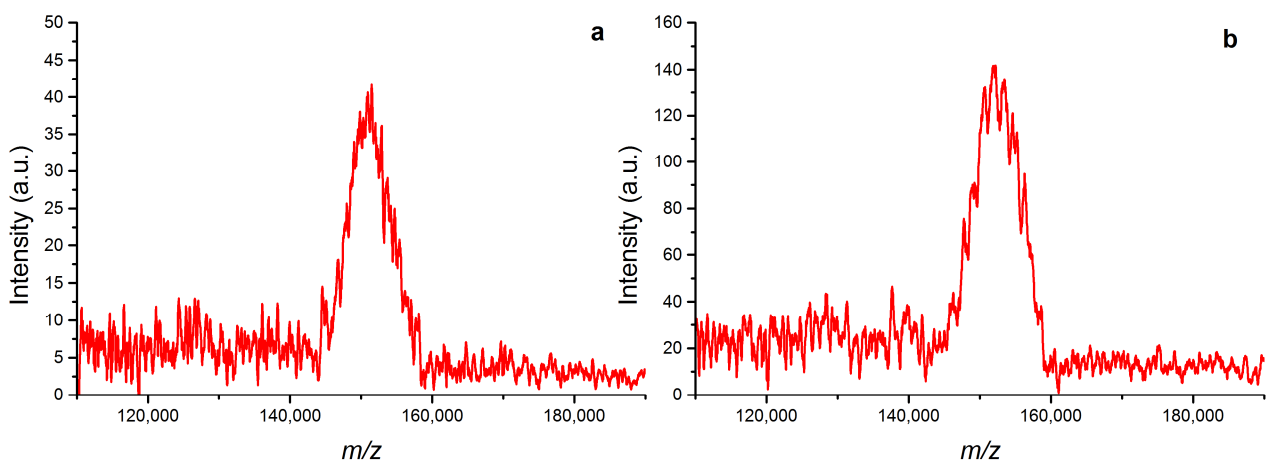


Figure 4. MALDI-MS spectra obtained during IgE-Abs extraction by immunomagnetic separation from a control solution using (a) 10 μL of elution buffer, pH 1.5 and (b) 10 μL of elution buffer, pH 2.4. Parameters: 50 μg of MBs, 100 μL of 2 $\mu\text{g mL}^{-1}$ IgE-Abs control solution, 15 min incubation.

As explained above, the signal-to-noise ratio and intensity are low for large molecules such as antibodies with typical MALDI-MS instrument. Having more concentrated eluates allows obtaining more intensive IgE-Abs peaks in the MALDI-MS spectra. To increase the IgE-Abs concentration in the eluate, nitrogen supported solvent evaporation is a method of choice. With this technique, 10 μL of eluate are reduced to 2 μL in two minutes at room temperature using a constant flow of nitrogen, increasing the concentration of IgE-Abs by a factor of 5. Figure 5.a corresponds to the MALDI-MS spectrum of the concentrated eluate obtained from a 10 μL eluate (Figure 5.b). This method may be used as an extra step before the analysis of eluates by MALDI-MS. Meanwhile, direct decreasing of the elution buffer volume was not possible, as volumes smaller than 10 μL are hard to be manipulated and do not provide a suitable dispersion of the MBs. Based on the above tests, extraction of control IgE-Abs using 100 μg of MBs with an incubation time of 15 minutes followed by 10 μL acidic elution resulted in satisfactory signal in MALDI-MS spectra.

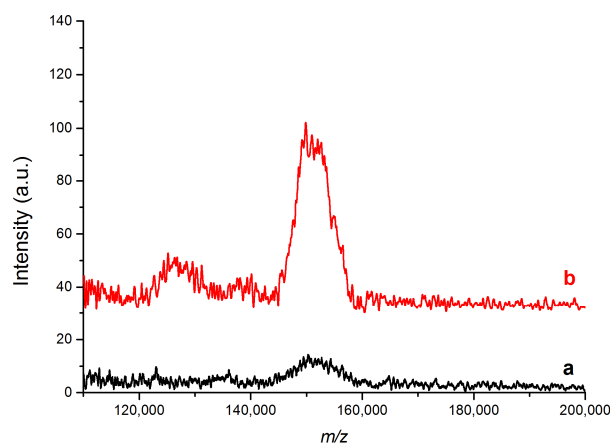


Figure 5. MALDI-MS spectra obtained during IgE-Abs extraction by immunomagnetic separation from a control solution **(a)** after concentration to 2 μL and **(b)** before concentration of eluate. Parameters: 100 μg of MBs, 100 μL of 2 $\mu\text{g mL}^{-1}$ IgE-Abs control solution, 15 min incubation, 10 μL of elution buffer, pH 2.4.

2.3.2 Optimization of immunomagnetic separation for IgE-Abs extraction from blood serum

Blood plasma is a complex matrix containing thousands of different proteins. Nevertheless, only 22 different proteins compose approximately 99 % of the total protein content of plasma. (35) Removing the clotting factors, such as fibrinogen, from the blood plasma yield blood serum, with protein concentration between 60 and 80 g of proteins per liter. The concentration of

antibodies (*i.e.* immunoglobulins) in blood serum varies significantly from person to person, depending on a high number of parameters such as the age, sex, residence, past diseases, *etc.* In general, all the combined immunoglobulins present in blood serum represent 11-15 % of its total protein content. (36) IgE-Abs, the target of the designed immunomagnetic separation procedure, account only for approximately 0.002 % of the total immunoglobulins. (37,38) Due to the extremely low IgE-Abs concentration, parameters such as the sample volume, washing procedure and the amount of MBs become extremely important and need to be optimized. The optimization of the IgE-Abs IMS was carried out using a commercial blood serum of a patient with a known allergy to milk.

As for all diagnostic procedure, it is interesting to decrease the sample volume required for each analysis to the minimum. For this reason, various blood serum volumes were tested while using the basic parameters previously optimized using control IgE-Abs solutions. Utilization of 200 and 100 μL of blood serum resulted in very similar MALDI-MS spectra (Figure 6.a.2 and 6.a.3, respectively) with low intensity for IgE-Abs, whereas application of 50 μL provided no distinctive peak (Figure 6.a.1). Therefore, 100 μL of blood serum was chosen as a reasonable sample volume for further manipulations.

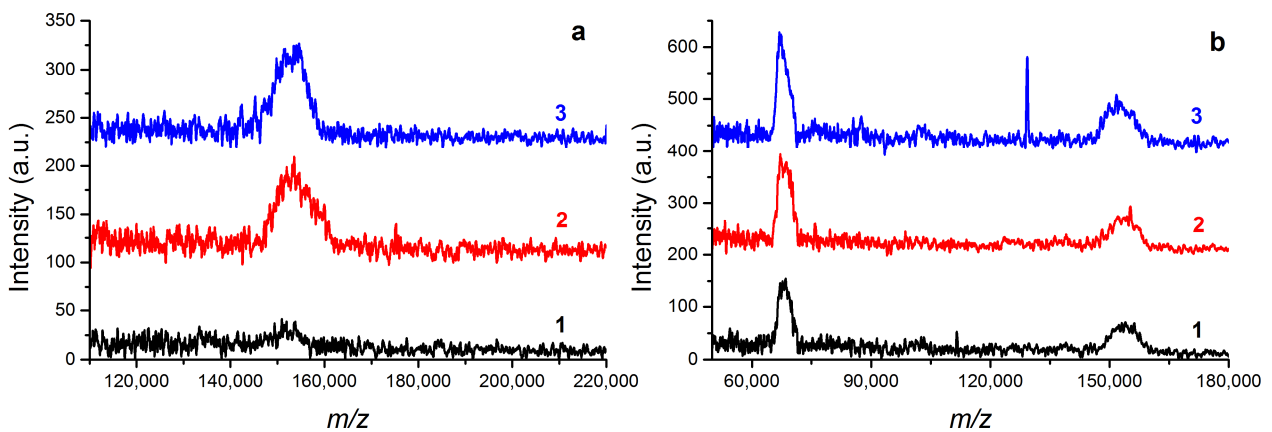


Figure 6. (a) MALDI-MS spectra obtained during IgE-Abs extraction by immunomagnetic separation using (1) 50 μL , (2) 100 μL and (3) 200 μL of blood serum of an allergic patient. Parameters: 100 μg of MBs, 15 min incubation, 10 μL of elution buffer, pH 2.4. (b) MALDI-MS spectra obtained during IgE-Abs extraction by immunomagnetic separation with (1) 5 min, (2) 15 min and (3) 30 min of reaction mixture incubation. Parameters: 100 μg of MBs, 100 μL of blood serum, 10 μL of elution buffer, pH 2.4, sinapinic acid matrix containing 5 $\mu\text{g mL}^{-1}$ of BSA.

Incubation time of the blood serum with the MBs is also essential for the efficiency of the IgE-Abs extraction by immunomagnetic separation. Three incubation times (5, 15 and 30 min) were tested (Figure 6.b). To determine the optimal time, bovine serum albumin (BSA) was added as an external standard to the sinapinic acid matrix with a concentration of $5 \mu\text{g}\cdot\text{mL}^{-1}$. The MALDI-MS spectra obtained showed that 5 min of incubation (Figure 6.b.1) was long enough for efficient IgE-Abs extraction.

The signals of IgE-Abs obtained in Figure 6 have low intensities and reveal that only small quantities of IgE-Abs were present in the eluate. To extract the maximum IgE-Abs from the blood serum, the quantity of MBs and the washing procedure were then modified. As expected, decreasing the amount of MBs to $50 \mu\text{g}$ (Figure 7.a.3) resulted in no distinguishable signals. Whereas, increasing this amount from $100 \mu\text{g}$ (Figure 7.a.2), as was firstly suggested for control IgE-Abs solution, to $150 \mu\text{g}$ (Figure 7.a.1) resulted in a large increase in the intensity of the IgE-Abs, at m/z 151,732. In these MALDI-MS spectra, several signals were detected at m/z below 120000 resulting from non-specific interactions between blood serum proteins (e.g. human serum albumin at m/z 67628) and the anti-human IgE-Abs coating (Figure 7.a.1-2).

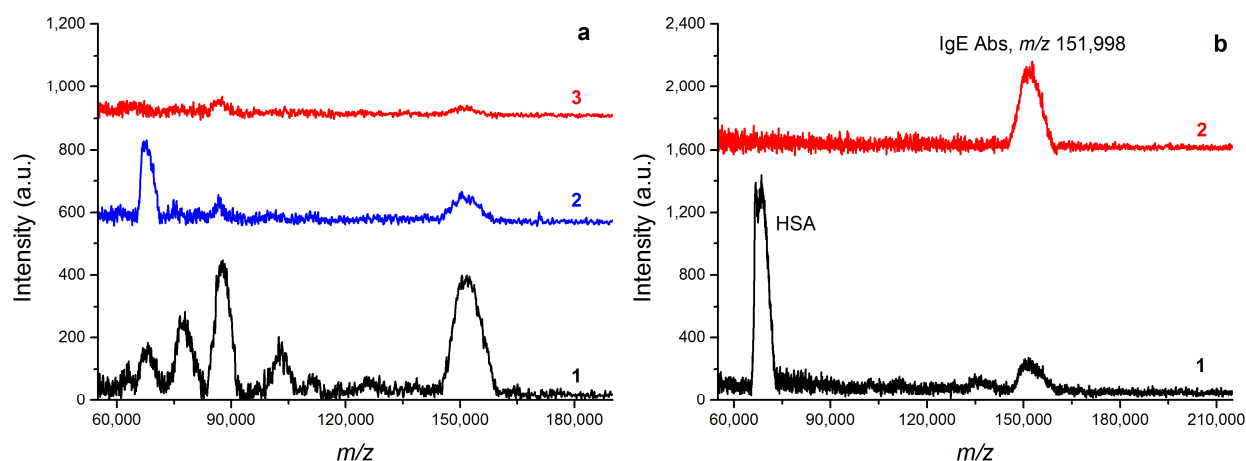


Figure 7. (a) MALDI-MS spectra obtained during IgE-Abs extraction by immunomagnetic separation with (1) $150 \mu\text{g}$, (2) $100 \mu\text{g}$ and (3) $50 \mu\text{g}$ of MBs. Parameters: $100 \mu\text{L}$ of blood serum, 5 min incubation, $10 \mu\text{L}$ of elution buffer, pH 2.4. (b) MALDI-MS spectra obtained during IgE-Abs extraction by immunomagnetic separation with (1) only three washing cycles with washing buffer or (2) three washing cycles with washing buffer and one with deionized water. Parameters: $150 \mu\text{g}$ of MBs, others are the same as indicated for the left panel.

The proteins extracted alongside IgE-Abs during the immunomagnetic separation procedure and detected in Figure 7.a.1-2 do not overlap with the IgE-Abs signal but may overlap with

allergens in the second part of the allergy CRD. Minimizing the non-specific interactions is, therefore, crucial to obtain a reliable diagnosis. In the immunomagnetic separation experiments described above, the washing step after the MBs separation from the blood serum was performed only by rinsing the MBs three times with 60 μL of washing buffer (10 mM PBS solution, 0.05 % Tween 20, pH 7.4). A slightly more acidic solution (pH \sim 5-6) as an additional washing buffer may remove the proteins responsible for the non-specific interactions. This pH range is below the pI of the most abundant serum proteins like human serum albumin and IgG Abs, causing their partial conformational unfolding and lateral repulsion between adsorbed molecules. (39) Meanwhile, such pH is not strong enough to disrupt antigen-antibody interaction and keeps the IgE-Abs bound to the anti-human IgE-Abs-coated MBs. The pH of pure deionized water is neutral in perfect conditions but absorption of atmospheric carbon dioxide can decrease the pH by several units, (40) making it a good candidate for a washing solution in immunomagnetic separation. Hence, MBs pellets were washed an additional time with deionized water at pH 5.5 before elution and MALDI-MS analysis. The resulting spectrum (Figure 7.b.2) showed a large decrease in non-specific interactions compared to the application of three rinsing cycles with washing buffer only (Figure 7.b.1).

The MALDI-MS spectrum corresponding to the optimized immunomagnetic separation of IgE-Abs from blood serum is shown in Figure 7.b.2 and the procedure with optimized parameters is described in the *Materials and methods* section 2.2.

It is worth mentioning that the concentration of the IgE-Abs in the blood serum used was determined by IACE-UV and classical commercial ELISA as $1940 \pm 140 \text{ ng}\cdot\text{mL}^{-1}$ and $2150 \pm 160 \text{ ng}\cdot\text{mL}^{-1}$, respectively. (41) For the developed immunomagnetic separation with MALDI-MS detection, we obtained a LOD (defined as 3 signal-to-noise ratio) of $2000 \text{ ng}\cdot\text{mL}^{-1}$ (Figure 7.b.2). However, the allergy diagnostic threshold is only $240 \text{ ng}\cdot\text{mL}^{-1}$. (42) Therefore, due to the low sensitivity of MALDI-MS detection towards IgE-Abs, developed immunomagnetic separation method is not reliable for total IgE-Abs quantification but can be successfully used for the milk allergy CRD as further discussed in this work.

2.3.3 Allergy CRD of a commercial blood serum by immunomagnetic separation using common allergenic milk protein solutions

The complete CRD of milk allergy was tested first on the same blood serum used for the optimization of IgE-Abs above. This blood serum, purchased from a specialized company, was col-

lected from a man suffering from milk allergy. More specifically, this patient was previously found to be sensitive to bovine serum albumin (BSA), lactoferrin (LF), α S1- and α S2-caseins. (41)

The CRD procedure was tested first using pure proteins solutions. β -lactoglobulin (β -LG, A and B isoforms), BSA and LF, which are known to be common milk allergens, (2) were chosen to perform the diagnostic procedure with the optimized conditions, as detailed in the *Materials and methods* section 2.2. The collected MALDI-MS spectra indicated that BSA-specific and LF-specific IgE-Abs were present in the patient blood serum, causing his sensibility to these proteins (Figure 8.a, b). The LODs (3 signal-to-noise ratio) for BSA and LF during diagnostic procedure were determined as 200 and 100 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. These values depend not only on the individual protein ionization efficiency in MALDI-MS, but also on the amount of corresponding specific IgE-Abs, present in the blood serum. Meanwhile, no β -LG signal was detected at m/z 18400 (Figure 8.c), pointing out that the patient blood serum contained no β -LG-specific IgE-Abs. These results are in agreement with the data previously obtained with the blood serum of the same allergic patient, (41) and prove the efficiency of the developed immunomagnetic separation, taking only 45 min, for an accurate and fast allergy CRD.

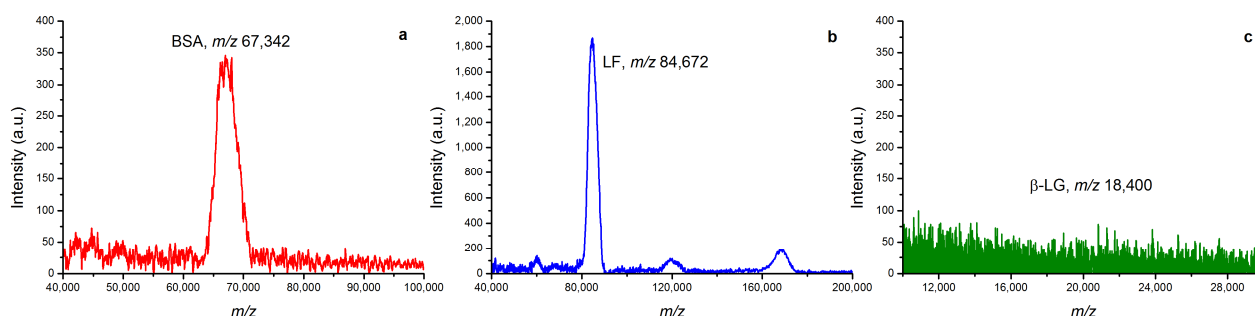


Figure 8. MALDI-MS spectra obtained during the milk allergy CRD using individual protein solutions: **(a)** BSA, **(b)** LF and **(c)** β -LG. Other parameters were as follow: 150 μg of MBs without IgE-Abs cross-linking, 200 μL of 200 $\mu\text{g}\cdot\text{mL}^{-1}$ protein solution, 5 min incubation, 10 μL of elution buffer, pH 2.4.

To decrease the analysis time and reagent consumption, the milk allergy CRD was carried out by IMS-MALDI-MS with a mixture of common milk allergens, namely α -lactalbumin (α -LA), β -LG, BSA and LF. A solution of these four proteins was prepared with a concentration of 250 $\mu\text{g}\cdot\text{mL}^{-1}$ for each protein. On the MALDI-MS spectrum of this solution (Figure 9.b), the peaks corresponding to the protein monomers were observed alongside the singly charged dimer and trimer of the

β -LG due to its superior ionization properties in contrast with other proteins. Meanwhile, the intensity of the α -LA peak is weak, potentially owing to the ion suppression from β -LG.

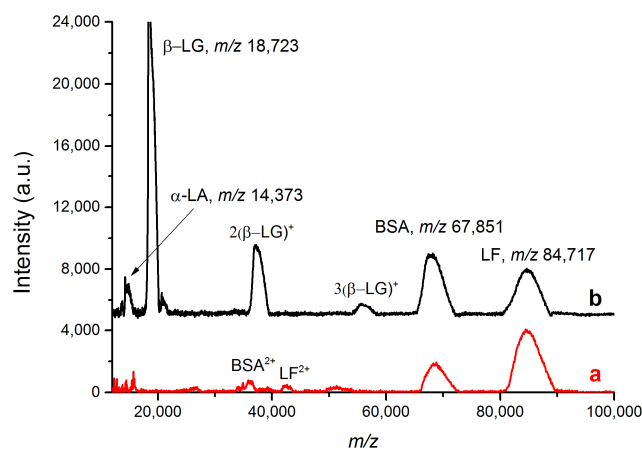


Figure 9. MALDI-MS spectra of **(b)** initial mixture of α -LA, β -LG, BSA and LF ($250 \mu\text{g}\cdot\text{mL}^{-1}$ each) used for diagnosis and **(a)** CRD of milk allergy using this potential allergen mixture. Other parameters are the same as indicated for Figure 8.

Application of the chosen proteins mixture for the CRD performance resulted in the MS spectrum presented in Figure 9.a. The monomer peaks of BSA and LF are easily detected confirming the CRD results obtained with the individual protein solutions. Moreover, the doubly charged forms of these two proteins were also detected at the noise level with the signals around m/z 34000 and m/z 42000. Based on the presented results, we can conclude that the proposed allergy CRD by immunomagnetic separation can provide not only accurate diagnosis from the individual allergen solutions, but also from a more complex allergen mixture in an efficient and unambiguous manner within only 45 min.

2.3.4 Chemical cross-linking of extracted IgE-Abs to MBs

The immunocomplexes of anti-human IgE-Abs and patient IgE-Abs obtained on the MBs surface after the IgE-Abs extraction are formed only by non-covalent antigen-antibody interactions. Therefore, they tend to dissociate or degrade over time and should be used right after the extraction step. In addition, co-elution of IgE-Abs or IgE subunits (heavy chain: 70 kDa, light chain: 23 kDa) may superpose themselves with allergens in the MALDI-MS spectra. To increase the stability of these complexes and prolong their lifetime for several days, the IgE-Abs were chemically cross-linked to the MBs coating using dimethyl suberimidate (DMS). This compound possesses two

imidoester groups, separated by approximately 11 Å, that reacts with primary amine at pH 8-10 to form an amidine bond (Figure 10).

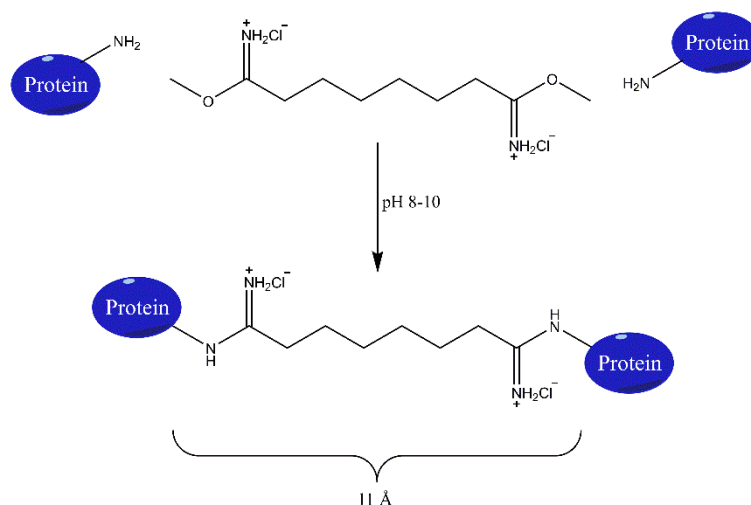


Figure 10. Dimethyl suberimidate (DMS) reaction with primary amines for protein cross-linking.

After the IgE-Abs cross-linking procedure (*Material and methods* section 2.2.3), 10 µL of elution buffer were added to the MBs and incubated during 5 minutes under medium stirring. The MALDI-MS spectrum of the obtained eluate displayed no peaks (data not shown) confirming that the IgE-Abs were efficiently bound to the MBs surface. Then, the cross-linked MBs were stored at 4°C and used 5 days after the cross-linking reaction to perform the CRD of milk allergy using a LF solution (200 µL, 200 µg·mL⁻¹). As shown in Figure 11.b, the intensity of the LF main peak at m/z 84553 decreased by a factor of 3 with respect to the LF extraction with the non-cross-linked MBs (Figure 11.a). This effect can be explained by the DMS random binding with the amino groups of the IgE-Abs paratopes. Without cross-linking, the degradation of the immunocomplex results in a total loss of IgE-Abs binding properties after storing them at 4°C for 5 days.

Despite the decrease in the sensitivity, cross-linking reaction successfully preserves in time the immunocomplex structure and biological activity. The necessity of a cross-linking step should be mainly judged by the complexity of allergens mixture or allergens extract employed. In addition, cross-linked MBs may be used several times as the patient IgE-Abs are not eluted from the MBs during the CRD procedure.

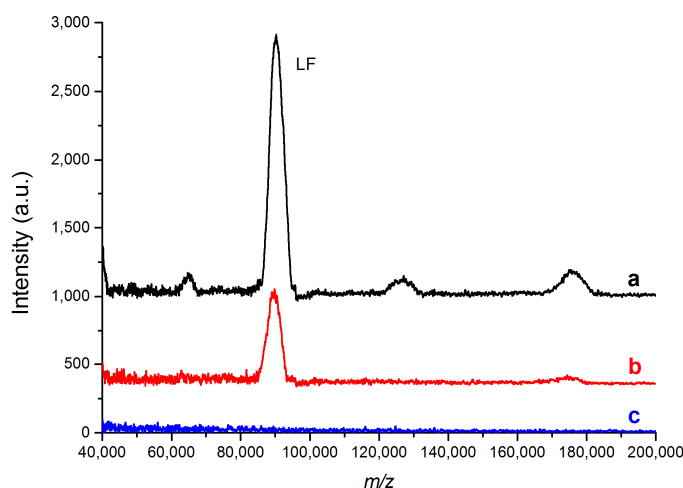


Figure 11. MALDI-MS spectra obtained from CRD of milk allergy by immunomagnetic separation with LF solution (200 μL , 200 $\mu\text{g}\cdot\text{mL}^{-1}$) using: **(a)** MBs without cross-linking immediately after IgE-Abs extraction, **(b)** cross-linked MBs stored for 5 days and **(c)** MBs without cross-linking stored for 5 days after IgE-Abs extraction. Other parameters are the same as indicated for Figure 8.

2.3.5 Control CRD of a commercial blood serum using soy milk and BSA-fortified soy milk

Soy milk, a beverage obtained from soybeans, is usually recommended as cow's milk substitute for people suffering from milk allergy. As soy proteins do not share similar amino acid sequences or patterns with milk proteins, no cross-reactivity between soy and cow's milk has been reported. Nevertheless, it is possible that some patients have a co-sensitization to soy and cow's milk proteins. (43) For these patients, rice-based or hydrolyzed milks are recommended. To test the selectivity of the developed method for milk allergy diagnosis, negative control diagnostic experiments were performed using soy milk.

In the MS spectra of diluted soy milk (Figure 12.a), only soy proteins with mass lower than 30 kDa were detected. As expected, no proteins were extracted during the CRD of milk allergy using 100 μL of soy milk diluted in 100 μL of water (Figure 12.b).

Meanwhile, fortifying soy milk with BSA to its final concentration of 200 $\mu\text{g mL}^{-1}$ (Figure 12.c) led to successful extraction and identification BSA during the CRD of milk allergy (Figure 12.d) without detection of any soy proteins. These control tests confirmed the efficiency and selectivity of the developed IMS-MALDI-MS technique and showed that the patient, whose blood serum was used, is not sensitive to soy proteins.

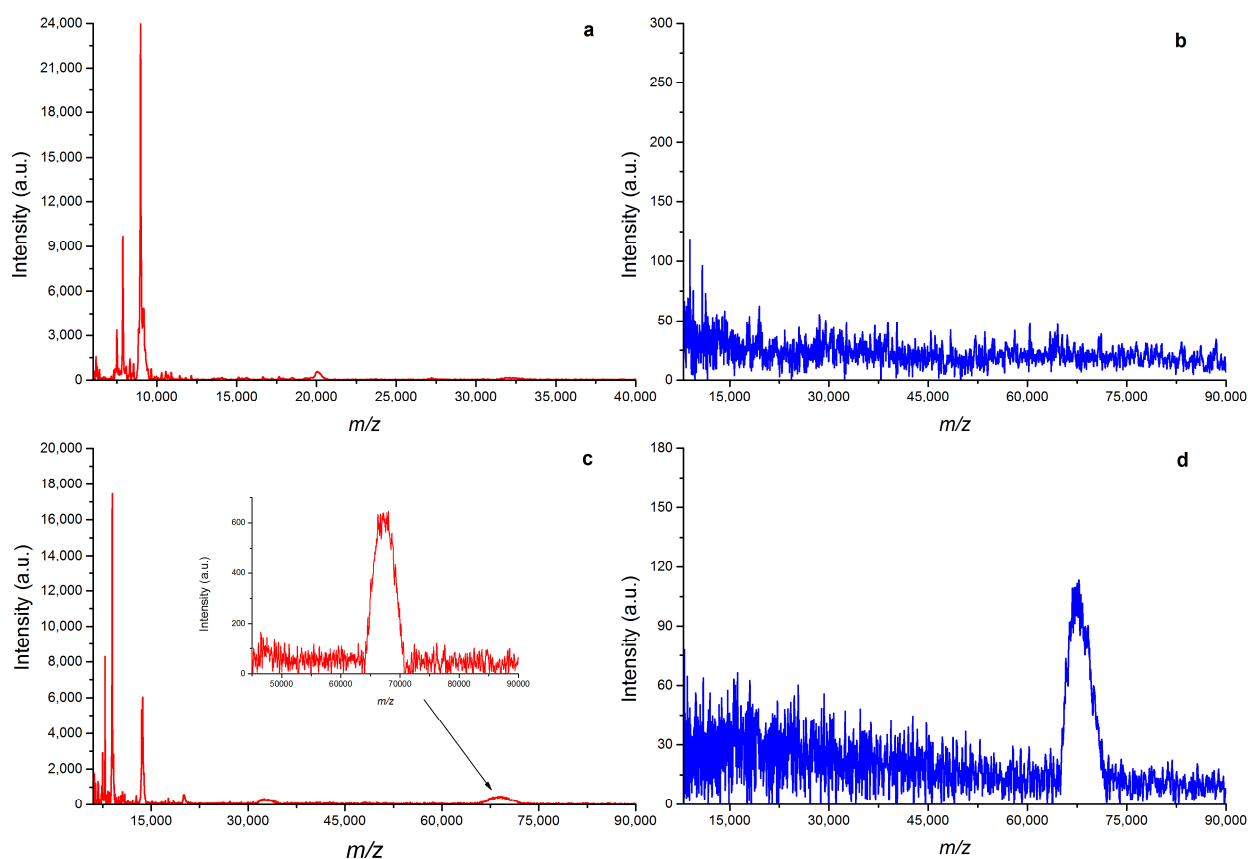


Figure 12. MALDI-MS spectra obtained from **(a)** soy milk diluted tenfold with water, **(b)** the CRD of milk allergy by immunomagnetic separation using 100 μL of soy milk diluted twice with water, **(c)** soy milk fortified with BSA ($200 \mu\text{g mL}^{-1}$) with zoomed BSA mass range as an insert, **(d)** the CRD of milk allergy by immunomagnetic separation using 100 μL of enriched soy milk diluted twice with water. Other parameters are the same as indicated for Figure 8.

2.3.6 Allergy CRD of a commercial blood serum by immunomagnetic separation using milk fractions

Application of commercially available pure allergens for the CRD potentially has several drawbacks: relatively high allergens cost, possible modifications induced by allergen production/isolation from natural products, and the absence of natural post-translational modifications due to production by recombinant technologies. In this case, diagnostic procedure is also limited only to the list of well-known allergens. Hence, the direct use of food extracts for the CRD by IMS-MALDI-MS is an interesting alternative.

Straight application of milk samples is challenging due to a very complex milk composition consisting of proteins ($\sim 35 \text{ g}\cdot\text{L}^{-1}$), minerals, carbohydrates, vitamins and fat. (2,44,45) The milk samples were therefore separated into their respective casein and whey fractions. Moreover, a

methanol/water mixture was utilized as an elution solution for the immunomagnetic separation in this case to avoid the casein precipitation.

Before the application, the whey fraction collected from the whole UHT milk was analyzed by MALDI-MS for quality control. Several proteins were clearly observed on the MS spectrum (Figure 13.a): α -LA at m/z 14263, β -LG at m/z 18423 and an unidentified protein at m/z 12286, potentially corresponding to the proteose-peptone component 5. (46) A dimer of α -LA is also detected at m/z 28482. However, it was impossible to detect BSA and LF directly in the whey fraction due to their lower concentrations and/or ionization efficiency in comparison with α -LA and β -LG. (2) Meanwhile, the MALDI-MS spectrum, originated from the proposed CRD with this fraction, demonstrated clear signals of BSA at m/z 68106, LF at m/z 77108 and α S2-casein at m/z 25072, indicating the patient sensibility to these proteins (Figure 13.b).

The control analysis of the casein fraction showed one large unresolved signal of α S1- and β -caseins at m/z 24643, their dimer, trimer and doubly charged species (Figure 13.c). The CRD carried out using this casein fraction yielded a peak of α -S2 casein at m/z 25243 and a small signal for the casein dimer in the corresponding MS spectrum (Figure 13.d). Similar to α S2-casein detection during whey fraction analysis (Figure 13.b), BSA traces, present in the casein fraction, were also extracted by the patient IgE-Abs resulting in the signals at m/z 68972 at the noise level. These data not only confirmed the patient sensitization to α S2-casein and BSA, but also highlighted the overall high sensitivity of the developed allergy test.

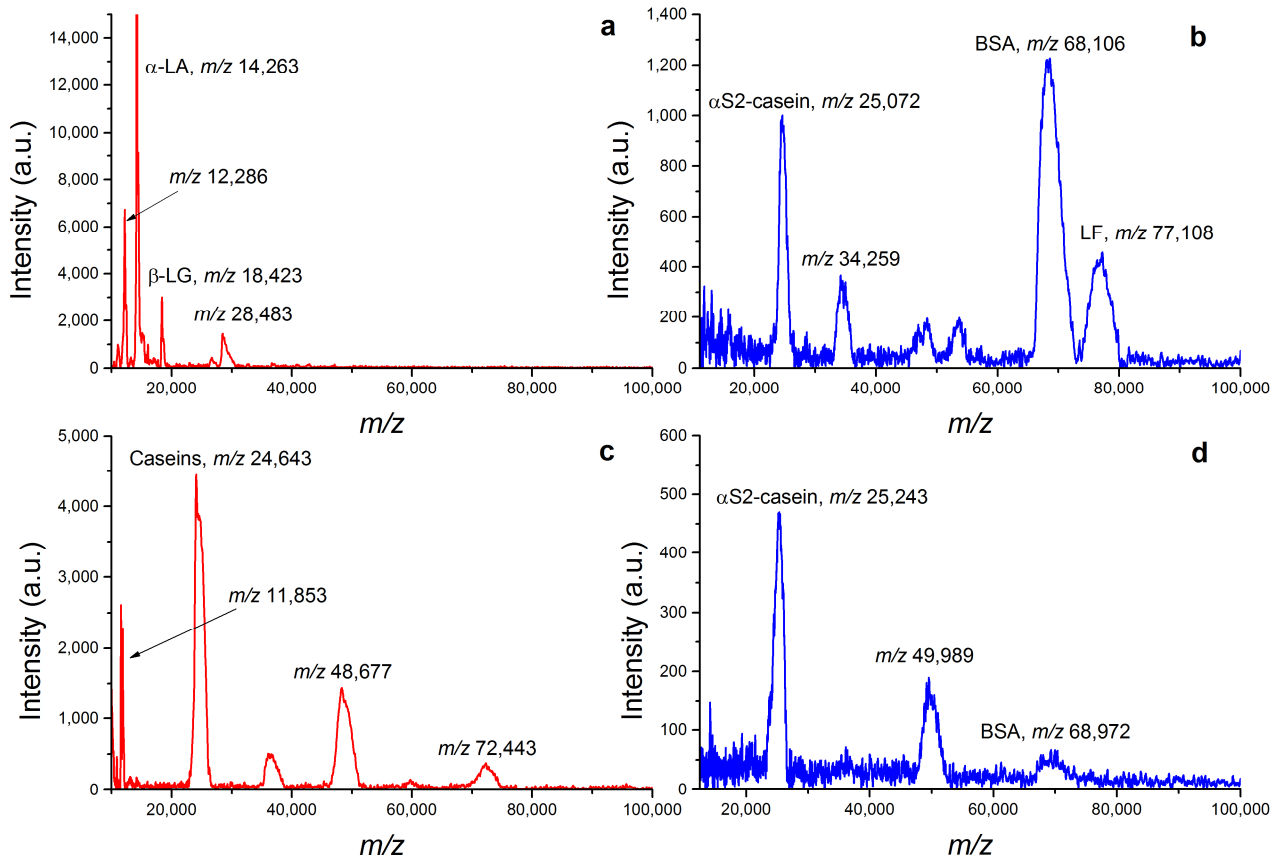


Figure 13. MALDI-MS spectra obtained during the milk allergy CRD using milk extracts. The MS spectra correspond to **(a)** whey fraction of UHT milk and **(b)** milk allergy CRD using this whey fraction, **(c)** the casein fraction of UHT milk and **(d)** milk allergy CRD using this casein fraction. Other parameters are as follow: 150 μg of MBs, 20 min incubation at room temperature, milk fraction dilution 3 times with water, 10 μL of methanol/water elution solution.

The CRD of milk allergy using immunomagnetic separation procedure was also repeated with fractions from skimmed milk powder solution. The control MS spectrum of the whey fraction (Figure 14.a) contained only peaks of α -LA at m/z 14551, β -LG at m/z 18814 and an unidentified protein at m/z 12286 that corresponds probably to the component 5 of the proteose-peptone. (46) The immunomagnetic separation for CRD using this fraction (Figure 14.b) showed clear signals corresponding to BSA and α -casein, present in traces in the whey fraction, at m/z 68161 and at m/z 24939, respectively. LF signal was not observed on the MS spectrum (Figure 14.b) owing to its very low concentration in the milk used. It is a typical situation as the amount of LF in the cow's milk varies greatly from one cow to another according to several parameters: age of the dairy cow, stage of lactation, number of milk somatic cells and presence of pathogens. (47,48) Therefore, to confirm the presence or absence of the LF allergy, it is necessary to utilize an individual solution for the described CRD by immunomagnetic separation.

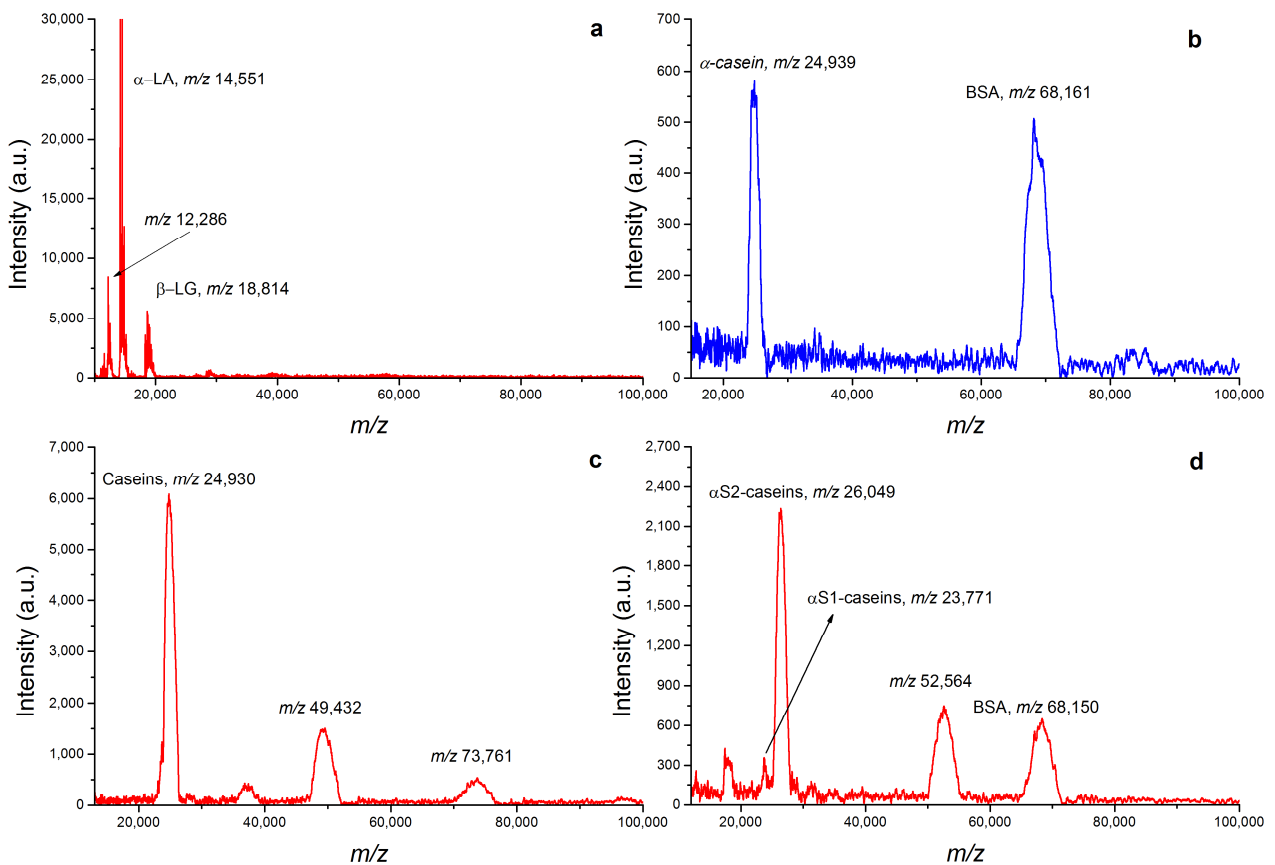


Figure 14. MALDI-MS spectra obtained from (a) the whey fraction of skimmed milk powder solution, (b) the CRD of milk allergy by immunomagnetic separation using 200 μ L of the whey fraction, (c) the casein fraction of skimmed milk powder solution and (d) the CRD of milk allergy by immunomagnetic separation using 200 μ L of the casein fraction. Other parameters are the same as indicated for Figure 13.

The MALDI-MS spectra of casein fraction from skimmed milk powder solution and eluate collected after allergy CRD with this fraction are displayed in Figure 14.c and 14.d. Similar to the results with whole milk, this casein fraction showed signals at m/z 24930, m/z 49432 and m/z 73761 corresponding to the α S1- and β -caseins (singly charged species, dimer and trimer, Figure 14.c). Meanwhile, the MS spectrum from CRD procedure revealed the patient sensibility to α S1 and α S2-caseins together with BSA (Figure 14.d). Despite the BSA presence in trace amounts in the casein fraction, the proposed immunomagnetic separation method allowed its successful identification as a sensitizer for the chosen patient.

Overall, performed immunomagnetic separation experiments with various cow's and soymilk fractions demonstrated good efficiency and sensitivity of proposed allergy CRD. The data presented are in a good accordance with each other and confirm the results obtained by the appli-

cation of individual protein solutions. As in a previously published work, (41) the chosen patient was shown to be potentially allergic to BSA, LF, α S1 and α S2-caseins. Moreover, if the concentration of BSA- and LF-specific IgE-Abs in his blood serum is estimated to be approximately at the same level, the amount of α S2-casein-specific Abs is higher than the one of α S1-casein-specific Abs. Meanwhile, the allergy CRD with the direct application of milk extracts did not reveal any unknown or rare allergens for this patient.

In general, the milk extracts application in the proposed CRD protocol can potentially suffer from the restrictions of MALDI-MS detection: MALDI-MS operated in a linear mode is a perfect method for fast protein analysis over a large mass range but the resolution decreases quickly with the increase of mass range. (29) The resolution drop results in the peak broadening at high m/z , poor mass determination and discrimination between the molecules with close m/z values, like for example α and β -caseins. To compensate for this effect and improve the accuracy of developed diagnostic technique, extracted allergens identification was realized by mass fingerprinting of proteolytic peptides generated from the allergens' tryptic digestion upon their elution from MBs. For keeping an overall CRD experimental time reasonably short, the allergens digestion was conducted by trypsin immobilized into MOSF, insuring fast, less than 1 h long, proteolysis with low level of trypsin autolysis. (49)

After the allergy CRD using the UHT milk whey fraction, allergen digestion resulted in the MALDI-MS spectrum presented in Figure 15. Among the observed peaks, 9 peptides corresponding to LF, 11 to BSA, 6 to α S1-casein and 9 to α S2-casein were detected (*Appendix Tables I-IV*) indicating the patient sensibility to these allergens. For β -LG and α -LA, no peptides were detected despite the high concentrations of these proteins in the whey fraction in comparison with other components, (44,45) owing to an effective non-specific interaction suppression during developed CRD. Other unidentified signals come from patient IgE-Abs, anti-human IgE-Abs from the MBs coating and typical human keratin contaminants.

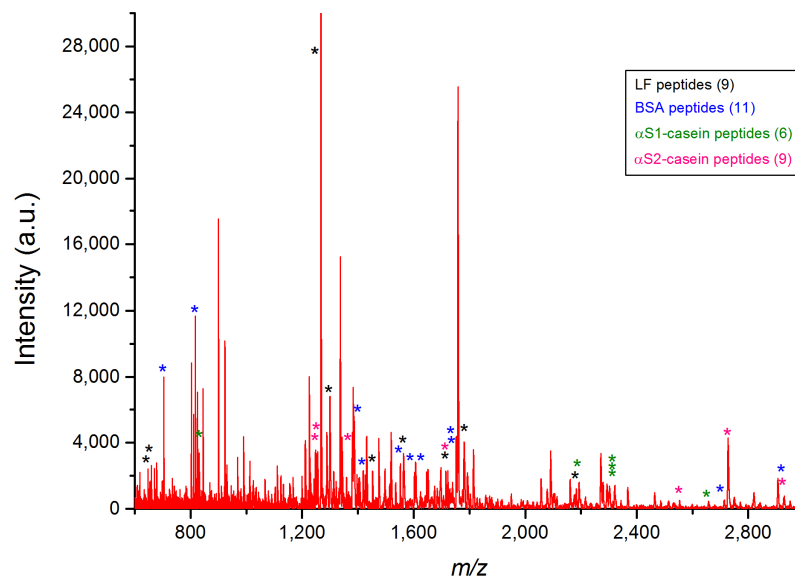


Figure 15. MALDI-MS spectrum obtained during milk allergy CRD using peptide mass fingerprinting for captured allergens identification. The immunomagnetic separation was carried out using the diluted UHT milk whey fraction and followed by digestion with trypsin-loaded MOSF. Other parameters are the same as indicated for Figure 13. All recognized peptides are marked with an asterisk of corresponding colour.

The peptide mass fingerprinting (PMF) displayed better detection sensitivity for α -S1 casein and can be very useful for the definitive identification of captured allergens, while conducting described CRD directly with allergenic food extracts. Moreover, the developed diagnostic technique does not require complex materials (50,51) or a time consuming electrophoretic separation step. (41) In contrast with recently introduced allergy diagnosis by lateral flow assay with recombinant allergens, (52) which lasts only 10 min, but may suffer from erroneous results, the designed immunomagnetic separation-based technique assures within 45 min (or 100 min with trypsin digestion) accurate allergy diagnosis owing to the MS detection. At the same time, such approach opens the possibility to easily perform cross-reactivity studies, identify new or rare allergens from natural food extracts and resolve the complex allergy cases, where classical diagnostic methods fail, while using only standard allergen list.

2.3.7 Additional allergy CRD for milk allergy

In addition to the commercial blood serum, the CRD procedure has been tested on a second case of clinically proven milk allergy. This blood serum was provided by the Immunology and Allergology department of Institut Central des Hôpitaux (Sion, Switzerland). For this patient, the

specific IgE levels were measured at 19.4 and 1.35 kU/L for the complete milk extract and caseins, respectively, using the standard ImmunoCap tests (Phadia/ThermoScientific, Sweden).

The CRD procedure was performed using the whey and casein fraction of whole UHT milk as described above. Application of the whey fraction revealed a low intensity signal in the MALDI-MS spectrum (Figure 16.a) corresponding to LF. Identification of this protein was further confirmed by PMF (Figure 16.b) with 4 detected peptides, listed in *Appendix Table V*. The CRD performance with casein fraction of UHT milk (Figure 16.c) revealed the sensibility of the patient to α - and β -caseins. PMF confirmed the identity of these proteins with 5, 6 and 16 peptides detected for β -casein, α S1-casein and α S2-casein, respectively (Figure 16.d and *Appendix Table VI*).

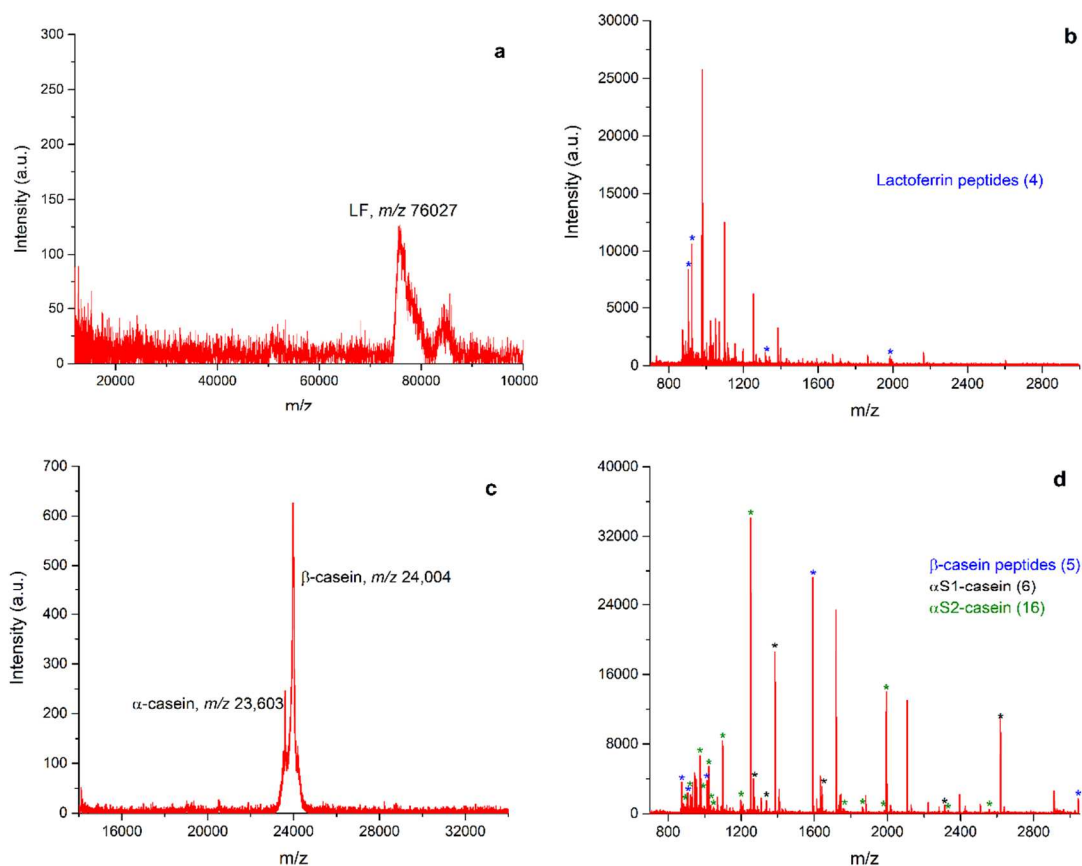


Figure 16. MALDI-MS spectra obtained from the milk allergy CRD using (a) the whey fraction and (c) the casein fraction of UHT milk. The diagnosis was confirmed by PMF for each milk fraction (b, d). All recognized peptides are marked with an asterisk of corresponding color. Other parameters are the same as indicated for Figure 13 and 15.

Those results show that the developed allergy CRD by IMS-MALDI-MS allows the identification of the complete allergenic profile of patients using milk extracts. As a liquid allergenic ex-

tract is required, the application of this method to other food extracts may include further steps including protein extraction and purification.

2.3.8 Allergy CRD applied to egg white allergy

Egg allergy is the second most common food allergy in children. (3,8) The four major proteins present in egg white are ovalbumin (54 % of total egg white proteins), ovotransferrin (12 %), ovomucoid (11 %) and lysozyme (3.4 %) and they are the main egg allergens (*Gal d 1-4*). (8)

The developed CRD method was further tested the using blood sera of 3 patients with proven egg white allergy and the blood serum of a patient without any egg allergy, used as a negative control. All blood sera were tested using ImmunoCAP tests (Phadia/ThermoScientific, Sweden) against the complete egg white extract and ovomucoid. Ovomucoid has been systematically tested as it is the major egg white allergen and is considered as a marker for egg allergy persistence. Patient N°1, N°2 and N°3 were positive to egg white with measured levels of specific-IgE of 1.14, 63 and 11 kU/L respectively, whereas patient N°4 was negative (0 kU/L). Ovomucoid specific-IgEs were measured at 0.64, 17 and 13 kU/L for patient N°1, N°2 and N°3, respectively.

The CRD was performed with a solution of egg white in 10 mM PBS solution (pH 7.4) with a protein concentration of approximately 1 mg·mL⁻¹. To avoid non-specific interactions with lipids and other components, two additional washings of the MBs with pure water were performed before the elution of the allergens. The MALDI-MS spectra obtained for the CRD of the four blood sera are shown in Figure 17. These results indicated that patients N°1, 2 and 3 are sensitive to ovomucoid, as proven by the ImmunoCap tests. In addition, patient N°2 has IgE-Abs specific to lysozyme and ovotransferrin (Figure 17.b and insert) and patient N°3 to ovalbumin (Figure 17.c), as the signals of these allergens were observed in the MALDI-MS spectra. As expected for a negative control, no protein signals were detected in the MALDI-MS spectrum corresponding to the CRD of patient blood serum N°4 (Figure 17.d). For patient N°1 and N°3, the protein signals have a relatively low intensity with a high background noise, potentially due to a low amount of allergic specific-IgE and/or poor ionization of these proteins in MALDI-MS. For this reason, the allergenic profile determination for patients N° 1 and 3 is not certain.

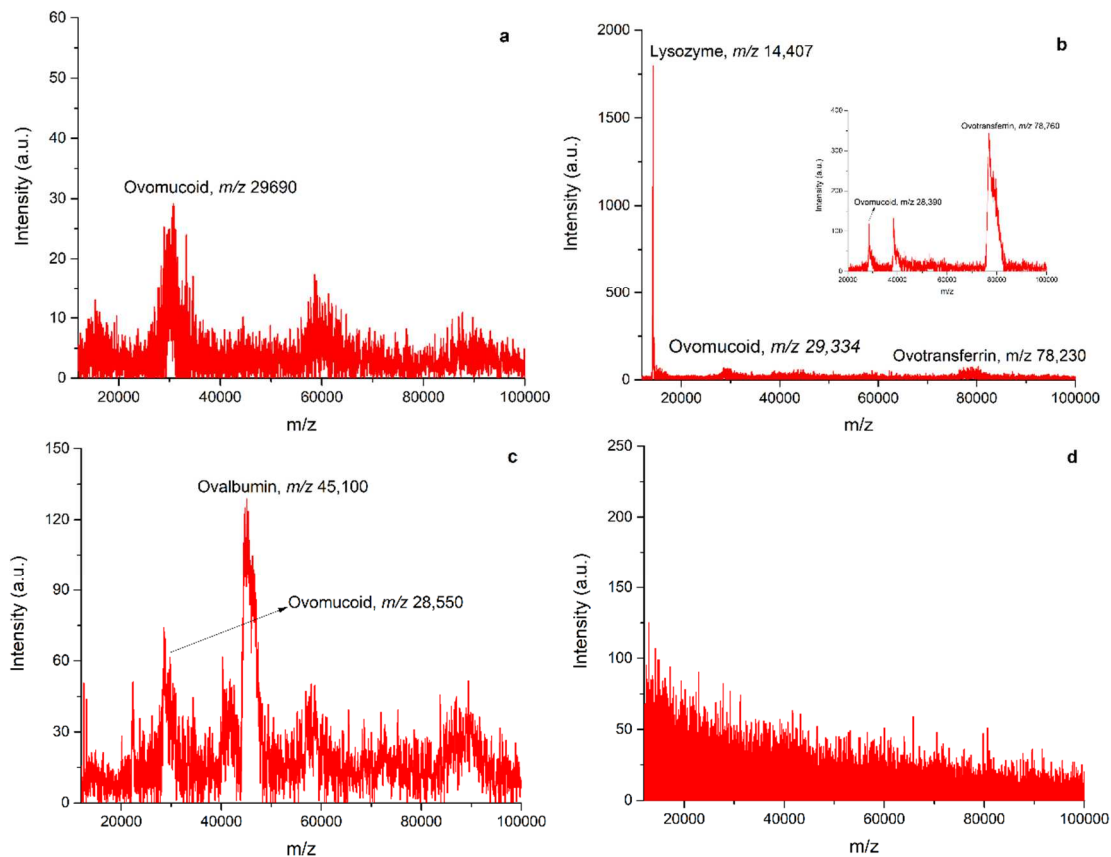


Figure 17. MALDI-MS spectra obtained from the egg white allergy CRD using a solution of egg white in PBS solution with a protein concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$ for patients (a) N°1, (b) N°2, (c) N°3 and (d) N°4 (negative control). The CRD procedure was modified to include two extra washings steps using pure water. Other parameters are the same as indicated for Figure 13.

In order to identify with certainty the extracted allergens during CRD, PMF was further performed. In comparison with milk allergens, lysozyme and ovomucoid are well-known, respectively, for their trypsin resistant (53) and trypsin inhibitor properties. (54) For this reason, the digestion of the egg allergens by trypsin immobilized in MOSF was preceded by the reduction and alkylation of disulfide bonds in denaturing buffer (6M urea), as reduction and alkylation are responsible for the total or partial loss of tertiary structure resulting in an increased digestibility of proteins and decreased trypsin inhibition. Performed PMF (Figure 18) confirmed the CRD of egg allergy obtained in Figure 17. Ovomucoid peptides were detected for patients N°1, 2 and 3, confirming their sensibility to this protein. Patient N°2 was confirmed to possess IgE-Abs specific to ovotransferrin and lysozyme, as 15 and 4 corresponding peptides were identified for these two allergens. Allergy to ovalbumin was confirmed for patient N°3, as 10 peptides specific to this allergen were detected. Application of PMF for patient N°4 yielded only peptides originating from ker-

atin contaminants and trypsin autolysis. All peptides identified in these PMF analyses are listed in *Appendix Table VII*.

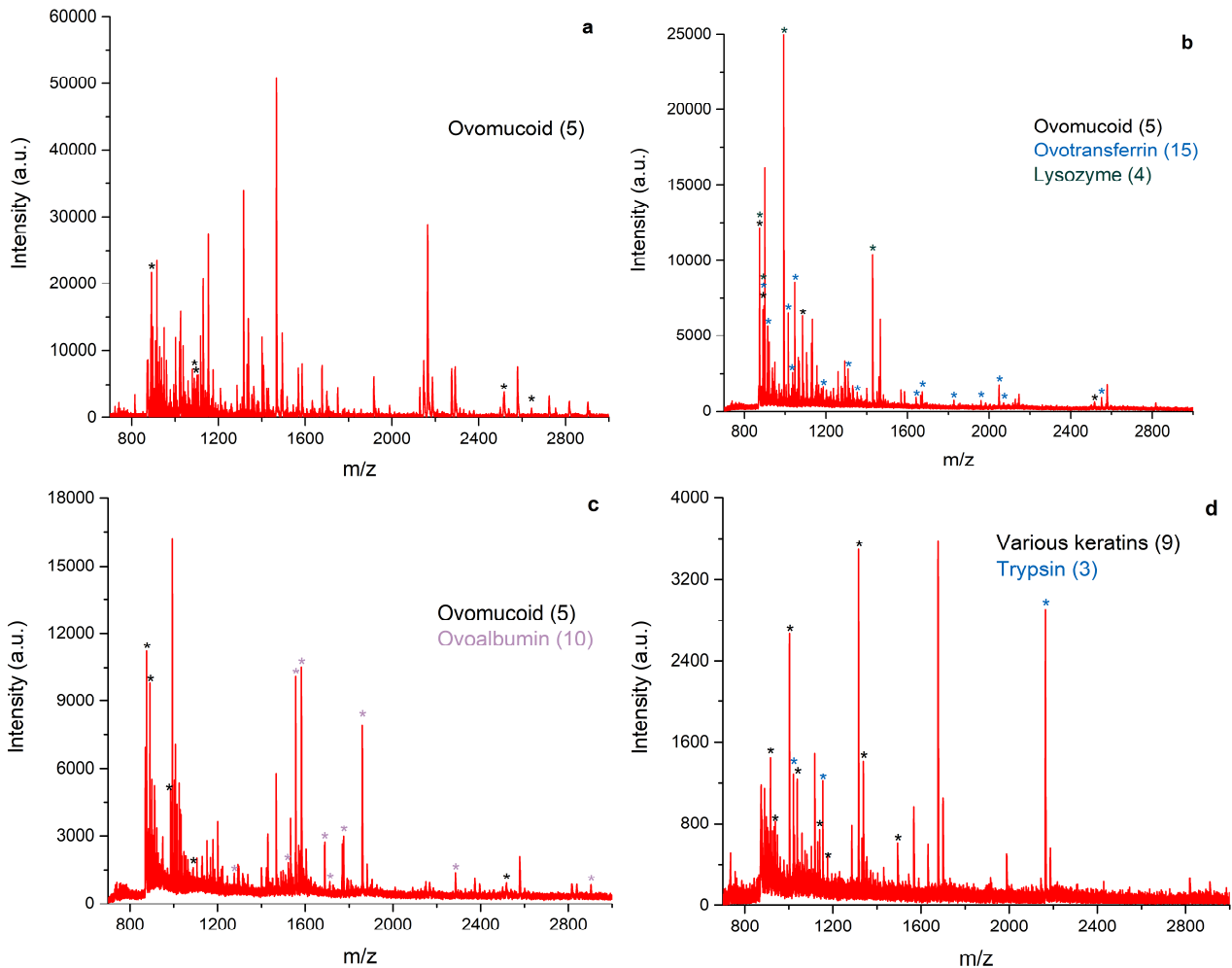


Figure 18. MALDI-MS spectra obtained during egg white allergy CRD using peptide mass fingerprinting for captured allergens identification for patients **(a)** N°1, **(b)** N°2, **(c)** N°3 and **(d)** N°4 (negative control). All recognized peptides are marked with an asterisk of corresponding color. All parameters are the same as indicated for Figure 17.

With these tests on egg allergy, the IMS-MALDI-MS method originally developed for milk allergy is shown to easily be transposed to other allergies with minimal modifications. Nevertheless, it is important to take into account some particular aspects such as the composition of the extract or the intrinsic properties of the allergens before applying it to other natural extracts.

2.3.9 Allergy CRD applied to a clinical blood serum with potential milk allergy

As a proof-of-concept, the present IMS-MALDI-MS procedure was applied to determine the sensitization profile of a clinical patient suffering from protein contact dermatitis, when exposed to the cow's whey fraction. In the hospital, trained professionals confirmed the allergy diagnosis by skin prick test using this particular whey fraction. However, standard *in vitro* analyses performed in clinical settings with common commercial milk allergens yielded inconclusive results. The department of Immunology and Allergology of the Institut Central des Hôpitaux (Sion, Switzerland), therefore, provided both the patient's blood serum and whey fraction to perform the allergy CRD by the present technique.

The patient was a 57 year old male, working as cheese-maker. He complained of recurrent and severe cutaneous lesions of his fingers and hands. These lesions worsened during periods of work activity and disappeared during periods of inactivity. He was avoiding milk because of systematic diarrhea appearing 30 to 45 minutes after drinking milk, whereas cheese has always been tolerated. This patient was not known for hay fever or for asthma. He never presented urticaria, rash or breathing difficulties. Because of these skin lesions, he consulted the allergology center of the Institut Central des Hôpitaux (Sion, Switzerland).

The clinical presentation was compatible with a severe chronic eczema of the hands. As the patient mentioned quick exacerbation of the symptoms (redness and pruritus) after contact with milk and particularly with the whey fraction of milk, a protein contact dermatitis to milk or whey proteins was suspected. Skin prick tests were positive for milk and the whey fraction of milk. Prick tests were negative for standard pneumallergens (grass, birch, ash, olive, *Dermatophagoides pteronyssinus*, farina and latex). Epidermal tests with the European Standard Series (True Test), including milk and whey fraction of milk, were positive only for epoxy resin (weak reaction), which was considered as non-pertinent. Finally, specific IgE-Abs tests (ImmunoCAP, Phadia/ThermoScientific, Sweden) were negative for the following milk proteins: α -lactalbumin (nBos d 4), β -lactoglobulin (nBos d 5), casein (nBos d 8) and bovine serum albumin (nBos d 6).

The serum of the patient has also been tested with ImmunoCAP ISAC (Phadia/ThermoScientific, Sweden) against a panel of allergens including the above-mentioned proteins and lactoferrin (nBos d LF). This test yielded no positive IgE-Abs specificity. The patient total IgE-Abs level was measured at 86 U/L. This clinical situation is compatible with a protein contact

dermatitis and the patient received a prescription for a tapered treatment of topic clobetasol, associated with emollient. He was instructed for complete contact avoidance with milk products during his work. Three levels of gloves were used by the patient from that period: silk gloves, vinyl gloves and large rubber gloves covering the entire arms. With this treatment and allergen avoidance, he was able to continue his work without recidivism of skin lesions.

First, the allergenic whey fraction has been analyzed by MALDI-MS (Figure 19.a). As expected, the main peaks detected at m/z 14205 and 18414, correspond to α -LA and β -LG, respectively. BSA and LF were not observed directly because of their low concentration in the whey fraction and probable competitive ionization of smaller molecules. The allergy CRD by immunomagnetic separation with this fraction yielded a strong signal at m/z 85345 corresponding to LF (Figure 19.b), indicating the presence of LF-specific IgE-Abs in the blood serum. We also detected a second weak peak with m/z around 55000 (Figure 19.c). Its intensity below 3 times the signal-to-noise ratio was probably caused by low concentration of trapped proteins in the analyzed whey fraction and/or by low amount of corresponding specific IgE-Abs in the patient's blood serum. This signal was hypothesized to correspond to BSA, partially degraded during the analysis.

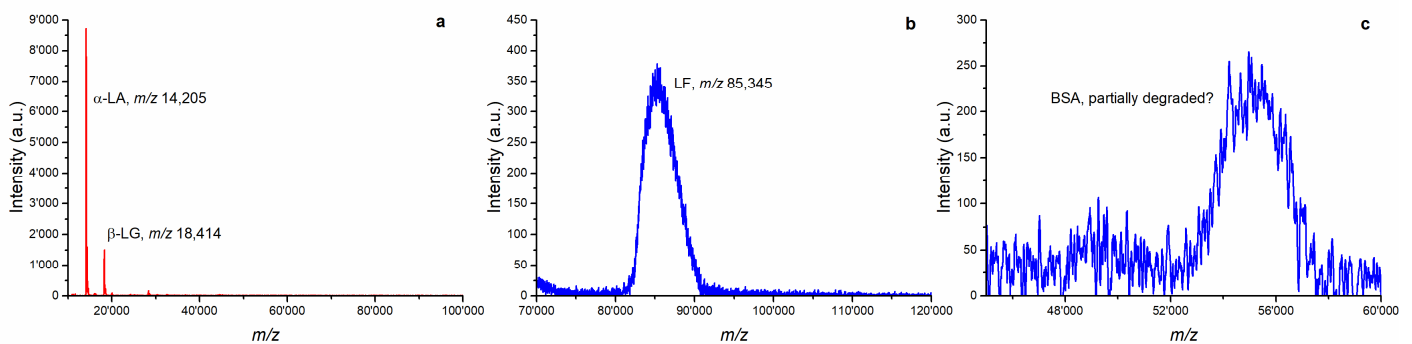


Figure 19. MALDI-MS spectrum obtained during the milk allergy CRD for clinical sample using whey fraction. The MS spectra correspond to **(a)** allergenic whey fraction used and **(b-c)** milk allergy CRD using 100 μ L of diluted whey fraction. Other parameters are the same as indicated for Figure 13.

To confirm the patient sensitization to LF and BSA, and verify the captured allergens identity, peptide mass fingerprinting was carried out using trypsin-loaded MOSF for proteolytic digestion (Figure 20). In order to obtain a better identification, two MALDI-MS spectra were recorded with partially overlapping mass ranges. In total, 16 and 12 unique peptides were identified for LF

and BSA, corresponding to 23.1 and 26.8 % of sequence coverage, respectively (Appendix Tables VIII-IX). Other signals are originated from human keratin contaminants and trypsin autolysis.

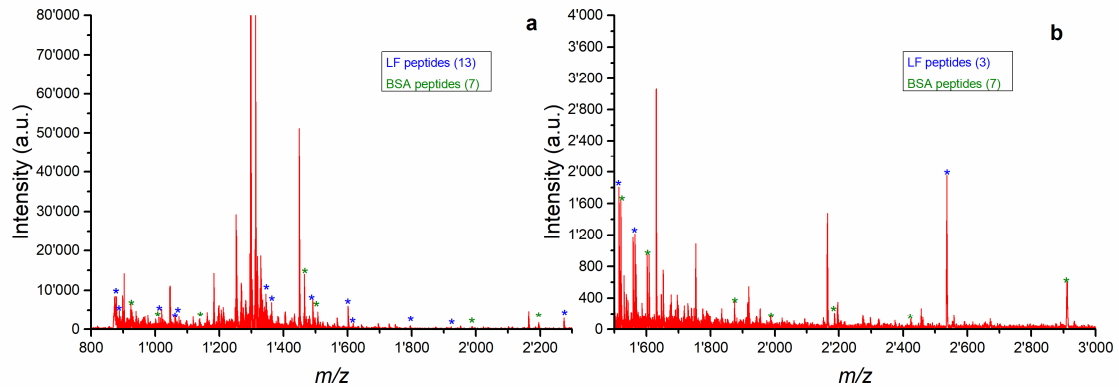


Figure 20. MALDI-MS spectra obtained during CRD of milk allergy by immunomagnetic separation using the allergenic whey fraction followed by digestion with trypsin loaded MOSF. Other parameters are the same as indicated for Figure 13 and 15. All recognized peptides are marked with an asterisk.

In addition, solutions of pure commercial LF and BSA have been prepared and used to perform the CRD. Application of BSA solution yielded, as previously, a barely detectable peak at m/z 54940 (Figure 21.a), which has previously been identified as degraded BSA. It is important to note that no native BSA peak was detected around m/z 67000. The low intensities of the degraded BSA signal (Figure 19.c and Figure 21.a) might indicate a low amount of BSA-specific IgE-Abs in the blood serum of the patient or cross-reactivity with another protein. With pure LF, the CRD resulted in an intense peak at m/z 84144 (Figure 21.b) confirming the diagnosis.

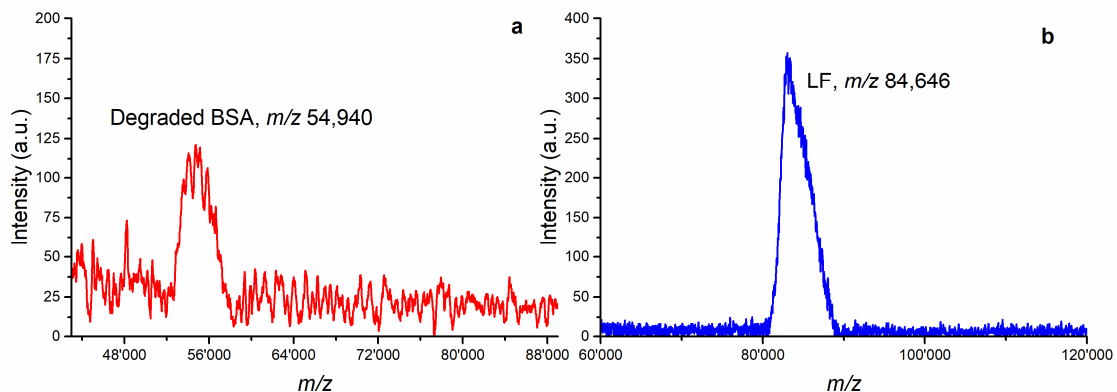


Figure 21. MALDI-MS spectra obtained during the CRD of milk allergy by immunomagnetic separation using 200 μL of 200 $\mu\text{g}\cdot\text{mL}^{-1}$ (a) BSA and (b) LF solutions. All parameters are the same as indicated for Fig. 13.

For a complete allergy diagnosis, we also performed the cross-reactivity studies with the clinical sample under consideration. The cross-reactivity between various allergens is quite common and arises from the similarities in their structures and amino acid sequences, resulting in shared antigenic properties. Therefore, the patient sensibility to goat and sheep milks was tested, as they are sometimes recommended as cow's milk substitutes, despite potential cross-reactivity.

Application of the whey fractions from goat and sheep milks (Figure 22.a, c) for the developed allergy CRD resulted in signals at m/z 67286 and m/z 67979 (Figure 22.b, d), corresponding to the serum albumins of these two species. These data pointed out the potential cross-reactivity for the patient between goat, sheep and bovine albumins, caused by their high sequence homologies (> 90 %). Co-sensitization to these allergens also should not be excluded, as it is hard to differentiate it from cross-reactivity. Nevertheless, the main conclusion is that neither goat, nor sheep milks are suitable as cow's milk substitutes for the patient under consideration.

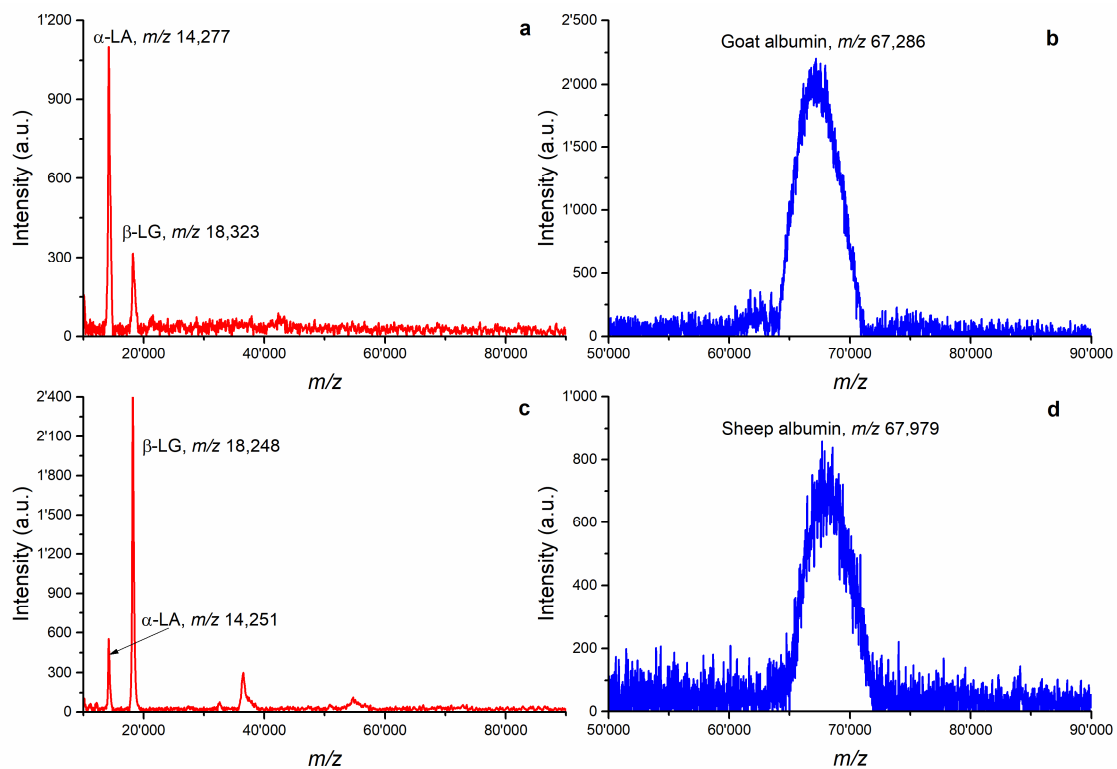


Figure 22. MALDI-MS spectra obtained for cross-reactivity studies during the milk allergy CRD for a clinical sample using milk extracts. The MS spectra correspond to (a) goat milk whey fraction, (b) milk allergy CRD using diluted goat milk whey fraction, (c) sheep milk whey fraction and (d) milk allergy CRD using diluted sheep milk whey fraction. Other parameters are the same as indicated for Figure 13.

The analysis of this clinical sample, for which commercial tests in hospital settings were inconclusive, clearly demonstrates the diagnostic potential of the developed immunomagnetic separation for personalized allergy CRD. Its main advantage consists in a direct application of allergenic food extract, possible due to the MALDI-MS implementation as a detection method. Both LF and BSA, detected as a degradation product, were identified as sensitizers for this patient, while directly applying the allergenic whey fraction. As LF is often regarded as a minor milk allergen, standard diagnostics in hospital settings are mainly conducted using only the most common milk allergens like caseins, α -LA, β -LG and BSA, in individual manner. Therefore, in the present clinical case, the patient was simply not tested for LF sensitivity. Meanwhile, standard clinical test for BSA sensitivity failed potentially because of variations in structural modifications (like post-translational modifications or partial protein degradation) between commercial proteins, used in the test, and the one, present in allergenic whey fraction. The proposed immunomagnetic separation with this whey fraction for allergy diagnosis permitted omitting such problems and provided comprehensive diagnostic results.

2.3.10 Allergy CRD applied to bolete allergy

Boletus edulis, usually referred as cep, king bolete or porcini, is an edible mushroom commonly used in French and Italian cuisine. *B. edulis* is usually well tolerated by the immune system and only a few cases of allergic reactions were reported after ingestion or inhalation of mushroom dust or spores. (55–59) Allergens from *B. edulis* have mainly been detected and partially characterized by immunoblots; nevertheless, Castillo *et al.* have recently used mass spectrometry and DeNovo sequencing to identify bolete allergens. (60)

The developed IMS-MALDI-MS method for the CRD of food allergy was carried out using the blood serum of a female patient from the Institut Central des Hôpitaux (Sion, Switzerland). This patient suffered from allergic reactions after ingestion and handling fresh and dry bolete. She was tested negative for allergy to common mushroom (*Agaricus hortensis/Agaricus bisporus*) using a standard ImmunoCAP test. This test does not allow testing other species of mushrooms. The patient responded positively to skin prick test using fresh bolete and display high level of circulating IgE-Abs. Blood serum from this patient was kindly provided by the Immunology and Allergology department of the Institut Central des Hôpitaux (Sion, Switzerland) in order to test it with the developed CRD by IMS-MALDI-MS.

First, a protein extract was prepared by extracting 0.5 g of freshly ground king bolete in PBS buffer followed by protein purification and lyophilisation, as detailed in *Material and Methods* section 2.2. The CRD was carried out using the same procedure as for the previously discussed milk and egg allergy cases. The MALDI-MS spectrum obtained at the end of the CRD display a single signal at 15.7 kDa (Figure 23.a).

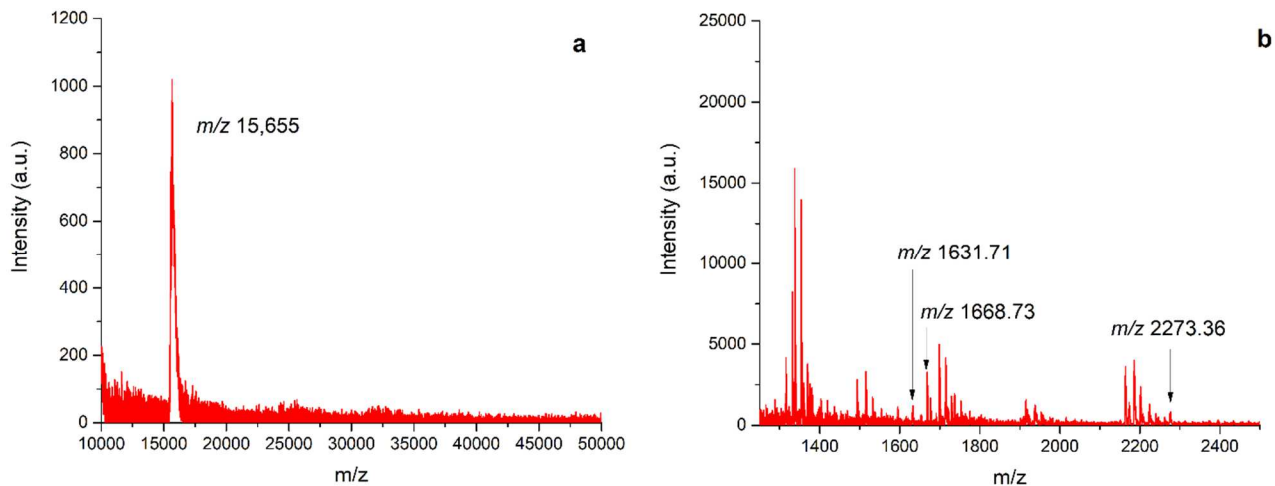


Figure 23. MALDI-MS spectra obtained for the CRD of *B. edulis* allergy (a) before and (b) after overnight digestion using trypsin. Parameters were as follow: 150 μg of MBs with IgE-Abs cross-linking, 200 μL of 200 $\mu\text{g}\cdot\text{mL}^{-1}$ protein solution, 5 min incubation, 10 μL of elution buffer, pH 2.4. Digestion was carried out overnight using trypsin (1:30 w/w ratio with respect to proteins) after denaturation at 95°C for 10 minutes, reduction with DTT (final concentration 5 mM) at 56°C for 30 minutes and alkylation with IAA (final concentration 10 mM) at room temperature for 30 minutes in the dark.

In order to identify the protein, a PMF analysis was carried out after digestion using trypsin immobilized in MOSF as previously described. The detected peptides originated only from trypsin autolysis and contaminants such as keratins, owing perhaps to a partial trypsin resistance of the protein. The digestion procedure was therefore modified to include a 10 minutes denaturation at 95°C, standard reduction and alkylation using DTT and IAA, and an overnight trypsin digestion using a 1:30 mass ratio of trypsin to proteins. The obtained MALDI-MS spectrum of the digest (Figure 23.b) display 3 peptides (listed in Table 2) originating from a lectin from *B. edulis*. A sequence coverage of 36.6 % is obtained with these peptides and allows identifying with certainty this lectin as a potential allergen.

Table 2. Detected peptides from *B. edulis* after CRD of a king bolete allergic patient using a fresh bolete extract.*

Observed m/z	Corresponding peptide sequence	Position	Protein	UniprotKB
1631.71	GTHLTMGGSSTSGVLR	40-56	Lectin	F2Z266
1668.73	TVFHYANGGTWSEAK	25-39	Lectin	F2Z266
2273.36	FEVKYTVVEGNNLEANVIFS	124-143	Lectin	F2Z266

* Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin was conducted as indicated in Figure 23.

Lectins are sugar-binding proteins widely present in plants and mushrooms. They are also known for their various biomedical and biotechnological properties, including antiproliferation of human cancer cell lines, bacterial and fungal growth inhibition, cell aggregation, immunomodulation and toxic effects. (61–64) In addition, lectins are known to be relatively resistant to heat and digestion, (65) explaining the poor digestibility and low number of detected peptides in Figure 23.b.

B. edulis allergens have not yet been formally identified, as this allergy is relatively rare. In their study, Castillo *et al.* proposed two allergens, a 16 kDa fruit body lectin and a 46 kDa protein of the glycoside hydrolase family. (60) However, the reported sequence and database accession number correspond to a lectin (UniProtKB Q8WZC9) from another mushroom species, *Xerocomellus chrysenteron*. This protein is, nevertheless, very similar to a lectin from *B. edulis* (UniProtKB F2Z266). Using the ExPASy (28) tool for protein similarity analysis based on the SIM algorithm, (66) a 82.5 % similarity are obtained on the 143 amino acids of these proteins. Despite the high similarity between both lectins, the three peptides used by Castillo *et al.* to identify the 16 kDa allergen show some variation in the amino acids sequence compared to the *B. edulis* lectin as shown in Table 3.

Table 3. Peptides used to identify lectin by Castillo *et al.* and corresponding *B. edulis* peptides with highlighted differences.

Protein	UniProtKB	Organism	Total amino acids	Peptide 1	Peptide 2	Peptide 3
Lectin	Q8WZC9	<i>Xerocomellus chrysenteron</i>	143	AN <u>GA</u> HTLT <u>Q</u> GGSGTSGVLR	EKQLAEY <u>S</u> VTS	<u>A</u> IG <u>K</u>
Lectin	F2Z266	<i>Boletus edulis</i>	143	AK <u>G</u> IHTLT <u>M</u> GGSGTSGVLR	EKQLAEY <u>N</u> VTS	<u>V</u> VG <u>R</u>

Based on our results, we can confirm that a 15.7 kDa protein is potentially recognized by patients IgE-Abs and may result in allergic symptoms. With the developed CRD method, this allergen was identified as a member of the family of mushroom fruiting body-specific lectins that was fully characterized previously. (67) Common mushrooms (*A. hortensis*/*A. bisporus*) also possess a similar lectin (UniProtKB Q00022). Nevertheless, the similarity with *B. edulis* lectin is limited to 52 %, explaining the negative response of the patient to *A. hortensis*/*A. bisporus* extracts in the ImmunCAP tests.

These results display the effectiveness of the developed CRD method based on IMS-MALDI-MS using natural extract. In addition to have successfully diagnosed the patient, a new allergen of *B. edulis* was formally identified thanks to the mass spectrometry methods used. Using natural extract for the allergy diagnosis allows the detection and identification of new allergens, whereas standard *in vitro* tests are limited to a given list of known allergens.

2.4 Conclusion

A fast and effective in-tube personalized allergy diagnosis was developed using immunomagnetic separation with MALDI-MS detection and applied to various allergy cases. Its key steps are the IgE-Abs extraction from the blood serum of an allergic patient by anti-human IgE-Abs-coated MBs, followed by the probing of immunocomplexes, obtained on MBs surface, with individual allergen solutions and/or allergenic food extracts. Within 45 min and consuming only 100 μ L of blood serum, the proposed technique provides a list of sensitizers for a chosen patient, as was demonstrated on a commercial blood serum of a milk allergic patient and clinical blood sera with sensitivity to milk, egg and king bolete. To further increase the accuracy of the developed allergy test, the allergens identification was carried out by mass fingerprinting of their tryptic peptides. In this case, directly after their extraction, allergens were digested with trypsin-loaded MOSF, which accelerates the trypsinolysis to 60 min instead of a standard overnight reaction. In specific cases, a standard overnight digestion protocol is required due to the intrinsic properties of the allergens. As a further proof-of-concept, we successfully applied the developed procedure to a clinical case of milk allergy, in which the standard *in vitro* diagnostics in hospital settings were inconclusive. It not only revealed the sensitization profile of the concerned patient, using directly the allergenic food extract, but also provided important cross-reactivity information. In addition, a new allergen was identified by using the blood serum a patient sensitive to *B. edulis*.

Overall, the proposed immunomagnetic separation with MALDI-MS detection provides an efficient and rapid allergy test with both types of allergen sources, individual allergen solutions and allergenic food extracts. Although in the present work, it was applied only to milk, egg and mushroom allergy diagnosis, the described procedure can be easily extended to any kind of food allergies because of its comprehensive and simple design. It assures within less than an hour a precise allergy diagnosis owing to the MS detection application, which also opens a possibility for identifying new, rare or modified allergens from natural food extracts. It is believed that the designed IMS-MALDI-MS method can overcome some issues of standard diagnostic methods, as it is not limited to a standard allergens list and also facilitates cross-reactivity studies, and may be used in tandem with other *in vitro* and *in vivo* methods to identify the complete sensitization profile of a patient.

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2.6 Appendix

Appendix I. Approbation letter from the ethics committee.



Commission cantonale valaisanne
d'éthique médicale (CCVEM)

Medizinisch-ethische Kommission
des Kantons Wallis (MEKKW)

Sion, le 18 octobre 2016

Monsieur
Dr Lionel Arlettaz
Médecin adjoint
Immunologie-Allergologie
Institut Central des Hôpitaux
Av. Grand-Champsec 86
1951 Sion

Concerne : sérum sanguin anonyme délivré au Laboratoire d'Electrochimie
Physique et Analytique de l'EPFL.

Monsieur le Docteur,

Suite à votre demande, la Commission Cantonale Valaisanne d'Ethique Médicale vous délivre une autorisation présidentielle de fournir, de manière anonyme, au Laboratoire d'Electrochimie Physique et Analytique de l'EPFL un échantillon de sérum sanguin d'un patient allergique au lait, duquel vous avez obtenu le consentement.

En vous remerciant d'en prendre note, nous vous présentons, Monsieur le Docteur, nos salutations les meilleures.

**Commission d'éthique
Le Président
Prof. P Ravussin**

Secrétariat Institut Central des Hôpitaux Valaisans (ICHV) • Av. Grand-Champsec 86 • 1951 Sion
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Table I. LF peptides detected after CRD of milk allergy using UTH whey fraction with immobilized trypsin digestion*

Detected mass [Da]	Peptide sequence	Position	Known modifications
647.5	CVPNSK	534-539	
659.6	AIAEKK	67-72	
1266.5	RWQWRMCK	40-47	(1xMSO, 2xTPO)
1298.5	CGLVPVLAENRK	424-435	
1452.9	ETAEEVKARYTR	352-363	
1563.0	AFALECIRIAIEKK	59-72	
1715.4	WCTISQPEWFKCR	27-39	(2xTPO)
1780.4	YTRVVWCAVGPEEQK	361-375	(1xTPO)
2183.0	VDSALYLGSRYLTLKNLR	333-351	

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Table II. BSA peptides detected after CRD of milk allergy using UHT whey fraction with immobilized trypsin digestion*

Detected mass [Da]	Peptide sequence	Position	Known modifications
703.4	VLISSAR	212-218	
817.2	SLGKVGTR	452-459	
1386.4	YICDNQDTISSK	286-297	
1418.3	LKECCDKPLLEK	298-309	
1550.9	CASIQKFGERALK	223-235	(1xSUCC)
1601.8	FWGKYLVEIARR	157-168	
1607.2	IETMREKVLASSAR	205-218	(MSO: 208)
1751.6	RHPEYAVSVLLRLAK	360-374	
1751.6	VPQVSTPTLVEVSR	438-451	(3xPHOS)
2713.4	LVTDLTKVHKECCHGDLLECADDR	257-280	
2905.5	ATEEQLKTMENFVAFVDKCCAADDK	562-587	

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Table III. α S1-casein peptides detected after CRD of milk allergy using UHT whey fraction with immobilized trypsin digestion*

Detected mass [Da]	Peptide sequence	Position	Known modifications
831.2	EDVPSEER	99-105	
2192.8	VPQLEIVPNSAEERLHSMK	121-139	(1xMSO)
2321.1	QMEAESISSSEEIVPNSVEQK	74-94	
2321.1	EDVPSERYLGYLEQLLRK	99-117	
2321.1	LKKYKVPQLEIVPNSAEER	116-134	(PHOS: 130)
2656.7	QMEAESISSSEEIVPNSVEQK	74-94	(1xMSO, 4xPHOS)

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Table IV. α S2-casein peptides detected after CRD of milk allergy using UHT whey fraction with immobilized trypsin digestion*

Detected mass [Da]	Peptide sequence	Position	Known modifications
1246.3	ITVDDKHYQK	86-95	
1246.3	LNFLKKISQR	176-185	
1251.5	EQLSTSEENSK	141-151	
1251.5	TKVIPYVRYL	213-222	
1379.3	EQLSTSEENSKK	141-152	
1722.8	KTVDMESTEVFTKK	152-165	(1xPHOS)
2553.1	TVDMESTEVFTKKTKLTEEEK	153-173	(1xPHOS)
2725.6	FPQYLQYLYQGPIVLPWDQVK	107-128	(TPO: 124)
2927.8	NANEEYSIGSSSEESAEVATEEVK	61-85	(3xPHOS)

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Table V. Peptides detected after CRD of milk allergy using bovine whey fraction with immobilized trypsin digestion*

Protein	Observed m/z	Peptide sequence	Position	Known modifications
Lactoferrin, LF	904.4	CVPNSKEK	534-541	
	922.4	AFALECIR	59-66	
	1320.5	SFQLFGSPPGQR	304-315	
	1982.9	CVPNSKEKYYGYTGAFR	534-550	

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Table VI. Peptides detected after CRD of milk allergy using bovine casein fraction with immobilized trypsin digestion*

Protein	Observed m/z	Peptide sequence	Position	Known modifications
B-casein	873.5	VKEAMAPK	113-120	
	911.3	EAMAPKHK	115-122	
	1013.4	HKEMPFPK	121-128	
	1591.9	VLPVPQKAVPYPQR	185-198	
	3042.7	RELEELNVPGEIVESLSSSEESITR	16-40	(3xPHOS)
α S1-casein	1267.6	YLGYLEQLLR	106-115	
	1337.6	HIQKEDVPSER	95-105	
	1384.6	FFVAPFPEVFGK	38-49	
	1641.8	FFVAPFPEVFGKEK	38-51	
	2312.3	FFVAPFPEVFGKEKVNELSK	38-57	
	2616.6	RPKHPIKHQGLPQEVLENLLR	106-115	
α S2-casein	904.3	NRLNFLK	174-180	
	922.3	ISQRYQK	182-188	
	975.4	TKVIPYVR	213-220	
	979.4	FALPQYLK	189-196	
	1022.4	VIPYVRYL	215-222	
	1032.5	NRLNFLKK	174-181	

αS2-casein	1050.5	KISQRYQK	181-188
	1098.5	AMKPWIQPK	204-212
	1195.5	NAVPITPTLNR	130-140
	1251.6	EQLSTSEENSK	141-151
	1251.6	TKVIPYVRYL	213-222
	1761.9	LTEEEKNRLNFKK	168-181
	1864	FALPQYLKTVYQHQQ	189-203
	1983.1	TVYQHQQAMKPWIQPK	197-212
	1991.2	KTKLTEEEKNRLNFKK	165-180
	1991.2	TKLTEEEKNRLNFKK	166-181
	2331.4	AMKPWIQPKTKVIPYVRYL	204-222
	2556.4	NAVPITPTLNREQLSTSEENSKK	130-152

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Table VII. Peptides detected after CRD of egg white allergy using egg white solution in PBS buffer with immobilized trypsin digestion*

Patient	Protein	Observed m/z	Corresponding peptide sequence	Position	Known modifications
N°1	Ovomucoid	891.5	VMVLCNR	107-113	CYS_CAM
		1088.5	VEQGASVDKR	137-146	
		1106.5	FPNATDKEGK	32-41	
		2515.0	CNFCNAVVESNGTLTSLHFGKC	189-210	3xCYS_CAM
		2640.9	AFNPVCGTDGVTYDNECLLCAHK	114-136	3xCYS_CAM
N°2	Ovomucoid	874.5	EHDGECK	81-87	CYS_CAM
		891.4	VMVLCNR	107-113	CYS_CAM
		1088.5	VEQGASVDKR	137-146	
		2515.1	CNFCNAVVESNGTLTSLHFGKC	189-210	3xCYS_CAM
N°2	Ovotransferrin	893.4	VAAHAVVAR	266-274	
		914.4	IRDLLER	623-629	
		1015.3	KSCHTAVGR	471-479	CYS_CAM

N°2	Ovotransferrin	1037.3	DEKSKCDR	372-379	CYS_CAM
		1047.4	YFGYTGALR	540-548	
		1186.4	DLLERQEKR	625-633	
		1308.6	GTEFTVNDLQ GK	120-131	
		1353.6	HTTVNENAPDQK	229-240	
		1641.8	FFSASCVPGATIEQK	174-188	CYS_CAM
		1671.7	NAPYSGYSGAFHCLK	204-218	CYS_CAM
		1826.8	NLQMDDFELLCTDGR	579-593	CYS_CAM
		1960	GAIEWEGIESGSVEQAVAK	155-173	
		2048.1	ECNLAEVPTHAVVVRPEK	602-619	CYS_CAM
		2070.1	GDVAFVKHTTVNENAPDQK	222-240	
		2550.3	TGTCNFDEYFSEG CAPGSPNSR	494-516	2xCYS_CAM
N°2	Lysozyme	874.5	HGLDNYR	33-39	
		893.4	CELAAAMK	24-31	CYS_CAM
		993.3	WWCNDGR	80-86	CYS_CAM
		1428.6	FESNFNTQATNR	33-39	
N°3	Ovalbumin	1275.6	KIKVYLPRMK	278-287	
		1522.8	YPILPEYLQCVK	112-123	CYS_CAM
		1555.8	AFKDEDTQAMPFR	188-200	
		1581.7	LTEWTSSNVMEER	265-277	
		1687.9	GGLEPINFQTAADQAR	128-143	
		1709.9	LTEWTSSNVMEERK	265-278	
		1774	ISQAVHAAHAEINEAGR	324-340	
		1859	ELINSWVESQTNGIIR	144-159	
		2284.5	VTEQESKPVQMMYQIGLFR	201-219	
		2284.5	LYAEERYPILPEYLQCVK	106-123	CYS_CAM
2901.6	FDKLPFGDSIEAQC GTSVNVHSSLR	60-85	CYS_CAM, PHOS		

N°3	Ovomucoid	874.5	EHDGECK	81-87	CYS_CAM
		891.4	VMVLCNR	107-113	CYS_CAM
		985.5	RHDGGCRK	146-153	CYS_CAM
		1088.5	VEQGASVDKR	137-146	
		2515.3	CNFCNAVVESNGTLTSLSHFGKC	189-210	3xCYS_CAM

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 18.

Table VIII. LF peptides detected after CRD of milk allergy using a clinical blood serum and bovine whey fraction with immobilized trypsin digestion*

Detected <i>m/z</i>	Peptide sequence	Position	Known modifications
875.3	NGKNCPDK	640-647	
888.3	SCHTAVDR	475-482	
1016.5	KSCHTAVDR	474-482	
1063.6	NLNREDFR	582-589	
1079.0	RAFALEICR	58-66	
1344.7	VVWCAVGPPEEQK	364-375	
1362.7	GSNFQLDDQLQGR	120-131	
1490.8	KGSNFQLDDQLQGR	119-131	
1513.9	NGKNCPDKFCLFK	640-652	
1563.9	SRSFQLFGSPPGQR	302-315	
1600.8	CGLVPVLAENRKSSK	424-438	
1616.8	ECHLAQVPSHAVVAR	263-277	
1793.9	NVRWCTISQPEWFK	24-37	
1922.0	KNVRWCTISQPEWFK	23-37	
2275.3	EDLIWKLLSKAQEKFGKKNK	283-301	
2535.3	NDTVWENTNGESTADWAKNLNR	564-585	

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Table IX. BSA peptides detected after CRD of milk allergy using clinical blood serum and bovine whey fraction with immobilized trypsin digestion*

Detected m/z	Peptide sequence	Position	Known modifications
927.5	YLVEIAR	161-167	
977.5	NECFLSHK	123-130	
1138.6	CASIQKFGER	223-232	
1465.7	VGTRCCTKPESER	456-468	
1490.8	FGERALKAWSVAR	229-241	
1519.9	LKPDNTLCDEFK	139-151	
1601.9	FWGKLYEAIIR	157-168	
1875.0	IETMREKVLASSARQR	205-220	
1987.0	VLASSARQLRCASIQK	212-228	(1xSUCC)
2185.2	KVPQVSTPTLVEVSRSLGK	437-455	(2xPHOS)
2424.4	DTHKSEIAHRFKDLGEEHFK	25-44	
2909.7	CCTESLVNRRPCFSALTPDETYVPK	499-523	(1xPHOS)

*Allergy CRD was performed using optimized immunomagnetic separation procedure with methanol/water elution, while the digestion using trypsin-loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*. Other parameters were the same as indicated in Figure 13 and 15.

Chapter 3.

Component-resolved diagnosis of nut allergies and identification of cross-reactive allergens for peanut-allergic patients

3.1 Introduction

Food allergies affect a large part of the population and are mainly caused by dairy products, eggs, peanuts, tree nuts, fish, shellfish, wheat and soya. (1) This group is often referred to the *Big 8* as they are responsible for approximately 90 % of all allergic reactions to food. (2,3) Food allergy prevalence varies greatly according to the data collection methods, as allergy self-reporting is sometimes included in studies and results in an overestimation of the food allergy prevalences. (4) Nevertheless, up to 2 % of adults and 8 % of children suffer from food allergy in western countries. (1,3,5) Not all food allergies result in the same risks and consequences for the patients. Peanut, tree nut, fish and shellfish allergies are associated with higher rates of anaphylaxis reactions compared to other allergenic foods. In addition, these allergies persist during the entire lifetime, whereas milk and egg allergies, for example, typically resolve themselves within a few years. (3,6,7) Allergies to peanuts and tree nuts have been extensively studied as they result in life-threatening reactions, occur early in life, are not commonly resolved, and are elicited by trace amounts of allergens. (8–15) Prevalences of those allergies (medically confirmed and not self-reported) are between 0.25 and 1.4 % in western countries. (3,16) Severe reactions, including anaphylaxis, may develop within minutes of allergen exposure. Tree nuts and peanuts alone are responsible for 60 to 80 % of the fatalities caused by food-related anaphylaxis. (17,18)

Cross-reactivity and cross-sensitization are commonly found between peanut and tree nut allergens. (19) Cross-reactivity is defined as the ability of a protein to be recognized by the IgE

antibodies (Abs) normally targeting another allergen, resulting in clinical symptoms. Similarities in the amino acid sequences and the tertiary structures result potentially in shared IgE-binding epitopes. (20) As proteins having the same roles in different organisms were highly conserved during the evolution process, cross-reactivity is mainly observed in between members of the same protein families. For example, the majority of nut allergens displays cross-reactivity as they are part of the seed storage proteins including legumins, conglutins and vicilins. (21) Nut allergic patients are thus advised to avoid peanuts and all tree nuts, significantly restricts their diet. Although, true cross-reactivity (*i.e.* causing clinical symptoms) is relatively occasional compared to cross-sensitization, which corresponds to positive specific-IgE-Abs or skin prick tests without clinical allergy symptoms upon ingestion or contact with cross-reactive allergen. (22) *In vitro* component-resolved diagnoses (CRD) have become important to determine specific-IgE sensitization and their combination with oral food challenges (OFCs) allows distinguishing cross-sensitization from cross-reactivity. Recently, a European clinical trial entitled “*Tree Nuts Allergies: Does a Single Nut Allergy Necessitate the Dietary Eviction?*”, usually referred to *ProNut*, was performed to distinguish the cross-reactivity from the cross-sensitization of nut allergens and to determine the correct dietary evictions for children suffering from nut allergies (clinicaltrials.gov, identified: NCT01744990). The 130 enrolled participants with diagnosed clinical nut allergy were subjected to skin prick tests (SPT), basophil activation tests, specific-IgE-Abs determinations and OFCs with all types of nuts in order to answer the main question of this study. The participants were recruited in Valencia (Hospital Infantil La Fe, Spain), Geneva (University Hospital Geneva, Switzerland), Chertsey (St. Peter’s Hospital, UK) and London (St. Thomas’ Hospital, UK). The study was limited to 1-16 years old children, with a minimum of 75 children younger than 6 years old spreaded over the different hospitals. The inclusion criteria were the confirmation of an allergic reaction after consumption of one or more nuts within the last 12 months with positive SPT (wheal size superior to 3 mm) and/or specific-IgE levels higher than $0.1 \text{ kU}\cdot\text{L}^{-1}$. Children without a convincing history of IgE-mediated allergic reaction after nut consumption but displaying positive SPT, specific-IgE tests and OFCs were included in the study. Uncontrolled asthma, chronic urticarial, chronic systemic diseases and the dependence on daily antihistamine use were set as the exclusion criteria. The evaluation of cross-reactivity in nut allergy was the primary outcome of the study. Meanwhile, the identification of predictive factors and the quality of life for nut-allergic patients were also considered in the *ProNut* study. Its outcome may lead to the better understanding of nut allergen cross-reactivity and the correct recommendations of dietary avoidance for allergic patients.

Herein, the sensitization profiles of 22 peanut-allergic patients of the *ProNut* study were identified by CRD, using a method modified from the procedure detailed in Chapter 2. The protein extracts from various nuts (*i.e.* almond, cashew nut, hazelnut, peanut, pecan nut, pistachio, walnut) were prepared directly in-house from commercially available nuts and the presence of allergens in these extracts was verified by bottom-up and top-down proteomics. Then, the previously developed CRD procedure based on immunomagnetic separation and mass spectrometry detection (IMS-MALDI-MS) was modified and adapted to peanut and tree nut allergies. The blood sera of the 22 peanut-allergic patients were tested against the 7 nut extracts using the new CRD procedure. The obtained sensitization profiles were then compared to clinical results of OFCs and specific-IgE-Abs determination in order to identify potential cross-reactive allergens.

3.2 Materials and methods

3.2.1 Chemical and materials

Organic unsalted peanuts and tree nuts were purchased in a local store. Acetone, polysorbate surfactant Tween 20, boric acid, potassium chloride, iodoacetamide, trifluoroacetic acid, protease inhibitor cocktail for plant cells and tissues, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Buchs, Switzerland). Sodium dihydrogen phosphate, sodium hydrogen phosphate, sodium chloride, ammonium acetate, ammonium bicarbonate, DL-dithiothreitol and formic acid (FA) were purchased from Fluka (Buchs, Switzerland). Methanol (hypergrade for LC-MS, LiChrosolv) and sodium hydroxide were obtained from Merck (Darmstadt, Germany) and RectoLab SA (Servion, Switzerland), respectively. Hydrochloric acid (32 %) was obtained from Fischer Chemicals AG (Zurich, Switzerland), while n-hexane and acetonitrile (ACN, Rotisolv HPLC) were purchased from Carl Roth AG (Karlsruhe, Germany). Tris-(hydroxymethyl)aminomethane (Tris) was obtained from Acros Organics (Basel, Switzerland). Trypsin from bovine pancreas was acquired from AppliChem GmbH (Darmstadt, Germany). Estapor tosyl-activated superparamagnetic polystyrene beads with a diameter of 1.29 μm were obtained from Merck Chimie SAS (Fontenay-sous-Bois, France). Mouse monoclonal anti-human IgE-Abs (0100-0414) were acquired from AbD Serotec (Oxford, United Kingdom). Dimethyl sulfoxide (DMS) were purchased from Pierce Biotechnology (Rockford, IL, USA). MOSF was kindly offered by Prof. Baohong Liu, Fudan University (Shanghai, China).

Ultrapure water was obtained from a Milli-Q Integral Water Purification System (Merck-Millipore, Zug, Switzerland) and used in all experiments. An Agilent 8453 G1103A spectrophotometer (Agilent Technologies, Waldbronn, Germany) was used for all UV-Vis analyses with quartz cuvettes (200–2500 nm spectral range). Protein quantification was carried out using a commercial BCA protein assay (Pierce, ThermoFisher Scientific, Rockford, USA) with BSA as a standard.

3.2.2 Preparation of nut flours

Almonds, walnuts, hazelnuts and pecan nuts were blanched for 10 seconds in boiling water before putting them in cold water (4°C) for a couple of minutes in order to easily remove the skin/pellicle of the nuts. All the nuts were then frozen in liquid nitrogen before grinding them in an electric kitchen grinder. The obtained powders/pastes were defatted by adding n-hexane with a 1:5 (w/v) ratio and the mixtures were stirred for 20 minutes at room temperature. The defatted flours were then filtered and dried overnight before storing them at 20°C until further use.

3.2.3 Protein extraction from flours and further purification procedure

For each type of nuts, the protein extraction was carried out by incubating 0.5 g of flour with 40 mL of 100 mM PBS buffer (pH 7.2) for 1 hour at room temperature with 16.7 µL of protease inhibitor cocktail. After the incubation, the solution was centrifuged at 6000 g for 10 minutes and the solid matter was discarded. The supernatant was filtered through a syringe filter (Chromafil Xtra PVDF-45/25, Macherey-Nagel Ag, Oensingen, Switzerland) in order to remove the remaining small particles. Cold acetone (–20°C) was added to the sample with a 5:1 (v/v) ratio and incubated overnight at –20°C in order to precipitate the proteins present in the sample. After centrifugation, the protein pellet was washed two times with cold acetone and redissolved in 25 mM ammonium bicarbonate buffer (pH 8.2). Thus, obtained individual protein extracts were then lyophilized using a commercial lyophilisation instrument (Alpha 2-4 LSC, Martin Christ, Osterode an Harz, Germany) and stored at –20°C until further use. When required, the protein extracts were reconstituted in 25 mM ammonium bicarbonate buffer (pH 8.2).

3.2.4 Mass spectrometry analysis of the nut protein extracts by top-down proteomic approach

The protein extracts were injected on an ACQUITY UPLC Protein BEH C₄ column (1 x 150 mm, 1.7 µm, Waters, Baden-Dättwil, Switzerland) using a Dionex Ultimate 3000 HPLC system (Thermo Fischer Scientific, San Jose, USA) coupled to Q Exactive HF Hybrid Quadrupole-Orbitrap

mass spectrometer (Thermo Fisher Scientific, San Jose, USA) set to Protein mode. The column oven was set to 60°C and a fixed flowrate of 0.09 mL·min⁻¹ was used. Eluents A and B consisted, respectively, in 99.9:0.1 % H₂O:FA and 99.9:0.1 % ACN:FA. The concentration of eluent B was kept at 5 % for the first 3 minutes as equilibration step, before increasing it to 45 % in 60 minutes as effective separation gradient. It was then further increased to 80 % in 1 minute and kept at this concentration for 5 minutes as a washing step. Finally, the eluent B was decreased to 5 % in 1 minute and maintained for 5 minutes to reequilibrate the column. A blank injection was performed between each sample analysis.

For the full MS scans, the resolution was set at 120'000 (at 400 *m/z*) with a maximum injection time of 200 ms, an automatic gain control (AGC) target of 3E6 and a source-induced dissociation of 15. The full MS spectra were recorded from 300 to 1900 *m/z* in profile mode by averaging 4 microscans. The MS² spectra were recorded in profile mode with Top2 method and the resolution 60'000 at 400 *m/z*, while averaging 4 or 8 microscans. The maximum injection time was fixed at 800 ms with an AGC target of 1E6. Proteins were isolated with a window of 15 *m/z* and fragmented using normalized collision energy (NCE) of higher-energy collisional dissociation (HCD) of 24 with default charge 10.

The open-source softwares MS-Deconv and TopPIC were used for the top-down proteomic data processing. (23,24) The MS spectra were deconvoluted using MS-Deconv with maximum charge set to 50, a limit monoisotopic mass of 85 kDa and a *m/z* error tolerance of 0.05. TopPIC was then used to search the deconvoluted proteoforms against the nut protein databases, with limit values for the signal to noise ratio of MS and MS² scans set to 3 and 1, respectively. The error tolerance for the precursor and the fragment masses was set to 15 ppm and unexpected mass shifts were allowed with a 500 Da limit. The proteoforms spectrum matches (PrSMs) were validated using E-values with a 1E-4 cutoff. The protein databases used were obtained from the Universal Protein Resource (UniProt, release January 2017) and contained the complete reviewed (SwissProt) and unreviewed (TrEMBL) proteomes of the corresponding nut species.

3.2.5 Mass spectrometry analysis of the nut protein extracts by bottom-up proteomic approach

The lyophilized protein extracts were resolubilized in 25 mM ammonium bicarbonate buffer (pH 8.2) to working concentrations of 2 mg·mL⁻¹. 150 µL of these extracts were then re-

duced by adding 5 μL of 200 mM dithiothreitol (DTT) in the same ammonium bicarbonate buffer and incubated for 30 minutes at 56 $^{\circ}\text{C}$. After letting the solutions to cool down, 10 μL of iodoacetamide (IAA, 100 mM, in ammonium bicarbonate buffer) were added and the alkylation reactions were performed in the dark for 30 minutes at room temperature. Trypsin ($2.5 \text{ mg}\cdot\text{mL}^{-1}$) was finally added with a ratio 30:1 (w/w, protein:trypsin) and the solutions were incubated overnight at 37 $^{\circ}\text{C}$ under moderate stirring. The tryptic reactions were stopped by acidification using trifluoroacetic acid to a final acid concentration of 0.1% and pH < 4. Tryptic peptides were extracted and concentrated using ZipTip C₁₈ pipette tips (Merck Millipore, Darmstadt, Germany) following the manufacturer protocol.

Tryptic peptides obtained after ZipTip C₁₈ purification were injected on a ZORBAX Eclipse Plus C₁₈ column (2.1 x 150 mm, 5 μm , Agilent, Waldbronn, Germany) using a Dionex Ultimate 3000 HPLC system (Thermo Fischer Scientific, San Jose, USA) coupled to Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA). The column oven was set to 30 $^{\circ}\text{C}$ and a fixed flowrate of 0.25 $\text{mL}\cdot\text{min}^{-1}$ was used. Eluents A and B consisted, respectively, in 99.9:0.1 % H₂O:FA and 99.9:0.1 % ACN:FA. The concentration of eluent B was kept at 3 % for the first 5 minutes as equilibration step, before increasing it to 40 % in 40 minutes as effective separation gradient. It was then increased to 80 % in 2 minutes and kept at this concentration for 5 minutes as washing step. Finally, the eluent B was decreased to 3 % in 1 minute and maintained for 5 minutes to reequilibrate the column. A blank injection was performed between each sample analysis.

A standard full MS/dd-MS² method was used for the analysis of the tryptic peptides. The resolution was set at 60'000 (at 400 m/z) for the full MS scans, with an AGC target of 1E6 and maximum injection time of 100 ms. The full MS spectra were recorded from 300 to 1900 m/z in profile mode. The MS² spectra were recorded in centroid mode with Top10 method and the resolution 30'000 at 400 m/z . The maximum injection time was fixed at 100 ms with 4 microscans averaging and an AGC target of 2E5. A 2 m/z isolation window was used for the parent ions and the NCE of HCD fragmentation was set at 27 with default charge 2. Only peptides with an assigned charged between 2 and 7 were allowed for selection and subsequent fragmentation.

The obtained data were processed using the open-source softwares SearchGUI and PeptideShaker (25,26) and the Trans-Proteomic Pipeline. (27) The protein databases used were ob-

tained from the Universal Protein Resource (UniProt, release February 2017) and contained the complete reviewed and unreviewed nut proteomes (both SwissProt & TrEMBL), common keratin contaminants and bovine trypsin. Carbamidomethylation of cysteine residue (+ 57.0215 Da) was set as a fixed modification, whereas acetylation of protein N-terminus and lysine (+ 42.0106 Da), deamidation of asparagine and glutamine (+ 0.9840 Da), and oxidation of methionine (+ 15.9949 Da) were set as potential modifications. A maximum of 4 missed cleavages was set with a precursor m/z tolerance of 10 ppm and a fragment tolerance of 0.04 Da. The peptide identifications were validated with a false discovery rate (FDR) of 1 %.

3.2.6 Functionalization of the magnetic beads with anti-human IgE Abs

The functionalization of magnetic beads (MBs) was performed according to the manufacturer protocol. (28) First, 20 μL of MBs ($100 \text{ mg}\cdot\text{mL}^{-1}$) were washed three times with a coating buffer (0.1 M sodium borate buffer, pH 9.5) and resuspended in 20 μL of the same buffer. 80 μL of anti-human IgE-Abs ($1 \text{ mg}\cdot\text{mL}^{-1}$), 260 μL of coating buffer and 166 μL of 3 M ammonium sulfate in coating buffer were added to the MBs suspension. The obtained solution was incubated overnight at room temperature under continuous moderate stirring to avoid MBs sedimentation. Afterwards, the supernatant was removed and 500 μL of the blocking buffer (10 mM PBS solution, 0.1 % Tween 20, pH 7.4) were added to the MBs for 1 h of incubation at room temperature. Then, the MBs were washed three times with washing buffer (10 mM PBS solution, 0.05 % Tween 20, pH 7.4) and resuspended in the storage buffer (10 mM PBS solution, 0.025% Tween 20, 0.02% NaN_3 , pH 7.4) to obtain a final concentration of $5 \text{ mg}\cdot\text{mL}^{-1}$. All the functionalized MBs were stored at 4°C and used within a period of 3 weeks after their coating.

3.2.7 Preparation of trypsin-loaded MOSF

1 mg of macroporous ordered silica foam (MOSF) was added to 100 μg of trypsin dissolved in 1 mL of 25 mM ammonium bicarbonate buffer (pH 7.8) and incubated at room temperature for 30 min. After the incubation, the suspension was centrifuged, the supernatant was discarded and the MOSF particles were washed twice with ammonium bicarbonate buffer. The obtained trypsin loaded MOSF was stored at 4°C in 200 μL of ammonium bicarbonate buffer, at a final working MOSF concentration of $5 \text{ mg}\cdot\text{mL}^{-1}$.

3.2.8 CRD procedure for the diagnosis of nut allergies by immunomagnetic separation

150 µg of anti-human IgE-Abs-functionalized MBs were transferred into an Eppendorf tube and washed three times with the washing buffer. 50 µL of the blood serum from an allergic patient were diluted to 100 µL with pure water and added to the MBs pellet. The incubation time for the IgE-Abs capture was set to 5 minutes at room temperature under medium stirring. After the incubation, the MBs were separated from the supernatant and washed three times with the washing buffer. The IgE-Abs were then covalently linked to the MBs using dimethyl suberimidate (DMS) as a cross-linker, using a procedure adapted from the DMS manufacturer protocol. (29) After the 150 µg of the resulting MBs were dispersed in 200 µL of 0.2 M triethanolamine (pH 9.0), then, mixed with 1 mL of 0.2 mM DMS in 0.2 M triethanolamine and incubated for 30 minutes at room temperature. To stop the reaction, 200 µL of 50 mM Tris-HCl buffer (pH 7.5) were added to the mixture and incubated for 15 minutes. After rinsing with washing buffer, the MBs with cross-linked IgE-Abs were directly used to perform the CRD.

Before performing the CRD, the lyophilized protein extracts were reconstituted in ammonium bicarbonate buffer (25 mM, pH 8.2) to obtain a final protein concentration of 1 mg·mL⁻¹. Then, 200 µL of the protein extracts were added to the cross-linked MBs and incubated for 15 minutes at room temperature under medium stirring. The MBs were then washed twice with washing buffer and two times with 100 mM PBS buffer (pH 7.2). The captured proteins were eluted by adding 10 µL of a 1:3 (v/v) methanol/water solution to the MBs pellet. The eluate was recovered and the MBs were washed three times with 100 µL of washing buffer. The allergen extraction procedure was then repeated twice using the same cross-linked MBs in order to accumulate more sample material, and the eluates were then combined.

3.2.9 Tryptic digestion of the protein extracted in the CRD procedure

The eluates obtained after the CRD procedure were concentrated to 1 µL using a rotational-vacuum concentrator (RVC 2-25, Martin Christ, Osterode an Harz, Germany) and rebuffered in 10 µL of 25 mM ammonium bicarbonate buffer (pH 8.2). 100 mM solution of DTT in the same ammonium bicarbonate buffer was added to the protein solutions to a final DTT concentration of 5 mM and the solutions were incubated at 56°C for 30 minutes. After letting the solutions to cool down, IAA (100 mM, in ammonium bicarbonate buffer) was added to a final concentration of 10 mM and the alkylation reaction was performed in the dark for 30 minutes at room temperature. 1 µL of the trypsin-loaded MOSF was added to the protein solutions and incubated at 37°C for

60 min. The tryptic reactions were stopped by acidification using trifluoroacetic acid to a final acid concentration of 0.1% and pH < 4 and the MOSF particles were removed by centrifugation. The tryptic peptides were extracted and concentrated using ZipTip C₁₈ pipette tips (Merck Millipore, Darmstadt, Germany) following the manufacturer protocol.

3.2.10 Mass spectrometry analysis by peptide mass fingerprinting of the extracted allergens

The tryptic peptide analysis were performed on a Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) by direct infusion using a TriVersa NanoMate chip-based nanoelectrospray ionization (nanoESI) source (Advion Biosciences, Ithaca, USA). Five to ten µL of the samples were injected via the NanoMate chip by applying a 1.2 - 1.4 kV ionization voltage. The mass spectra were recorded in positive mode for to 2 to 20 minutes, with 45'000 or 60'000 resolution at 400 *m/z*, depending on the quality of the MS spectra obtained. The maximum injection time was set to 200 ms with an AGC target of 3E6 and averaging of 4 microscans. The doubly charged peptide LGEDNINVEGNEQFISASK (*m/z* 1082.0318), originating from trypsin autoproteolysis, was used as an internal lock mass in all experiments.

The obtained *.raw* mass spectra were converted to the *.mzXML* format using the MsConvert tool from the ProteoWizard Toolkit. (30) The MS spectra were deconvoluted using MS-Deconv with maximum charge set to 10, a limit monoisotopic mass of 5 kDa and *m/z* error tolerance of 0.05. The protein identification was then performed by peptide mass fingerprinting using MsPI v1.2, an open source Perl procedure. (31) The peptides masses were searched against the nut protein databases obtained from the Universal Protein Resource (UniProt, release February 2017), both SwissProt & TrEMBL. Randomized sequence databases were created by the software for the validation of the results using a *p*-value threshold of 0.05. The mass tolerance, the maximum number of missed cleavages, and the number of allowed post-translational modifications (PTMs) were set to 1 ppm, 4 and 4, respectively. Trypsin and standard contaminants, such as keratins, were automatically removed from the results. Carbamidomethylation of cysteine residue (+ 57.0215 Da) was set as a fixed modification whereas acetylation of protein N-terminus and lysine (+ 42.0106 Da), deamidation of asparagine and glutamine (+ 0.9840 Da), and oxidation of methionine (+ 15.9949 Da) were set as potential modifications.

3.2.11 Alignment and scoring of the nut allergens proteoforms

The amino acids sequences of all the nut allergens proteoforms were obtained from the Universal Protein Resource (UniProt) web servers. The alignment were performed using the Needleman-Wunsch and the Smith-Waterman algorithms in Matlab (v. R2015b) using the corresponding build-in commands. BLOSUM50 was set as the scoring matrix and the penalty for opening gaps was set to 8. The scores for all alignment pairs were assembled in a single matrix and represented as a heat map (Appendix Figure I).

3.3 Results and discussion

3.3.1 Nut flour preparation and protein extraction

Nuts are naturally encased in a fibrous skin called the pellicle, which contains high level of antioxidants and antimicrobial chemicals. (32–34) These pellicles were removed from pecan nuts, walnuts, almonds and hazelnuts by blanching them for 10 seconds and cooling them rapidly, to avoid the degradation of heat-labile allergens. Peanut and cashew nut were bought already peeled, whereas pistachio skin was not removed. The nut flours were then prepared as detailed in *Materials and method* section 3.2.2. The freezing process during flour preparation is important to avoid the thermal stress and the extensive protein modifications induced normally by grinding. (35–37) n-Hexane and diethyl ether are commonly used organic solvents for the defatting of food products thanks to their high extraction efficiencies. (38,39) Recently, Walczyk *et al.* showed the superiority of n-hexane over diethyl ether for the defatting of peanut flour, resulting in better allergen extraction. (40) For this reason, n-hexane was chosen as the defatting solvent in all the experiments. The samples were weighted before and after the defatting procedure and compared to the natural fat content of each nut in order to ensure a total defatting of the flours. A second defatting procedure was required for pistachios, almonds, hazelnuts and pecan nuts.

A large variety of aqueous buffers, pH, temperatures and incubation times has been previously used for plant protein extraction. (41–46) In these studies, neutral or slightly basic buffers (pH 7-9) showed the best extraction efficiency, with incubation times ranging from minutes to 1 hour. To increase the protein yield, the use of elevated temperatures and/or chaotropic agents is suggested. Meanwhile, milder conditions are required in the present work in order to avoid excessive modification, fragmentation and protein denaturation that could affect further CRD experi-

ments. The protein extractions were therefore performed at room temperature in a neutral PBS buffer (100 mM, pH 7.2), as detailed in *Material and Method* section 3.2.3. For pistachio, almond, hazelnut, pecan nut, walnut, cashew nut and peanut, the protein concentration were measured after the extraction procedure at 2.8, 3.8, 2.8, 0.7, 3.7, 3.0 and 3,3 mg·mL⁻¹, respectively.

3.3.2 Mass spectrometry analysis of the nut extracts by top-down proteomics

The quality of each protein extract and the presence of allergens have been assessed first by a top-down proteomic (TDP) approach, as detailed in *Materials and Methods* sections 3.2.4. TDP analyses, complementary to classical bottom-up proteomics (BUP), subject intact proteins to LC separation followed by full MS/dd-MS² analysis directly without enzymatic digestion allowing collection of unique information at proteoforms level avoiding protein inference problem and without introduction of artificial modifications caused by trypsinolysis. (47) The MS¹ and MS² spectra obtained may display an extremely high complexity due to the potential presence of various proteoforms and the large number of high-mass fragment ions. For this reason, TDP procedures require high mass accuracy and resolution to separate spectrally the peaks and identify precisely small mass-shifts between proteoforms. (48) TDP is therefore carried out on either Fourier-transform ion cyclotron resonance (FT-ICR) or Orbitrap mass analyzers. Compared to other proteomic approaches, TDP allows analysing intact proteins, identifying easily post-translational modifications (PTMs), and detecting proteoforms or degradation products. (49) Additionally, as no protein digestion is required, TDP procedures result usually in a full protein coverage without the loss of PTM sites. (49) However, the complexity of the generated data and various technical limitations make TDP more challenging than other MS-based procedures. (50) TDP methods are complementary and alternative to other MS procedures and have been recently proposed for food authentication and quality control based on allergen identification. (51–53)

The TDP analysis was first tested on a peanut extract obtained following the developed extraction procedure. 33 proteins in total were identified, 8 of which are known allergen isoforms, *i.e.* proteoforms cause by polymorphisms (Table 1). However, partial or even extensive degradation of the proteins was observed for this extract. For example, the allergen *Ara h 10* (isoform Q647G5) was identified, with a E-value of 1.35E–11, based only on the small fragment AA 155-169, with a mass of 2802.4894 Da and detected in the MS¹ scan bearing 5 charges. The tandem spectrum corresponding to the *Ara h 10* product ions obtained in the TDP analysis is displayed in Figure 1, whereas the identified ions are listed in Appendix Table I. The expectation value, called E-value,

is a statistical tool for the evaluation of confidence measures and refers to the expected number of random hits having an equal or better score than the observed score of the identified proteins. (54) The most classical methods to evaluate E-values are based on multiple hypothesis testing methods using Poisson-based probability with Bonferroni correction of proteoform assignment being incorrect. (54–56) E-values are calculated for each proteoform detected by TopPIC and give an estimation of the quality of the protein identification, depending on multiple parameters, including the length of the protein/fragment and the number of matched peaks and fragment ions. (24)

Table 1. Allergens identified by TDP approach ^(a) in peanut flour, extracted in the presence of 200 and 16.7 μ L of PIC.

Allergen Code	Protein (UniProt N°)	E-values ^(b) obtained using:	
		200 μ L of PIC	16.7 μ L of PIC
<i>Ara h 1</i>	P43238	3.45E-08	3.50E-07
<i>Ara h 3</i>	A1DZF0	3.91E-07	4.81E-17
	B5TYU1	1.33E-16	1.33E-11
	E5G077	6.73E-07	1.44E-07
	O82580	1.89E-05	
	Q8LKN1		6.40E-08
	Q9SQH7	8.95E-10	3.51E-07
	Q647H3	1.15E-15	-
	Q6T2T4	6.45E-19	-
	Q9FZ11	6.00E-10	-
<i>Ara h 6</i>	Q647G9	-	5.84E-06
<i>Ara h 10</i>	Q647G5	-	1.35E-11
Total number of identified proteins		27	33
Total number of identified allergenic proteoforms		20	19
Total number of identified allergens isoforms		9	8

(a) TDP analyses were performed with the averaging of 4 microscans for MS² spectra, whereas other parameters are listed in *Materials and Methods* section 3.2.4.

(b) The E-values listed corresponds to the best protein-spectrum matches (PrSMs).

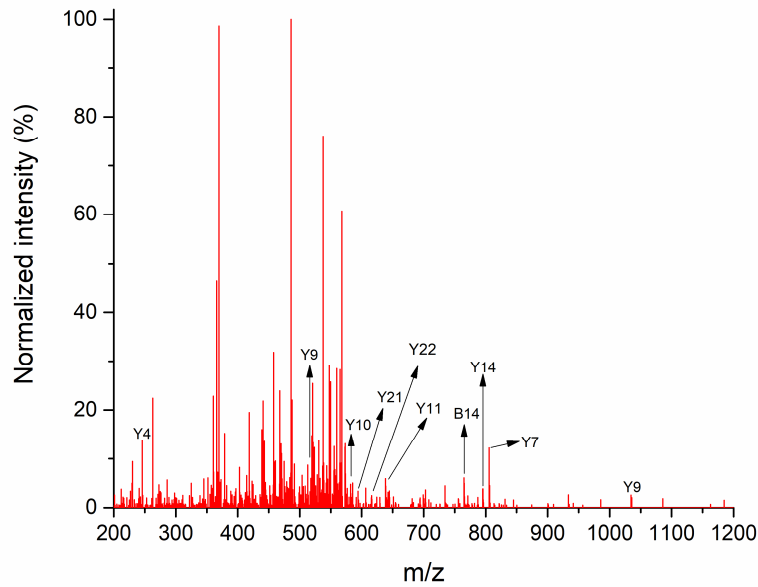


Figure 1. Tandem spectrum of *Ara h 10* fragment AA 155-169 obtained during the TDP analysis of the peanut extract. The MS² spectrum was measured with a resolution of 60'000 at 400 *m/z* and with 4 averaged microscans. Other parameters were set as in *Materials and Methods* section 3.2.4. The identified product ions are listed in Appendix Table I. The numbering of the product ions begin at the amino acid 155.

Protein degradation is to be expected during the extraction procedure due to the release and activation of proteases present in the nuts, the mechanical stress induced by the grinding, as well as all temperature changes, especially during liquid nitrogen treatment. Proteases are key proteins of seeds and nuts as they are required during the germination process and various biological pathways (57,58) The protease inhibitor cocktail (PIC) added to the extraction buffer contains various chemical able to block the four groups of plant proteases (cysteine, aspartic, serine and metal proteases). As the PIC amount used in this first experiment (16.7 μL) was potentially too low to inhibit efficiently the proteases, the peanut extraction was repeated in the presence of 200 μL of PIC. The TDP analysis resulted in the identification of 27 proteins and 9 allergen isoforms (detected as proteoforms) in this case (Table 1) and degradation was still detected to a similar extend. In both extraction procedures, various isoforms of *Ara h 1* and *Ara h 3* were identified, meanwhile *Ara h 6* and *Ara h 10* identification is limited to the first extraction procedure with 16.7 μL of PIC (Table 1). In both samples, *Ara h 2* (isoform Q6PSU2) was detected but excluded during the validation process due to its E-values slightly higher than the $1\text{E-}4$ cutoff. The E-values of the best protein-spectrum matches (PrSMs) are also compiled in Table 1 and demonstrates no clear improvement when using 200 μL of PIC instead of 16.7 μL . These data suggest that the protein degradation is a direct consequence of other factors arising from the extraction procedure itself and not

caused by the proteases. For this reason, 16.7 μL of PIC were added to the PBS buffer for all extraction procedures. It is worth mentioning that this information about allergen degradation upon widely used extraction procedure is crucial for the further CRD experiments: it revealed that a lot of allergens used for diagnostic procedure herein and in other studies were actually not in their intact form, but present as various fragments, thus resulting potentially in an incomplete sensitization profile determination by CRD. To the best of our knowledge, such observation was never reported before simply due to the fact that allergenic extracts were always characterized by BUP approach, in which the allergen are specially digested into peptides prior to any MS-based detection.

In this work, higher energy collisional dissociation (HCD) was used to obtain fragment ions from a precursor molecule during TDP analysis. HCD fragmentation is relatively similar to the standard collision induced dissociation (CID), as they both result in b and y-ions. (49) The collisional energy of HCD is higher than CID and gives usually more informative fragmentation spectra. (59) The level of fragmentation in the HCD cell is controlled by a parameter called the normalized collision energy (NCE). By modifying this parameter, the axial field and the offset of the RF rods of the HCD cell are changed resulting in stronger or weaker fragmentation. (60) The TDP analysis of the peanut protein extract was carried out at various NCE levels and the number of identified proteins/allergens was used to determine the optimal fragmentation conditions, crucial for this approach. Applying a stepped 20/25 NCE the best overall identification of peanut proteins, with 34 hits compared to the other tested NCE values (Table 2). This result was expected, as during HCD fragmentation with stepped NCE, the precursor ions are fragmented at several energy levels and the combined fragment ions are detected simultaneously. (61) However, setting the NCE to 24 resulted in a better allergen identification, with 8 detected allergen isoforms, whereas a NCE of 30 decreased drastically the quality of the protein identification due to extensive overfragmentation. Additionally, the major peanut allergen *Ara h 1* was not detected with a NCE set to 30, (Table 2). For these reasons, a NCE of 24 was used in all further TDP analyses.

Table 2. Allergens identified in peanut flour by a TDP approach ^(a) using different NCE parameters

Allergen code	Allergen detected (UniProt N°)	E-value with stepped NCE at 20/25	E-value with NCE at 24	E-value with NCE at 30
Ara h 1	P43238	4.08E-07	3.45E-08	-
	A1DZF0	9.38E-27	3.91E-07	3.22E-09
	B5TYU1	9.69E-07	1.33E-16	6.28E-06
	E5G077	4.72E-14	6.73E-07	
	O82580		1.89E-05	
Ara h 3				-
	Q647H3	5.45E-25	1.15E-15	3.95E-14
	Q6T2T4	1.06E-26	6.45E-19	6.75E-13
	Q9SQH7	1.47E-13	8.95E-10	6.67E-05
	Q9FZ11	-	6.00E-10	
Total number of detected proteins		34	33	13
Total number of allergenic proteoforms		18	19	12
Total number of detected allergens isoforms		7	8	5

(a) All TDP analyses were carried out as detailed in *Materials and Methods* section 3.2.4 with the averaging of 4 microscans.

Based on the results discussed above, proteins were extracted from all flours in the presence of 16.7 μ L of PIC and the extracts were analyzed by the TDP approach with a NCE set to 24. The TDP analysis was also repeated with the same parameters but varying the number of averaged microscans per MS² spectra. In general, averaging several microscans improves the signal-to-noise level and decreases the mass errors, leading to higher quality MS² spectra. However, the procedure of microscan averaging increases significantly duty cycle of full MS/dd-MS² analysis and leads to a drop in the number of identified proteins. (62) The optimal number of averaged microscans varies greatly depending on the instrument, the sample complexity, LC separation conditions and other MS parameters used. (62,63) In this work, the seven nut protein extracts were analyzed with 4 and 8 averaged microscans for MS² spectra. All allergens identified in the TDP analyses with 4 and 8 averaged microscans are listed in Table 3, whereas all identified allergen proteoforms and their respective E-values are detailed in Appendix Tables II and III. As expected, the total number of identified proteins decreased when 8 microscans were averaged, except for pecan and pistachio extracts, as shown in Figure 2. Meanwhile, the number of unique allergens and their different proteoforms identified by TDP is relatively similar for 4 and 8 microscans averaging with almost no influence on the E-values (Figure 2 and Appendix Tables II and III).

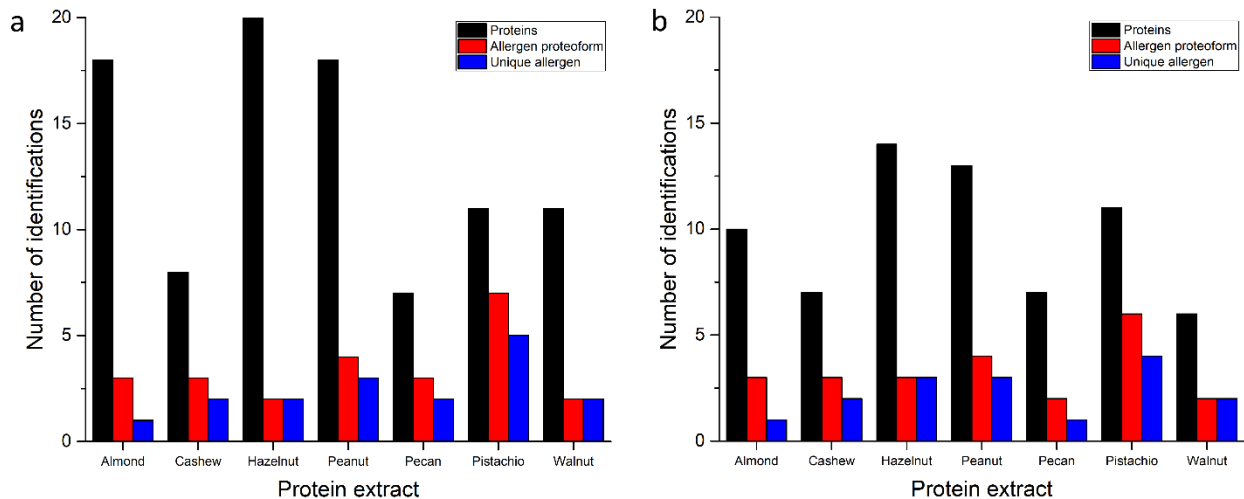


Figure 2. Number of identified proteins, allergen proteoforms including various isoforms and unique allergens obtained during the TDP analysis of the seven nuts protein extract using averaging of (a) 4 and (b) 8 microscans for MS² spectra. TDP parameters are as described in *Materials and Methods* section 3.2.4.

In fact, the results obtained from 2 sets of experiments yielded complementary allergen identifications, for example, for hazelnut and walnut allergens (Table 3). However, when combining both sets of results, only 21 of the 44 known allergens were detected and identified, corresponding to 48 % of allergen recovery. For pistachio, the complete sets of allergens were detected between both numbers of averaged microscans, whereas not all the allergens were identified for the other nut extracts. Only 4 allergens were detected in the peanut extract while 14 allergens are known. This may be explained by the large difference in allergen concentrations and the matrix effects resulting in different extraction efficiencies. Another important factor influencing TDP results is the quality of the databases used for data processing. Proteomes of all nut species are poorly characterized in contrast with human or mouse proteomes: most of the entries belong to TrEMBL section of UniProt database having many redundant entries with mistakes and missing amino acid residues and no information about potential PTMs. As in TDP approach the allergenic proteins are identified at intact proteoform level, application of poor quality database inevitably leads to rejection or poor scoring of good identifications. Significant protein degradation observed upon extraction procedure could also alter the TDP data analysis. Typical drawbacks of TDP approach like issues with large (> 40 kDa) protein identification on the chromatographic scale together with reduced efficiency of their fragmentation by HCD, problems with deconvolution of overlapping charge envelopes from the coeluting proteoforms, long duty cycles and repeated

fragmentation of the same proteoform at different charge states also have an impact on the obtained results.

Table 3. Allergens identified in nut protein extracts^(a-b) by a TDP approach with the averaging of 4 and 8 microscans for MS² spectra.

Nut extract	Allergen code	4 μ scans	8 μ scans
Almond	Pru du 3	✗	✗
	Pru du 4	✗	✗
	Pru du 5	✗	✗
	Pru du 6	✓	✓
Cashew	Ana o 1	✓	✓
	Ana o 2	✓	✓
	Ana o 3	✗	✗
Hazelnut	Cor a 1	✓	✗
	Cor a 2	✗	✓
	Cor a 6	✗	✗
	Cor a 8	✗	✗
	Cor a 9	✓	✓
	Cor a 10	✗	✓
	Cor a 11	✗	✗
	Cor a 12	✗	✗
	Cor a 13	✗	✗
	Cor a 14	✗	✗
Walnut	Jug r 1	✗	✓
	Jug r 2	✓	✓
	Jug r 3	✗	✗
	Jug r 4	✓	✗
	Jug r 5	✗	✗

Nut extract	Allergen code	4 μ scans	8 μ scans
Peanut	Ara h 1	✓	✓
	Ara h 2	✓	✗
	Ara h 3	✓	✓
	Ara h 5	✗	✗
	Ara h 6	✗	✓
	Ara h 7	✗	✗
	Ara h 8	✗	✗
	Ara h 9	✗	✗
	Ara h 10	✗	✗
	Ara h 11	✗	✗
	Ara h 12	✗	✗
	Ara h 13	✗	✗
	Ara h 14	✗	✗
	Ara h 15	✗	✗
	Pecan	Car i 1	✗
Car i 2		✓	✓
Car i 4		✓	✗
Pistachio	Pis v 1	✓	✓
	Pis v 2	✓	✓
	Pis v 3	✓	✓
	Pis v 4	✓	✗
	Pis v 5	✓	✓

(a) The symbol ✗ indicates that the corresponding allergen was not detected.

(b) The symbol ✓ indicates that the corresponding allergen was detected and identified by TopPIC with an E-value lower than the set 1E-2 cut-off.

3.3.3 Mass spectrometry analysis of the nut extracts by bottom-up proteomics

In complement to the TDP analysis discussed above, a standard BUP approach has been performed in order to obtain the complete overview of allergenic compositions of all the nut extracts. The proteolytic digestion and the BUP analyses were performed as detailed in the *Materials and Methods* section 3.2.5. The data analysis was carried out on two freely available softwares: the Trans-Proteomic Pipeline, TPP, (27) and SearchGUI/PeptideShaker, SGUI/PS, (25,26) with the X!TANDEM peptide search engine based on heuristic matching and scoring algorithm (64) and displayed similar outcomes. All the allergens identified by SGUI/PS and TPP are listed in Table 4, whereas the detailed BUP results are listed in Appendix Tables IV-V. Small variations in the search

and validation algorithms between the two softwares are responsible for the results difference. In general, the peptide validation process is stricter in TPP resulting in the rejection of several peptides validated by SGUI/PS and in the overall lower sequence coverages and numbers of identified peptides. The two softwares were able to identify most of the time the same allergens as shown in Table 4. The only exceptions are *Cor a 10*, *Ara h 11*, *Ara h 15* and *Pis v 4*, detected only by one software and rejected by the other, due to their low validation scores based only on 2 to 3 unique peptides. In total, 36 allergens were identified in the nut extracts by BUP, corresponding to an 82 % allergen recovery.

Table 4. Allergens identified in nut protein extracts ^(a-b) by a BUP approach using SearchGUI/PeptideShaker (SGUI/PS) and the Trans-Proteomic Pipeline (TPP).

Nut extract	Allergen code	SGUI/PS	TPP
Almond	<i>Pru du 3</i>	✗	✗
	<i>Pru du 4</i>	✗	✗
	<i>Pru du 5</i>	✗	✗
	<i>Pru du 6</i>	✓	✓
Cashew	<i>Ana o 1</i>	✓	✓
	<i>Ana o 2</i>	✓	✓
	<i>Ana o 3</i>	✓	✓
Hazelnut	<i>Cor a 1</i>	✓	✓
	<i>Cor a 2</i>	✗	✗
	<i>Cor a 6</i>	✗	✗
	<i>Cor a 8</i>	✓	✓
	<i>Cor a 9</i>	✓	✓
	<i>Cor a 10</i>	✗	✓
	<i>Cor a 11</i>	✓	✓
	<i>Cor a 12</i>	✓	✓
	<i>Cor a 13</i>	✓	✓
	<i>Cor a 14</i>	✓	✓
Walnut	<i>Jug r 1</i>	✓	✓
	<i>Jug r 2</i>	✓	✓
	<i>Jug r 3</i>	✓	✓
	<i>Jug r 4</i>	✓	✓
	<i>Jug r 5</i>	✗	✗
Peanut	<i>Ara h 1</i>	✓	✓
	<i>Ara h 2</i>	✓	✓
	<i>Ara h 3</i>	✓	✓
	<i>Ara h 5</i>	✗	✗
	<i>Ara h 6</i>	✓	✓
	<i>Ara h 7</i>	✓	✓
	<i>Ara h 8</i>	✓	✓
	<i>Ara h 9</i>	✓	✓
	<i>Ara h 10</i>	✓	✓
	<i>Ara h 11</i>	✗	✓
	<i>Ara h 12</i>	✓	✓
	<i>Ara h 13</i>	✓	✓
	<i>Ara h 14</i>	✗	✗
	<i>Ara h 15</i>	✗	✓
	Pecan	<i>Car i 1</i>	✓
<i>Car i 2</i>		✓	✓
<i>Car i 4</i>		✓	✓
Pistachio	<i>Pis v 1</i>	✓	✓
	<i>Pis v 2</i>	✓	✓
	<i>Pis v 3</i>	✓	✓
	<i>Pis v 4</i>	✓	✗
	<i>Pis v 5</i>	✓	✓

(a) The symbol ✗ indicates that the corresponding allergen was not detected.

(b) The symbol ✓ indicates that the corresponding allergen was detected and identified by TPP/SGUI/PS with a 1% FDR.

As was already mentioned, TDP data revealed crucial information at intact proteoform level about allergen degradation resulted from applied extraction procedure. Thus, both ap-

proaches seem to provide complementary information and based on their combined results it is possible to confirm the relatively good extraction of allergenic proteins. In total, 84 % of the known nut allergens were successfully extracted and identified. Overall, only 7 allergens were not detected at all: *Pru du 3*, *Pru du 4*, *Pru du 5*, *Cor a 6*, *Ara h 5*, *Ara h 14* and *Jug r 5* (Tables 3 and 4). Lipophilic allergens, such as members of the oleosin family, are especially hard to extract and specific procedures are required, (65) explaining the absence of *Ara h 14* and the poor detection of *Ara h 15*, *Cor a 12* and *Cor a 13*. Similarly, the allergens from the pathogenesis-related protein group (PR-10) were barely identified (*Ara h 8*, *Cor a 1*) or where not detected in the samples (*Jug r 5*). The failure to observe *Pru du 3*, *Pru du 4*, *Pru du 5*, *Cor a 6*, *Ara h 5* in the extracts is probably caused by their low concentrations, their poor extraction under the chosen conditions, matrix effects or short-comings of applied TDP and BUP procedures. All in all, a majority of the known allergens were successfully extracted from nut flours with the developed procedure. Despite partial or extensive allergen degradation observed, the global quality of the extracts was deemed sufficient for their use in the CRD of nut allergy. Meanwhile, optimization of the extraction procedure may be required for some allergens not detected here (Tables 3 and 4). Meanwhile, constant improvement both from hardware (MS instruments) and software (deconvolution and data processing algorithms) sides of BUP approach and especially TDP one, further optimization of analysis parameters can also help to improve the data discussed above.

3.3.4 Component-resolved diagnosis of nut allergies by immunomagnetic separation and peptide mass fingerprinting.

The personalized CRD procedure developed in Chapter 2, based on immunomagnetic separation (IMS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS), was modified and applied to nut allergies using protein extracts prepared and characterized as discussed above. The blood sera of 22 peanut-allergic patients subjected to this CRD analysis in order to identify their sensitization profile with prepared nut protein extracts were obtained from the *ProNut* study as mentioned above. Their specific-IgE-Abs levels and the OFCs outcomes were kindly supplied by one of the studies chairmans, Prof. Eigenmann of the Geneva University Hospital (HUG, Switzerland).

The IMS procedure was modified to comply with the requirements and limitations imposed by nut allergies. Firstly, the blood serum amounts were relatively limited. As each sample had to be tested against all the nut protein extracts, the blood sera were diluted with pure water

to maintain the 100- μ L volume for the IgE-Abs extraction of the original procedure. Because of the dilution, the IgE-Abs captured during the procedure were chemically cross-linked to the MBs in order to reuse multiple times the formed immunocomplexes and accumulate enough samples for subsequent allergen detection by peptide mass fingerprinting (PMF). In the original CRD procedure (Chapter 2), the detection of allergenic proteins extracted after IMS procedure was carried out by identifying either intact allergens themselves or their peptides upon tryptic digestion (PMF approach) using MALDI-MS. MALDI-MS analysis is rapid, relatively uncomplicated and low-cost in supplies making it perfect for routine diagnoses. However, in the present case, the identification of intact nut allergens by MALDI-MS is limited as some allergens in the same extracts have similar masses impossible to distinguish with this MS technique. For example, *Ara h 2*, *Ara h 6* and *Ara h 7* have masses of approximately 17, 15 and 15 kDa, respectively. Additionally, different tryptic peptides with similar masses may overlap partially due to the limited instrument resolution even in RF mode. For this reason, the allergens identification by PMF was carried out using a high-resolution mass spectrometer (HRMS, Q Exactive Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific) equipped with a nanoelectrospray ionization (nanoESI) source (Nanomate, Advion Biosciences). When operated in full scan mode, HRMS instruments scan continuously a given m/z range with higher resolution and better mass accuracy compared to other systems. (66) For routine analyses, a mass accuracy of 2 to 10 ppm is expected for peptides with a recently calibrated HRMS instrument. (67) A lock mass is commonly used in HRMS procedures in order to decrease the errors on the mass measurements by correcting the instrumental drifts. A lock mass is defined as the mass of a specific ion of known mass and m/z value, present naturally or added to the sample before the analysis, which is used to perform the recalibration by correcting the m/z shifts in real time. (68) Thus, using internal lock mass results in a higher mass accuracy, as the fluctuations of the ESI source and HRMS instrument will have the same impact on the analytes and the lock mass. On Orbitrap instruments, it is possible to reach sub-ppm mass accuracy for peptide analyses with the use of a lock mass. (69) Naturally present targets for lock mass are the polydimethylcyclosiloxanes (PCM), extremely common volatile ambient air contaminants that are easily ionized in ESI sources. (70) The PCM $(\text{Si}(\text{CH}_3)_2\text{O})_6$ is detected as a mono-protonated species at m/z 445.120025 and is commonly used as a lock mass in HRMS. (67,69,71) However, PCM signals have been showed to have relatively weak and more fluctuating intensities than the analytes ions. (67,72) Herein, the peptide LGEDNINVEGNEQFISASK was easily detected in all allergen extracts digested with cationic bovine trypsin upon IMS procedure and was therefore set as the lock mass, allowing the record-

ing of highly accurate HRMS spectra. This peptide (AA 73-92, 2162.0491 Da) arises from the bovine trypsin autoproteolysis and was detected as a doubly charged species (m/z 1082.0318) with good intensity. A typical HRMS spectrum obtained during the modified CRD procedure (IMS-nanoESI-HRMS) for a peanut-allergic patient with the lock mass signal at m/z 1082.0318 is displayed in Figure 3. The allergen-related peptides identified during the PMF data processing detailed in *Materials and Methods* section 3.2.10, are labelled with asterisks, whereas the unassigned peaks arose from normal spectral background noises, various contaminants, trypsin autolysis and digestion of keratins and other proteins.

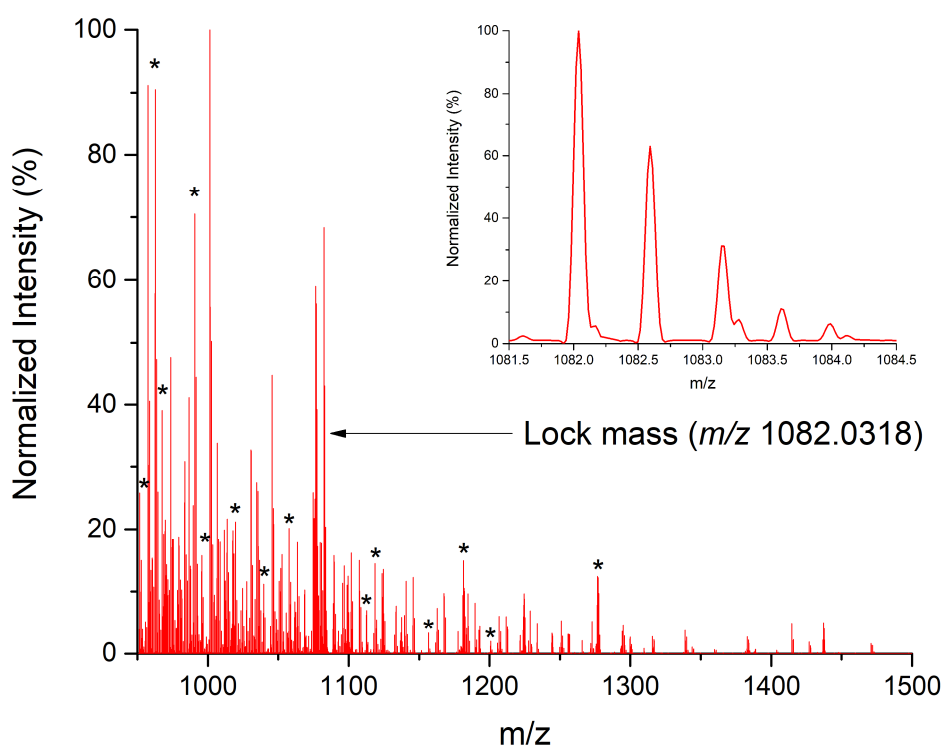


Figure 3. nanoESI-HRMS spectrum obtained during the modified CRD procedure for a peanut-allergic patient. The insert corresponds to a zoom on the used lock mass with m/z 1082.0318 for the monoisotopic peak. The HRMS spectrum was measured by direct infusion using HRMS instrument with a resolution of 60'000 at 400 m/z . The peptides identified during the PMF data processing are labelled with asterisks. The CRD procedure and the nanoESI-HRM analysis were performed as detailed in *Materials and Methods* section 3.2.8 and 3.2.10.

Described modified CRD procedure (IMS-nanoESI-HRMS) was applied to the blood sera of the 22 peanut-allergic patients and the allergens identified by this HRMS-based PMF are listed in Tables 5 and 6. As expected, all blood sera displayed sensitization to one or several peanut aller-

gens as all the patients participated in ProNut study had the clinically confirmed peanut allergy. Being the major peanut allergens, (73,74) *Ara h 1*, *Ara h 2* and *Ara h 3* were recognized as sensitizers for all the patients. Surprisingly, *Ara h 2* was identified as a potential allergen only for 18 % of the patients whereas the prevalences of *Ara h 1* and *Ara h 3* are 82 and 77 %, respectively. During the TDP analysis of the peanut extract (Table 3 and Appendix Tables II-III), *Ara h 2* was identified only when 4 microscans were averaged. Moreover, a partial degradation due to extraction procedure was observed for this allergen. The best E-value of all *Ara h 2* isoforms (mainly detected as single proteoform) was attributed to the truncated fragment AA 72-122 with a value of $1.1E-7$ and 12 identified product ions, whereas only 6 product ions were identified for the intact protein giving a $5.7E-5$ E-value. The corresponding tandem spectra are displayed in Figure 4 and the identified product ions are listed in Appendix Table VI. These results suggest that the majority of the *Ara h 2* has been truncated during the extraction procedure losing its major epitopes and went therefore unrecognized by IgE Abs of most of the patients during the CRD procedure, explaining the low *Ara h 2* prevalence observed.

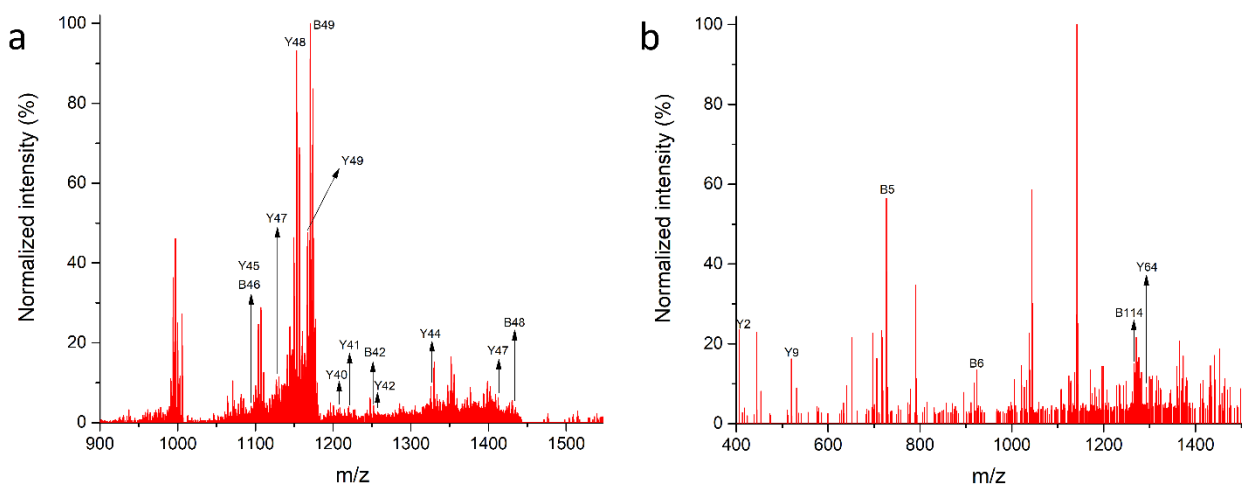


Figure 4. Tandem spectra obtained during the TDP analysis of peanut extract corresponding to the fragmentation of **(a)** *Ara h 2* fragment (AA 72-122) and **(b)** complete intact *Ara h 2*. The MS^2 spectra were measured with a resolution of 60'000 at 400 m/z and with 4 averaged microscans. Other parameters are as in *Materials and Methods* section 3.2.4. The identified product ions are listed in Appendix Table VI. Unknown modifications were identified on the *Ara h 2* fragment and the complete *Ara h 2*, with masse of 99.11 and 81.95 Da, respectively.

Table 5. Allergens identified during the CRD of nut allergies by IMS-nanoESI-HRMS for *ProNut* patients 1 to

 11^(a).

Nut extract	Allergen code	Patients											
		1	2	3	4	5	6	7	8	9	10	11	
Almond	<i>Pru du 3</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Pru du 4</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Pru du 5</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Pru du 6</i>	✓	✓	✓	x	✓	✓	x	✓	✓	✓	✓	✓
Cashew	<i>Ana o 1</i>	✓	✓	x	x	x	x	x	x	✓	x	x	x
	<i>Ana o 2</i>	✓	x	✓	x	x	✓	✓	✓	✓	x	x	x
	<i>Ana o 3</i>	x	x	x	x	x	x	x	x	✓	x	x	x
Hazelnut	<i>Cor a 1</i>	x	x	x	✓	x	✓	✓	x	x	x	x	x
	<i>Cor a 2</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Cor a 6</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Cor a 8</i>	x	x	x	x	x	x	✓	x	x	x	x	x
	<i>Cor a 9</i>	x	x	x	✓	✓	x	x	x	✓	x	✓	✓
	<i>Cor a 10</i>	x	x	x	x	x	✓	✓	✓	✓	x	x	x
	<i>Cor a 11</i>	x	x	x	x	x	✓	x	x	✓	x	x	x
	<i>Cor a 12</i>	x	x	x	✓	x	x	x	x	✓	x	x	x
	<i>Cor a 14</i>	x	x	x	✓	x	x	x	✓	x	x	x	✓
Peanut	<i>Ara h 1</i>	✓	✓	✓	✓	✓	x	✓	✓	✓	x	x	x
	<i>Ara h 2</i>	x	x	x	x	x	x	x	✓	x	x	x	x
	<i>Ara h 3</i>	✓	✓	✓	✓	x	✓	✓	x	✓	x	✓	✓
	<i>Ara h 5</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 6</i>	x	x	✓	✓	x	x	✓	x	✓	✓	✓	✓
	<i>Ara h 7</i>	x	x	x	✓	x	x	x	x	✓	x	x	x
	<i>Ara h 8</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 9</i>	x	x	x	x	x	x	x	✓	x	x	x	x
	<i>Ara h 10</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 11</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 12</i>	x	✓	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 13</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 14</i>	x	✓	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 15</i>	x	x	x	x	x	x	x	x	x	x	x	x
Pecan	<i>Car i 1</i>	x	✓	x	x	x	x	x	x	x	x	x	x
	<i>Car i 2</i>	x	✓	x	x	✓	x	✓	x	x	✓	✓	✓
	<i>Car i 4</i>	x	✓	x	x	x	x	✓	✓	x	x	✓	✓
Pistachio	<i>Pis v 1</i>	✓	x	x	x	x	✓	✓	✓	x	x	x	x
	<i>Pis v 2</i>	✓	✓	x	x	x	✓	x	x	✓	x	✓	✓
	<i>Pis v 3</i>	✓	x	x	x	x	✓	x	x	✓	x	✓	✓
	<i>Pis v 4</i>	x	x	x	x	x	x	x	x	✓	x	x	x
	<i>Pis v 5</i>	x	x	x	x	x	x	✓	✓	✓	x	✓	✓
Walnut	<i>Jug r 1</i>	x	x	x	x	x	x	x	✓	x	x	x	x
	<i>Jug r 2</i>	x	x	✓	✓	x	x	x	x	✓	x	✓	✓
	<i>Jug r 3</i>	x	x	x	x	x	x	x	x	x	x	x	x
	<i>Jug r 4</i>	x	✓	x	x	x	x	x	x	✓	x	✓	✓
	<i>Jug r 5</i>	x	x	x	x	x	x	x	x	x	x	x	x

(a) Allergens with the symbol ✓ and highlighted in green were identified as sensitizers for the patients, whereas the symbol x refers to undetected allergens.

Table 6. Allergens identified during the CRD of nut allergies by IMS-nanoESI-HRMS for *ProNut* patients 12 to 22^(a).

Nut extract	Allergen code	Patients										
		12	13	14	15	16	17	18	19	20	21	22
Almond	<i>Pru du 3</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Pru du 4</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Pru du 5</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Pru du 6</i>	x	✓	✓	x	✓	x	x	x	✓	x	✓
Cashew	<i>Ana o 1</i>	✓	x	✓	x	x	x	x	x	✓	x	x
	<i>Ana o 2</i>	✓	x	✓	x	x	x	x	x	x	✓	x
	<i>Ana o 3</i>	✓	x	x	x	x	x	x	x	x	x	x
Hazelnut	<i>Cor a 1</i>	✓	x	✓	x	✓	x	x	x	x	x	x
	<i>Cor a 2</i>	✓	x	x	x	x	x	x	x	✓	x	x
	<i>Cor a 6</i>	✓	x	x	x	x	x	x	x	x	x	x
	<i>Cor a 8</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Cor a 9</i>	x	x	x	✓	✓	✓	x	x	x	x	✓
	<i>Cor a 10</i>	x	x	✓	x	✓	x	x	x	x	x	x
	<i>Cor a 11</i>	x	x	x	x	x	x	x	x	✓	x	x
	<i>Cor a 12</i>	x	✓	x	x	x	x	x	x	x	x	x
	<i>Cor a 13</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Cor a 14</i>	✓	x	x	✓	x	x	x	x	✓	x	✓
Peanut	<i>Ara h 1</i>	✓	x	✓	✓	✓	✓	✓	✓	✓	✓	✓
	<i>Ara h 2</i>	x	x	x	✓	✓	x	✓	x	x	x	x
	<i>Ara h 3</i>	✓	✓	✓	x	✓	✓	✓	✓	✓	x	✓
	<i>Ara h 5</i>	x	x	x	x	x	x	x	x	✓	x	x
	<i>Ara h 6</i>	x	x	x	x	✓	✓	x	x	x	✓	x
	<i>Ara h 7</i>	x	x	x	x	✓	x	✓	x	x	x	x
	<i>Ara h 8</i>	x	✓	x	x	x	x	x	✓	x	x	x
	<i>Ara h 9</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 10</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 11</i>	✓	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 12</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 13</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Ara h 14</i>	x	✓	x	x	x	x	x	x	✓	x	x
	<i>Ara h 15</i>	x	x	x	x	x	x	x	x	x	x	x
	Pecan	<i>Car i 1</i>	✓	x	x	x	x	x	x	x	x	x
<i>Car i 2</i>		✓	x	✓	x	x	✓	✓	x	✓	✓	x
<i>Car i 4</i>		✓	x	x	x	x	x	✓	x	✓	x	x
Pistachio	<i>Pis v 1</i>	x	x	x	x	x	x	x	x	✓	x	x
	<i>Pis v 2</i>	x	✓	✓	x	✓	✓	x	✓	✓	✓	x
	<i>Pis v 3</i>	✓	x	✓	x	x	x	x	x	✓	x	✓
	<i>Pis v 4</i>	x	✓	x	x	x	x	x	x	x	x	x
	<i>Pis v 5</i>	x	x	✓	x	x	x	✓	x	x	x	x
Walnut	<i>Jug r 1</i>	x	x	✓	x	x	x	x	✓	x	x	x
	<i>Jug r 2</i>	✓	x	x	x	✓	x	✓	x	x	x	✓
	<i>Jug r 3</i>	x	x	x	x	x	x	x	x	x	x	x
	<i>Jug r 4</i>	x	x	x	x	✓	x	✓	x	✓	x	✓
	<i>Jug r 5</i>	x	x	x	x	x	x	x	x	x	x	x

(a) Allergens with the symbol ✓ and highlighted in green were identified as sensitizers for the patients, whereas the symbol x refers to undetected allergens.

Recently, *Ara h 6* has been suggested to be a major allergen, as it was found to be relatively similar in structure and sequence to *Ara h 2* (74) and both allergens were demonstrated to have comparable allergenic activities. (75,76) In the performed IMS-nanoESI-HRMS experiments (Tables 5 and 6), *Ara h 6* was indeed identified as a sensitizer for 9 patients (41 % prevalence) making it the third recognized protein after *Ara h 1* and *Ara h 3*. As for *Ara h 2*, *Ara h 6* was detected only once in the two TDP analyses carried out (Table 3). In contrast to *Ara h 2*, no degradation products were detected for *Ara h 6*, allowing the totality of its epitopes to be potentially recognized by the patient IgE-Abs. The minor peanut allergens were detected for some patients. However, *Ara h 10*, *Ara h 13* and *Ara h 15* were not identified as sensitizers for the complete set of 22 patients tested (Tables 5 and 6).

Hazelnut allergy is the most common nut allergy in Europe and the major allergens are *Cor a 1*, *Cor a 8* and *Cor a 9*. (77–80) It was therefore not surprising to identify *Cor a 1* and *Cor a 9* in 27 and 36 % of the performed CRD analyses, respectively. However, whereas *Cor a 8* was identified only for one patient. For this allergen, 12 peptides were identified by BUP (Table 4 and Appendix Tables IV and V), meanwhile no proteoforms at all were identified with the TDP approach (Table 3 and Appendix Tables II and III), explaining the low prevalence of *Cor a 8* in current experiments.

For the CRD of almond allergies by IMS-nanoESI-HRMS, only the allergen *Pru du 6* was extracted by the patients IgE-Abs. The three other almond allergens were not detected in the protein extracts (Tables 3 and 4) and were therefore not available to carry out successfully the CRD procedure. The same was found for *Jug r 3* and *Jug r 5*, two of the walnut allergens, not detected in their native forms in the protein extracts and thus not recognized by patients IgE-Abs.

Ana o 1, *Ana o 2* and *Ana o 3* are the only three known allergens in cashew nuts. In this work, *Ana o 2* was found to be the major recognized protein with a prevalence of 41 %. However, *Ana o 3* prevalence is potentially underrepresented in these results as only the fragment AA 42-78, corresponding to 30 % of the complete protein sequence, was detected in the performed TDP analyses but not validated due to its E-value higher than the 1E-4 cutoff. Meanwhile, the results obtained by IMS-nanoESI-HRMS for the pecan and pistachio extracts display no particular features.

3.3.5 Comparison of CRD by IMS-nanoESI-HRMS with specific-IgE-Abs levels and oral food challenges

During the ProNut study, the 22 peanut allergic patients, whose blood sera were used the described above CDR tests, were tested by OFCs against various nuts. Their specific-IgE-Abs levels were subsequently evaluated using the standard commercial *in vitro* test called ImmunoCAP (Phadia/ThermoScientific, Sweden). The outcomes of these tests are listed in Appendix Table VII. The OFCs are considered the “golden standards” for the diagnosis of food allergy. (81–83) They are based on the observation of allergic symptoms after ingestion of increasing quantities of the suspected food allergens. (84) As the OFCs result potentially in severe allergic reactions and anaphylaxis, it is required to perform them within hospital settings or in a place suitable to handle and treat the symptoms caused by the test. (85,86) As alternative, the determination of specific-IgE-Abs levels carried out using commercial systems, such as the ImmunoCAP, is a safe and relatively inexpensive tool in the diagnostic process. (87) Unfortunately, it is not possible to test all the known allergens with the ImmunoCAP test. For example, only 5 of the peanut allergens (*Ara h 1, 2, 3, 8* and *9*) and 4 for hazelnut (*Cor a 1, 8, 9* and *14*) are available on this diagnostic platform. In general, the specific-IgE-Abs determination is performed only with allergens known to be responsible for severe allergic reactions or possible allergy persistence. For example, patients displaying a sensitization to *Cor a 9* and *Cor a 14* suffer usually from more severe allergic reactions than patients sensitized to other hazelnut allergens. (88) In addition to specific allergens, ImmunoCAP also offers the possibility to test the complete protein extracts for all the nuts. The test outcomes are expressed in $\text{kUA}\cdot\text{L}^{-1}$, with $1 \text{ kUA}\cdot\text{L}^{-1}$ corresponding to $2.4 \text{ ng}\cdot\text{mL}^{-1}$. In general, this test is considered positive if the specific-IgE-Abs levels are higher than $0.7 \text{ kUA}\cdot\text{L}^{-1}$, whereas values between 0.35 and $0.7 \text{ kUA}\cdot\text{L}^{-1}$ are equivocal.

Direct comparison between the IMS-nanoESI-HRMS and ImmunoCAP diagnostic procedures at the allergen level is difficult and the different outcomes may be explained by the designs of the two CRD methods. In the ImmunoCAP test, most of the allergens are produced by recombinant technologies. Amongst all the available nut allergens, only *Cor a 9* is purified from a hazelnut extract. Recombinant allergens have the advantages of being highly pure and consistent from batch to batch. However, they have limitations of relatively high costs, the production complexity, the potential absence of natural post-translational modifications and protein folding issues. (89) In addition, the allergens are required to be immobilized on the surface of the ImmunoCAP plate.

Allergen immobilization, either by passive absorption or by the use of chemical linkers, results in the potential hindering of IgE epitopes and the loss of their IgE-binding potential. However, the use of allergens extracted from natural sources is limited by the possible degradation resulting from the extraction procedure and by the allergen concentrations in the allergenic source. The partial allergen truncation has a dual effect on IgE-Abs recognition. A loss of IgE-binding potential is possible if the degradation occurs at the epitope position. Meanwhile, it may result in the exposition of new IgE epitopes, normally not accessible in the protein native form.

The sensitization prevalence of each nut extract in the tested patient group is easily calculated based on the number of patients for which one or several allergens have been identified. ImmunoCAP and IMS-nanoESI-HRMS display relatively similar prevalences for the seven nut extracts as shown in Figure 5. All patients were found, by both methods, to be sensitized to at least one peanut allergens corresponding to a 100 % prevalence. IMS-nanoESI-HRMS exhibits a higher allergy prevalence for hazelnut, almond, pecan, pistachio and walnut compared to ImmunoCAP, whereas the opposite was observed for cashew nuts. These prevalences show that 51 to 62 % of the peanut allergic patients are sensitized to at least another nut extracts. As shown by the OFCs in Figure 5 and Appendix Table VII, only a limited number of peanut-allergic patients exhibit symptoms after ingestion of other nuts despite possessing specific-IgE-Abs against them. For cashew extracts, similar prevalences are obtained using the three methods of diagnosis. Meanwhile, the results vary significantly when looking at individual patients. For example, the cashew-specific IgE-Abs of patient N°17 were measured at $88.2 \text{ kUA}\cdot\text{L}^{-1}$ by ImmunoCAP (Appendix Table VII), but no allergens was detected by IMS-nanoESI-HRMS (Table 6) and the OFC was negative (Appendix Table VII). On the contrary, patient N°16 is not sensitized to cashew allergens according to the both *in vitro* tests (Table 6 and Appendix VII) but was positively tested by OFC. The same observations can be made for other patients and demonstrate the complexity of direct comparison between different diagnostic methods.

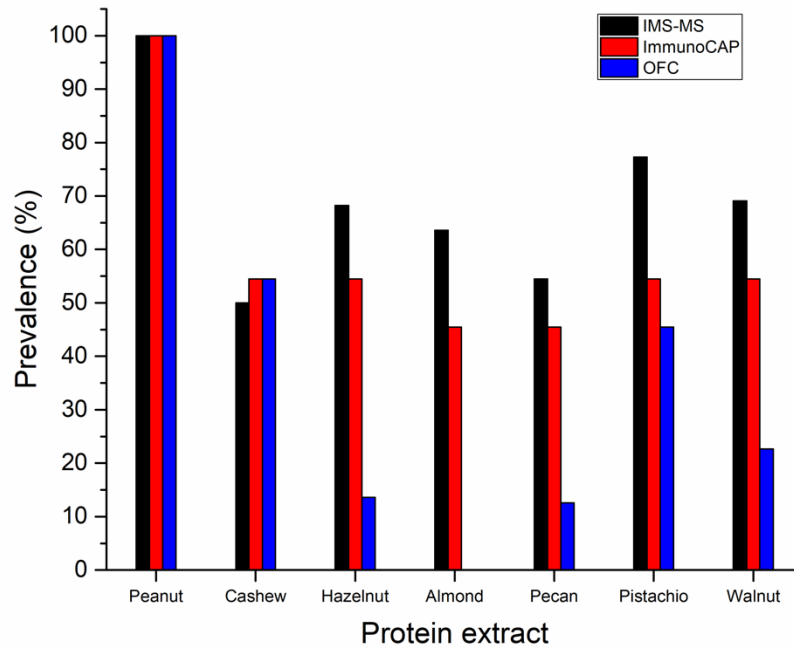


Figure 5. Allergy/sensitization prevalence for the seven extracts based on the IMS-nanoESI-HRMS, ImmunoCAP and OFC tests carried out on the 22 peanut allergic patients from ProNut study. For ImmunoCAP, a value of 0.35 kUA/L was used as the cutoff. Data are detailed in Tables 5-6 and in Appendix Table VII.

Despite their different designs, the comparison between ImmunoCAP and IMS-nanoESI-HRMS reveals several distinctive trends. For peanut allergy, the specific-IgE-Abs levels were identified by ImmunoCAP with 5 allergens: the three major allergens (*Ara h 1*, *Ara h 2*, *Ara h 3*) and the less common *Ara h 8* and *Ara h 9*. The results obtained by IMS-nanoESI-HRMS (Tables 5 and 6) differ sometimes from the outcomes of the ImmunoCAP tests (Appendix Table VII). *Ara h 2* was identified as sensitizers for only 4 patients by IMS-nanoESI-HRMS, whereas 95 % of the patients are shown to be sensitized to *Ara h 2* by ImmunoCAP. As previously explained, *Ara h 2* was partially truncated (Figure 3) during the extraction procedure resulting in possible loss of IgE epitopes, thus explaining the observed deviations from the ImmunoCAP results. Meanwhile, *Ara h 6* is known to have 59 % of sequence identity with *Ara h 2* (74) and similar allergenic properties. (75,76) *Ara h 6* was detected for 9 patients by IMS-nanoESI-HRMS (Tables 5 and 6); all of them were positively tested for *Ara h 2* in the ImmunoCAP tests (Appendix VII), confirming the *Ara h 2/6* cross-sensitization. For the other 4 peanut allergens tested with ImmunoCAP (*Ara h 1*, *3*, *8*, and *9*), the outcomes of both CRD methods were similar for several patients (N°4, 14, 16, 19, 20, 21, 22). In the other cases, only partial agreement was observed between ImmunoCAP and IMS-nanoESI-HRMS results for the peanut allergy. Importantly, performing the CRD by IMS-nanoESI-HRMS with

natural extracts and identifying accurately the allergens by PMF open the diagnostic procedure to the whole panel of allergens. Hence, the peanut allergens *Ara h 5, 7, 11, 12* and *14* were identified for several patients and displayed a low prevalence (4-14 %), as expected for minor allergens. Those allergens were always identified by proposed CRD simultaneously with at least one of the major allergens.

For the other nut protein extracts, more differences in the sensitization profiles of the patients were observed when comparing the ImmunoCAP test with IMS-nanoESI-HRMS (Tables 5 and 6). For example, a complete agreement was obtained only for 5 patients (N° 1, 2, 12, 13 and 18) based on the 4 individual hazelnut allergens tested (*Cor a 1, 8, 9* and *14*), whereas only a partial agreement between the CRD methods was reached for the other patients.

All in all, the CRD of nut allergies was successfully carried out by IMS-nanoESI-HRMS. The sensitization prevalences were similar to the standard commercial tests ImmunoCAP for complete protein extracts, while variations were observed at the individual allergens level. Identification of specific-IgE-Abs is not always correlated with positive OFCs, as the appearance of clinical symptoms after food ingestion follows complex and highly regulated biological reactions. However, the outcomes of *in vitro* CRD procedures yield precious information on the severity, the persistence and potential cross-reactivity with other allergens.

3.3.6 Cross-reactivity and cross-sensitization to nut allergens

Cross-reactivity between two allergens is caused by the ability of a given protein to be recognized by the IgE-Abs normally targeting another allergen. (20) True cross-reactivity produces clinical symptoms, whereas cross-sensitization corresponds to the presence of allergen-specific IgE-Abs without allergy symptoms upon allergen ingestion. (22) *In vitro* CRD methods have been developed to identify specific-IgE-Abs and are therefore ideal to study the cross-reactivity and cross-sensitization at the allergen levels. OFCs are then useful to observe the appearance of symptoms and to distinguish between cross-reactivity and cross-sensitization.

Typically, cross-reactivity is extremely common in nut allergens due to the evolutionary conservation of protein structures in different species and various studies confirmed the possible bindings of specific IgE-Abs to multiple nut allergens. (12,90–92) Because of the high frequency of anaphylaxis toward nuts, understanding and predicting the cross-reactivity of nut proteins is extremely important. In the last years, cross-reactivity prediction has been the subject of various

studies using bioinformatics and computational methods. (93–96) The 3D structures and the amino acid sequences are required to compare allergens and predict cross-reactivity successfully. However, the determination of tertiary structure is a complex process, which has been carried out only for a limited list of allergens.

As allergens known to cross-react with each other show a certain amount of homology and identity, the comparison of their amino acids sequence provides already some interesting information. Herein, two different algorithms were used to align nut allergens and identify homologous allergens based on the returned scoring values. The Smith-Waterman (97) and the Needleman-Wunsh (98) algorithms were used to align one by one all the combinations of nut allergen isoforms, corresponding to more than 13000 individual alignments. The results are displayed in Appendix Figure I as a color-coded heat map. The identification of several allergen groups with high homology/identity (*i.e.* with an elevated Bit-score) are listed in Table 6. As expected, all the allergens showing similarity in their amino acids sequences are members of the same protein families.

Table 6. Allergen groups displaying high homology/identity^(a)

Protein family ^(b)	Allergens
Vicilin (7S globulin)	<i>Ara h 1</i> <i>Pis v 3</i> <i>Cor a 11</i> <i>Jug r 2</i> <i>Car i 2</i> <i>Ana o 1</i>
Conglutin (2S albumin)	<i>Ara h 2</i> <i>Ara h 6</i> <i>Ara h 7</i> <i>Pis v 1</i> <i>Cor a 14</i> <i>Jug r 1</i> <i>Car i 1</i>
Legumin (11S globulin)	<i>Ara h 3</i> <i>Pis v 2</i> <i>Pis v 5</i> <i>Cor a 9</i> <i>Jug r 4</i> <i>Car l 4</i> <i>Ana o 2</i> <i>Pru du 6</i>
Profilin	<i>Ara h 5</i> <i>Cor a 2</i> <i>Pru du 4</i>
Pathogenesis-related protein (PR-10)	<i>Ara h 8</i> <i>Cor a 1</i> <i>Jug r 5</i>
Non specific lipid-transfer protein (nsLTP)	<i>Ara h 9</i> <i>Cor a 8</i> <i>Jug r 3</i> <i>Pru du 3</i>
Oleosin	<i>Ara h 10</i> <i>Ara h 11</i> <i>Ara h 14</i> <i>Ara h 15</i> <i>Cor a 12</i> <i>Cor a 13</i>
Defensin	<i>Ara h 12</i> <i>Ara h 13</i>

(a) Homology/identity was evaluated based on the scoring values returned by the two algorithms used for the alignment (Appendix Figure I).

(b) The protein families for each allergens were found on UniProt or the official Allergen Nomenclature website (allergen.org).

Various studies have been carried out to identify the potential cross-reactivity between several nut allergens. For example, the cross-reactivity of pistachio with cashew nut and walnut with pecan nut was thus demonstrated in several publications. (99–102) In one study, Barre *et al.* investigated the structure of vicilins of various nut allergens and showed the cross-reactivity of IgE-Abs of peanut-allergic patients against *Cor a 11*, *Ana o 1* and *Jug r 2*. (103) For its part, *Ara h 2*

was showed to cross-react with almond and brazil nut extracts (104) and with the pecan and walnut conglutins, *Car i 1* and *Jug r 1*. (105) Despite all of these studies, no large-scale cross-reactivity assessment was performed with *in vivo* and *in vitro* tests on a large panel of patients against all the nut allergens. The aim of the *ProNut* study is precisely to resolve this lack of understanding in nuts cross-reactivity.

The determination of cross-reactivity between peanut and the other tested nuts is possible with the OFCs data. Cross-reactivity was identified only for 13 patients, while 9 patients were only allergic to peanuts. Cashew nuts, pistachios, walnuts, hazelnuts, and pecan nuts were confirmed to be allergenic for 55.4, 45.5, 22.7, 13.6 and 13.6 % of the tested patients, respectively. Almond was the only extract not displaying cross-reactivity, as no symptoms were observed in the OFCs. The identification of specific IgE-Abs by IMS-nanoESI-HRMS and ImmunoCAP revealed no particular trend at the allergen level for the 13 cross-reactive cases. Indeed, it is complicated to identify accurately general cross-reactivity patterns for seven nut extracts with this limited number of patients. A larger panel of patients is required to deduce conclusive cross-reactivity data by removing variations and statistical errors.

The cross-sensitization is determined purely *in vitro* by ImmunoCAP and IMS-nanoESI-HRMS. In ImmunoCAP tests (Appendix Table VII), only 13 of the 44 nut allergens were evaluated against patient blood sera, limiting the cross-sensitization analysis. For example, *Ara h 1* is the only member of the vicilin family being tested and no specific cross-sensitization with non-vicilin allergens is observed. For the 20 patients with an *Ara h 2* allergy, a cross-sensitization with *Cor a 14* and *Jug r 1* was observed for 30 and 35 % of them. Meanwhile, 55 % of the patients with an *Ara h 3* allergy were sensitized to *Cor a 9*. In the case of *Ara h 8* and *Ara h 9* allergies, the number of patients is limited to 6 and 4, respectively. A larger number of patients is required to obtain a reliable evaluation of cross-sensitization. However, it is possible to observe that all the *Ara h 8*-sensitized patients recognized *Cor a 1*. All the patients with an *Ara h 9* allergy were sensitized to *Pru du 3* and *Jug r 3* whereas only 50 % also to *Cor a 8*. At the meantime, IMS-nanoESI-HRMS results provided a wider picture for potential cross-sensitization between members of the same allergenic protein families. Using this method it was found that patient with an *Ara h 1* allergy were sensitized to *Car i 2*, *Pis v 3*, *Jug r 2*, *Ana o 1*, and *Cor a 11* with prevalences of 50, 43, 39, 32 and 11 % respectively. For patients suffering from an allergy to *Ara h 3*, the sensitizers were *Pru du 6*, *Ann o 2*, *Cor a 9*, *Car i 4*, *Pis v 2*, *Pis v 5*, and *Jug r 4* with prevalences of 65, 41, 35, 35, 65, 29 and 41 % respectively.

For the other peanut allergens, the number of patients is too limited to identify reliably cross-sensitizers, as they are minor allergens or because of the protein extract quality.

3.4 Conclusion

The personalized CRD procedure developed in Chapter 2 (IMS-MALDI-MS) was modified and tested to diagnose nut allergies using patient blood sera collected for ProNut clinical study. In order to realize this, first the allergenic nut extracts were in-house prepared directly from the whole nuts *i.e.* almond, cashew nut, hazelnut, peanut, pecan nut, pistachio and walnut. Content and quality of these extracts were assessed by BUP and TDP approaches that yielded complementary information. The presence of the majority of the allergens in the extracts was confirmed by the BUP analyses, while partial or extensive protein degradation was observed in the TDP procedure. Then IMS-MALDI-MS technique was adapted to the requirements of nut allergies CRD. In addition to the IMS extraction parameter modifications, the allergen detection method was switched from direct intact protein analysis or PMF by MALDI-MS to PMF using a nanoESI source coupled to a HRMS instrument, allowing the fast and accurate identification of extracted allergenic proteins. Afterwards, the home-made characterized protein extracts of the seven nuts (were used to perform the CRD with this modified IMS-nanoESI-HRMS method using of the blood sera of 22 patients with a clinically proven peanut allergy from ProNut study. Obtained results provided full nut allergy sensitization profile for these 22 patients and were compared to the outcomes of the standard commercial *in vitro* test (ImmunoCAP) and provocation challenges (OFCs). At the general nut species level for all the tested blood sera, both *in vitro* methods were in a good agreement while OFC data were revealing true clinical allergy cases. On the individual allergen level, there was only partial agreement between ImmunoCAP and proposed CRD procedure. Observed dissimilarities between the two CRD methods are explained by their intrinsic designs and the differences in the allergenic sources used. The immobilization of recombinant allergens in ImmunoCAP results potentially in the partial hindering of various IgE epitopes. Meanwhile, protein degradation during the production of natural extracts is responsible for the loss of recognized IgE epitopes and the exposition of previously inaccessible epitopes.

As cross-reactivity and cross-sensitization was the main topic of the ProNut study, it was also analyzed herein. The data about clinical cross-reactivity between several nuts was observed in the OFCs carried out for each tested patient by the HUG (Switzerland), while the outcomes of ImmunoCAP tests and IMS-nanoESI-HRMS demonstrated cross-sensitization for the tested set of nut

extracts. The cross-sensitization prevalences of peanut-allergic patients towards other nuts ranged from 11 to 100 % depending on the individual allergen tested and the CRD methods. As ImmunoCAP tests are limited to a list of available allergens, the complete patient's sensitization profile is not obtainable. On the other hand, testing patient blood sera against natural extracts by IMS-nanoESI-HRMS made available the whole panel of nut allergens thus providing additional valuable information to clinical ProNut data. With this method, 7 and 5 individual allergens were identified as potential cross-sensitizers of *Ara h 3* and *Ara h 1*, respectively. As expected, all of these allergens are members of the same protein families, the legumins and the vicilins, and display high level of similarity/homology. However, the reliable identification of all potential cross-sensitizers is limited by the number of patients, especially for the minor allergens, and by the quality of the natural extracts. Further improvements of the protein extraction procedure are required to extract efficiently allergens, with limited amount of degradation. Additionally, the pre-concentration of the extracts and/or the selective allergens isolation by liquid chromatography could complete and clarify the obtained results. Nevertheless, proposed CRD procedure was demonstrated to be flexible and adaptive to the needs of particular allergy study and could bring potentially useful information even within a clinical study like ProNut.

3.5 References

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3.6 Appendix

Table I. Product ions identified for *Ara h 10* fragment AA 155-169 in the TDP analysis of peanut extract with 4 averaged microscans.

Calculated monoisotopic mass (Da)	Measured monoisotopic m/z	Charge	Ion	Position	Error (ppm)
490.2871	246.1508	2	Y4	21	1.37
1033.5534	517.784	2	Y9	16	1.67
1161.6119	581.8132	2	Y10	15	1.4
2359.2469	590.819	4	Y21	4	0.56
2458.3138	615.5857	4	Y22	3	-0.08
1274.6962	638.3554	2	Y11	14	1.5
1527.7896	764.9021	2	B14	14	0.21
1588.8168	795.4157	2	Y14	11	-0.07
804.4103	805.4175	1	Y7	18	1.56
1033.5524	1034.5596	1	Y9	16	0.68

Table II. Allergens identified in nut protein extracts by a TDP approach with the averaging of 4 microscans for MS² spectra.

Nut extract	Total number of detected proteins	Allergen detected (UniProt N°)	Allergen code	E-value of best PrSM ^(a)
Almond	18	E3SH28	<i>Pru du 6</i>	5.66E-19
		E3SH29	<i>Pru du 6</i>	3.09E-31
		S5LMS1	<i>Pru du 6</i>	1.13E-30
Cashew	8	Q8GZP6	<i>Ana o 2</i>	5.32E-26
		Q8L5L5	<i>Ana o 1</i>	1.22E-06
		Q8L5L6	<i>Ana o 1</i>	1.94E-05
Hazelnut	20	A0A0A0P7E3	<i>Cor a 9</i>	2.89E-06
		Q08407	<i>Cor a 1</i>	2.59E-07
Peanut	18	A1DZF0	<i>Ara h 3</i>	2.16E-13
		B5TYU1	<i>Ara h 3</i>	1.33E-07
		E5G076	<i>Ara h 1</i>	4.65E-07
		Q6PSU2-2	<i>Ara h 2</i>	1.10E-07
Pecan	7	B3STU4	<i>Car i 2</i>	2.81E-07
		B3STU7	<i>Car i 2</i>	3.40E-06
		B5KVH4	<i>Car i 4</i>	1.11E-05
Pistachio	11	B2BDZ8	<i>Pis v 4</i>	6.50E-06
		B2KN55	<i>Pis v 2</i>	4.09E-27
		B4X640	<i>Pis v 3</i>	8.15E-05
		B7P072	<i>Pis v 1</i>	1.60E-08
		B7P073	<i>Pis v 2</i>	3.57E-32
		B7P074	<i>Pis v 2</i>	9.68E-14
Walnut	11	B7SLJ1	<i>Pis v 5</i>	3.20E-25
		Q2TPW5	<i>Jug r 4</i>	3.32E-09
		Q9SEW4	<i>Jug r 2</i>	2.19E-05

(a) PrSM: proteoform spectrum match

Table III. Allergens identified in nut protein extracts by a TPD approach with the averaging of 8 microscans for MS² spectra.

Nut extract	Total number of detected proteins	Allergen detected (UniProt N°)	Allergen code	E-value of best PrSM ^(a)
Almond	10	E3SH28	<i>Pru du 6</i>	2.59E-27
		E3SH29	<i>Pru du 6</i>	6.98E-25
		S5LMS1	<i>Pru du 6</i>	3.17E-22
Cashew	7	Q8GZP6	<i>Ana o 2</i>	2.69E-26
		Q8L5L5	<i>Ana o 1</i>	5.17E-06
		Q8L5L6	<i>Ana o 1</i>	1.65E-06
Hazelnut	14	Q8W1C2	<i>Cor a 9</i>	4.40E-06
		Q9AXH5	<i>Cor a 2</i>	4.83E-05
		Q9FSY7	<i>Cor a 10</i>	4.87E-06
Peanut	13	A1DZF0	<i>Ara h 3</i>	6.45E-07
		P43238	<i>Ara h 1</i>	6.33E-07
		Q647G9	<i>Ara h 6</i>	2.16E-06
		Q9SQH7	<i>Ara h 3</i>	9.73E-12
Pecan	7	B3STU4	<i>Car i 2</i>	3.75E-05
		B3STU7	<i>Car i 2</i>	2.58E-05
Pistachio	11	B2KN55	<i>Pis v 2</i>	9.19E-26
		B4X640	<i>Pis v 3</i>	1.13E-05
		B7P072	<i>Pis v 1</i>	5.06E-20
		B7P073	<i>Pis v 2</i>	3.65E-28
		B7P074	<i>Pis v 2</i>	3.34E-28
Walnut	6	B7SLJ1	<i>Pis v 5</i>	9.89E-27
		P93198	<i>Jug r 1</i>	3.30E-06
		Q9SEW4	<i>Jug r 2</i>	5.78E-05

(a) PrSM: proteoform spectrum match

Table IV. Allergens identified in nut protein extracts by a BUP approach using the Trans-Proteomic Pipeline (TPP).

Nut extract	Total number of detected proteins	Allergen detected (UniProt N°)	Allergen code	Sequence coverage (%)	Number of unique validated peptides
Almond	15	E3SH28	<i>Pru du 6</i>	63.9	95
		E3SH29	<i>Pru du 6</i>	69.4	64
		S5LMS1	<i>Pru du 6</i>	84.3	24
Cashew	4	Q8GZP6	<i>Ana o 2</i>	76.8	88
		Q8H2B8	<i>Ana o 3</i>	65.2	27
		Q8L5L5	<i>Ana o 1</i>	34.1	26
		Q8L5L6	<i>Ana o 1</i>	34.1	26
Hazelnut	14	AOA0A0P7E3	<i>Cor a 9</i>	54.1	39
		D0PWG2	<i>Cor a 14</i>	33.3	12
		Q84T21	<i>Cor a 12</i>	10.7	2
		Q84T91	<i>Cor a 13</i>	17.9	2
		Q8S4P9	<i>Cor a 11</i>	35.9	20
		Q8W1C2	<i>Cor a 9</i>	50.7	36
		Q9ATH2	<i>Cor a 8</i>	53.9	12
		Q9FPK2	<i>Cor a 1</i>	7.5	1
		Q9FPK3	<i>Cor a 1</i>	7.5	1
		Q9FPK4	<i>Cor a 1</i>	7.5	1
Peanut	51	Q9FSY7	<i>Cor a 10</i>	1.0	1
		Q9SWR4	<i>Cor a 1</i>	7.5	1
		A1DZF0	<i>Ara h 3</i>	64.5	60
		A1DZF1	<i>Ara h 3</i>	21.7	7
		A5Z1R0	<i>Ara h 6</i>	57.9	32
		B0YIU5	<i>Ara h 8</i>	35.9	5
		B3EWP3	<i>Ara h 12</i>	47.9	6
		B3IXL2	<i>Ara h 1</i>	59.3	88
		B4XID4	<i>Ara h 7</i>	25.6	8
		B6CEX8	<i>Ara h 9</i>	44.8	2
		B6CG41	<i>Ara h 9</i>	56.5	5
		COHJZ1	<i>Ara h 13</i>	25.0	2
		E5G076	<i>Ara h 1</i>	42.6	67
		N1NG13	<i>Ara h 1</i>	53.5	87
		O82580	<i>Ara h 3</i>	42.0	34
		P43237	<i>Ara h 1</i>	59.3	88
		P43238	<i>Ara h 1</i>	53.5	87
		Q0GM57	<i>Ara h 3</i>	35.3	21
		Q45W86	<i>Ara h 11</i>	9.5	1
		Q5I6T2	<i>Ara h 3</i>	57.6	55
Q647G3	<i>Ara h 15</i>	14.5	3		
Q647G5	<i>Ara h 10</i>	19.5	4		
Q647G9	<i>Ara h 6</i>	57.9	32		
Q647H2	<i>Ara h 3</i>	22.7	9		
Q647H3	<i>Ara h 3</i>	64.1	60		
Q647H4	<i>Ara h 3</i>	54.9	51		
Q6IWG5	<i>Ara h 3</i>	35.3	21		
Q6PSU2	<i>Ara h 2</i>	58.8	37		
Q6PSU2-2	<i>Ara h 2</i>	58.8	37		
Q6PSU3	<i>Ara h 1</i>	59.3	88		
Q6PSU6	<i>Ara h 1</i>	60.1	42		
Q9FZ11	<i>Ara h 3</i>	60.9	58		

Pecan	3	B3STU4	<i>Car i 2</i>	21.7	24
		B5KVH4	<i>Car i 4</i>	4.6	3
		Q84XA9	<i>Car i 1</i>	22.4	10
Pistachio	9	B2KN55	<i>Pis v 2</i>	62.5	67
		B4X640	<i>Pis v 3</i>	43.4	34
		B7P072	<i>Pis v 1</i>	59.7	35
		B7P073	<i>Pis v 2</i>	67.1	68
		B7P074	<i>Pis v 2</i>	62.5	67
		B7SLJ1	<i>Pis v 5</i>	71.9	67
Walnut	7	C5H617	<i>Jug r 3</i>	36.1	4
		P93198	<i>Jug r 1</i>	46.0	18
		Q2TPW5	<i>Jug r 4</i>	44.4	26
		Q9SEW4	<i>Jug r 2</i>	32.4	34

Table V. Allergens identified in nut protein extracts by a BUP approach using SearchGUI and PeptideShaker.

Nut extract	Total number of detected proteins	Allergen detected (UniProt N°)	Allergen code	Sequence coverage (%)	Number of unique validated peptides
Almond	13	E3SH28	<i>Pru du 6</i>	73.5	105
		E3SH29	<i>Pru du 6</i>	69.4	99
		S5LMS1	<i>Pru du 6</i>	88.2	46
Cashew	4	Q8GZP6	<i>Ana o 2</i>	77.5	114
		Q8H2B8	<i>Ana o 3</i>	63.0	32
		Q8L5L5	<i>Ana o 1</i>	34.2	21
Hazelnut	10	AOA0A0P7E3	<i>Cor a 9</i>	67.5	92
		D0PWG2	<i>Cor a 14</i>	29.9	15
		Q84T21	<i>Cor a 12</i>	5.7	1
		Q84T91	<i>Cor a 13</i>	27.9	6
		Q8S4P9	<i>Cor a 11</i>	45.3	34
		Q8W1C2	<i>Cor a 9</i>	65.4	81
		Q9ATH2	<i>Cor a 8</i>	63.5	16
Peanut	43	Q9FPK2	<i>Cor a 1</i>	7.5	1
		A1DZE9	<i>Ara h 6</i>	27.6	14
		A1DZF0	<i>Ara h 3</i>	78.1	82
		B0YIU5	<i>Ara h 8</i>	49.7	6
		B3EWP3	<i>Ara h 12</i>	47.9	6
		B3EWP4	<i>Ara h 13</i>	36.7	4
		B4XID4	<i>Ara h 7</i>	29.9	7
		B6CG41	<i>Ara h 9</i>	56.5	5
		O82580	<i>Ara h 3</i>	58.2	46
		P43237	<i>Ara h 1</i>	58.8	88
		P43238	<i>Ara h 1</i>	41.4	64
		Q0GM57	<i>Ara h 3</i>	42.8	31
		Q5I6T2	<i>Ara h 3</i>	77.2	79
		Q647G4	<i>Ara h 10</i>	16.0	3
		Q647G5	<i>Ara h 10</i>	24.9	5
		Q647G8	<i>Ara h 7</i>	27.2	7
		Q647G9	<i>Ara h 6</i>	53.1	37
		Q647H2	<i>Ara h 3</i>	37.2	15
		Q647H3	<i>Ara h 3</i>	15.3	13
		Q647H4	<i>Ara h 3</i>	70.7	71
Q6PSU2	<i>Ara h 2</i>	49.4	43		
Q6PSU6	<i>Ara h 1</i>	66.3	42		
Q9FZ11	<i>Ara h 3</i>	71.3	75		
Q9SQH1	<i>Ara h 7</i>	15.0	3		
Q9SQH7	<i>Ara h 3</i>	57.0	66		
Pecan	4	B3STU4	<i>Car i 2</i>	48.4	63
		B5KVH4	<i>Car i 4</i>	24.0	8
		Q84XA9	<i>Car i 1</i>	48.3	38
Pistachio	10	B2BDZ8	<i>Pis v 4</i>	3.5	1
		B2KN55	<i>Pis v 2</i>	79.7	118
		B4X640	<i>Pis v 3</i>	62.2	51
		B7P072	<i>Pis v 1</i>	77.2	46
		B7P073	<i>Pis v 2</i>	79.4	118
		B7P074	<i>Pis v 2</i>	79.7	119
B7SLJ1	<i>Pis v 5</i>	82.5	96		

Walnut	7	C5H617	<i>Jug r 3</i>	39.5	7
		P93198	<i>Jug r 1</i>	66.9	44
		Q2TPW5	<i>Jug r 4</i>	70.6	68
		Q9SEW4	<i>Jug r 2</i>	51.3	57

Table VI. Product ions identified for *Ara h 2* and the *Ara h 2* fragment AA 72-122 in the TDP analysis of peanut extract with 4 averaged microscans.

Allergen	Calculated monoisotopic mass (Da)	Measured monoisotopic m/z	Charge	Ion	Position	Error (ppm)
<i>Ara h 2</i> ^(a)	809.3786	405.6966	2	Y7	132	0.74
	1037.4909	519.7527	2	Y9	130	1.77
	727.3417	728.349	1	B5	5	2.13
	922.3719	923.3792	1	B6	6	-0.89
	13805.1058	1256.0169	11	B114	114	-10.79
	7680.6832	1281.1211	6	Y64	75	9.04
<i>Ara h 2</i> fragment (AA 72-122) ^(b)	5459.4325	1092.8938	5	Y45	5	3.15
	5459.4325	1092.8938	5	B46	46	12.44
	5644.5055	1129.9084	5	Y47	3	0.55
	5740.54	1149.1153	5	Y48	2	-2.23
	5827.5652	1166.5203	5	Y49	1	-3.38
	5844.5775	1169.9228	5	B49	49	-0.26
	4812.1437	1204.0432	4	Y40	10	8.8
	4869.1037	1218.2832	4	Y41	9	-3.93
	5001.1681	1251.2993	4	B42	42	-4.07
	5024.2213	1257.0626	4	Y42	8	-0.05
	5296.3311	1325.0901	4	Y44	6	-3.94
	5643.4805	1411.8774	4	Y47	3	-3.48
	5731.5282	1433.8893	4	B48	48	5.8

(a) *Ara h 2* was identified with an unknown modification of 81.95 Da on the leucine residue 27.

(b) *Ara h 2* fragment (AA 72-122) was identified with an unknown modification of 99.11 Da, localized between AA 82 and 113.

Table VII. Detection of nut allergy for patients N° 1-22 using ImmunoCAP tests and oral food challenges. ^(a-d)

Allergen/total extract	Patients										
	1	2	3	4	5	6	7	8	9	10	11
Pru du 3	0.02	0.03	11.40	0.04	0.55	0.75	17.70	0.03	1.62	0.00	0.00
Almond	0.33	0.00	6.83	0.11	0.07	0.43	21.30	1.54	0.62	5.31	0.00
Ana o 3	0.00		0.21	0.01	0.01	0.65	21.50	6.06	0.87	88.60	0.00
Cashew	0.00	0.00	3.41	0.07	0.03	0.45	64.70	5.91	0.88	74.90	0.23
Cor a 1	0.00	0.00	0.21	0.00	0.63	5.83	0.01	0.00	4.79	101.00	0.00
Cor a 8	0.00	0.00	4.43	0.00	0.05	0.49	5.65	0.01	0.46	0.00	0.00
Cor a 9	0.00	0.00	9.01	0.03	0.02	0.09	33.20	1.09	0.16	9.43	0.00
Cor a 14	0.06	0.00	N/A	0.00	3.25	0.13	0.09	0.23	0.00	15.00	0.20
Hazelnut	0.06	0.01	11.80	0.15	1.66	4.53	40.90	N.A	3.85	84.40	0.11
Ara h 1	0.27	0.06	0.89	0.16	9.83	54.00	13.50	0.01	19.70	10.80	40.70
Ara h 2	5.16	1.00	0.72	21.90	34.70	101.00	46.50	1.31	69.40	3.70	101.00
Ara h 3	0.00	0.00	2.95	0.01	0.03	48.40	34.80	0.04	0.23	0.88	6.64
Ara h 8	0.00	0.00	0.45	0.00	0.17	2.07	0.04	0.01	3.96	16.20	0.00
Ara h 9	0.00	0.00	11.60	0.00	0.11	0.04	15.20	0.02	1.06	0.00	0.00
Peanut	3.35	1.15	15.80	29.10	34.80	101.00	>100	1.75	66.90	20.30	101.00
Pecan	0.02	0.01	3.06	0.06	1.23	0.06	3.43	0.68	1.17	6.00	0.00
Pistachio	0.01	0.01	5.24	0.14	0.07	0.59	65.70	5.28	1.27	101.00	0.24
Jug r 1	0.00	0.02	0.08	0.00	3.02	0.06	0.17	0.03	1.16	13.00	0.00
Jug r 3	0.01	0.02	7.43	0.02	0.29	0.62	9.58	0.03	1.10	0.00	0.00
Walnut	0.02	0.01	10.90	0.12	2.75	0.09	10.30	1.27	2.07	12.70	0.00

Allergen/total extract	Patients											
	12	13	14	15	16	17	18	19	20	21	22	
Pru du 3	0.00	0.00	0.00	0.00	0.00	3.23	0.00	1.22	0.00	0.00	0.00	
Almond	0.16	0.00	0.30	1.31	0.00	17.20	0.00	1.87	0.18	0.52	0.00	
Ana o 3	8.71	1.35	0.00	1.56	0.27	71.70	0.00	0.98	0.00	1.56	0.00	
Cashew	8.09	1.14	0.30	1.59	0.25	88.20	0.00	2.69	0.00	1.62	0.00	
Cor a 1	9.03	0.00	0.00	0.00	0.19	0.00	0.00	42.00	0.00	29.50	0.24	
Cor a 8	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.43	0.00	0.00	0.00	
Cor a 9	0.00	0.00	0.18	0.29	0.00	101.00	0.00	2.72	0.00	0.16	0.13	
Cor a 14	0.34	0.00	0.10	0.00	0.17	101.00	0.00	0.10	0.00	0.48	0.00	
Hazelnut	5.22	0.00	0.64	1.62	0.19	101.00	0.00	28.10	0.12	14.80	0.27	
Ara h 1	1.15	0.00	56.50	44.20	12.60	0.00	0.00	19.40	10.10	0.14	5.33	
Ara h 2	17.00	0.00	101.00	101.00	33.80	2.06	1.67	39.20	37.50	3.94	9.15	
Ara h 3	0.00	0.00	67.60	0.28	0.13	3.17	0.00	4.15	5.12	0.00	1.18	
Ara h 8	9.51	0.00	0.00	0.00	0.00	0.12	0.00	13.00	0.00	10.10	0.00	
Ara h 9	0.00	0.00	0.00	0.00	0.00	2.69	0.00	0.55	0.00	0.00	0.00	
Peanut	21.00	1.35	101.00	101.00	48.90	24.50	1.29	52.50	68.20	4.66	14.10	
Pecan	0.39	0.00	0.00	0.39	0.00	60.00	0.00	0.44	0.00	0.11	0.00	
Pistachio	8.77	1.19	0.16	2.36	0.36	101.00	0.00	4.39	0.00	2.19	0.00	
Jug r 1	1.68	0.00	0.00	0.47	0.85	55.40	0.00	0.24	0.00	0.00	0.00	
Jug r 3	0.00	0.00	0.00	0.00	0.00	2.06	0.00	1.22	0.00	0.00	0.00	
Walnut	1.33	0.00	0.00	1.75	0.82	95.10	0.00	0.53	0.00	0.59	0.00	

(a) ImmunoCAP and OFCs were performed in hospital settings by trained professionals.

(b) Positive oral food challenges are highlighted in green.

(c) The values for the ImmunoCAP are kUA/L.

(d) ImmunoCAP is a commercial allergy test sold by Phadia/ThermoScientific (Sweden).

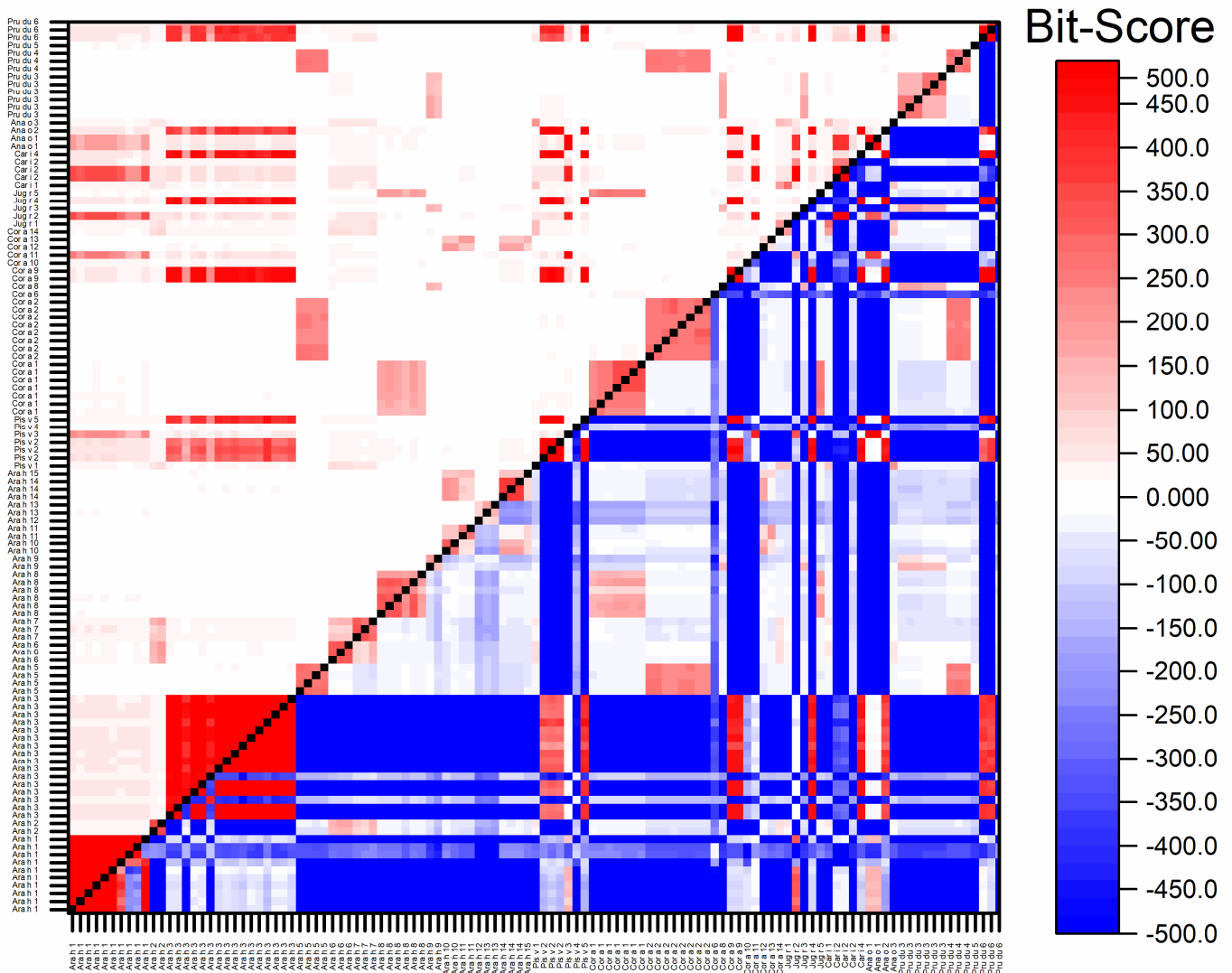


Figure I. Heat map obtained for the alignments of all nut allergen proteoforms with the Smith-Waterman (top-left corner) and Needleman-Wunsch (bottom-right corner) algorithms. The Bit-score represents the quality of the alignment and higher values correspond to higher similarity/homology in the allergens sequences.

Chapter 4.

***In vitro* allergen nitration: comparison of various nitrating agents**

4.1 Introduction

During the last decades, the prevalence of allergy has increased in industrial countries. This life-threatening disease affects nowadays around 25 % of the population. (1) Several hypotheses were proposed to explain this increase including genetic predisposition, reduced exposure to infections and pathogens during childhood, social/physiological stress, change in diet, and exposure to environmental pollution. (2)

Air pollution is a well-known factor that enhances pulmonary diseases by causing inflammation of the respiratory system. Asthma, chronic obstructive pulmonary diseases and respiratory infections are all exacerbated by air pollutants, such as fine particulate matter, ozone and nitrogen oxides. (3) Allergies are also greatly impacted by air quality. In the case of pollen allergy, hay fever, and allergy-induced asthma, the molecules responsible for the atopic reaction are present as airborne particles and exposed to pollution. In addition to the respiratory system exacerbation, pollutants induce chemical modifications of particulate matter and pollen allergens. (2,4)

Under smog conditions, the main chemical modification observed in allergens is the nitration of tyrosine (Tyr) residues caused by ozone and nitrogen monoxide. For example, Franze *et al.* have detected nitrated species in the dust collected on windows and roadsides in various large German cities. (5) The nitration of proteins by NO₂/O₃ was also studied in a laboratory setup by exposing proteins to the gas mixture or by bubbling it in a solution containing the dissolved protein. (6) In addition, nitration of proteins is also a post-translational modification occurring naturally *in vivo* and having a significant role in cardiovascular diseases, neurodegenerative disorders,

infections and oxidative stress. (7–9) The reaction involves a highly unstable radical Tyr intermediate that reacts with nitrogen dioxide, resulting in 3-nitrotyrosine (NTyr), as shown in Figure 1. In the biological pathway, the Tyr residue radical is formed by the scavenging of hydroxyl radicals, superoxide anions, and hydrogen peroxide produced by the cell metabolism. Then, the tyrosyl radical reacts with nitrogen dioxide liberated *in situ* from the decomposition of peroxynitrite (ONOO^-) and its derivatives. In the atmosphere, the nitration of proteins is still not completely understood and is the subject of intense studies. (5,10–13)

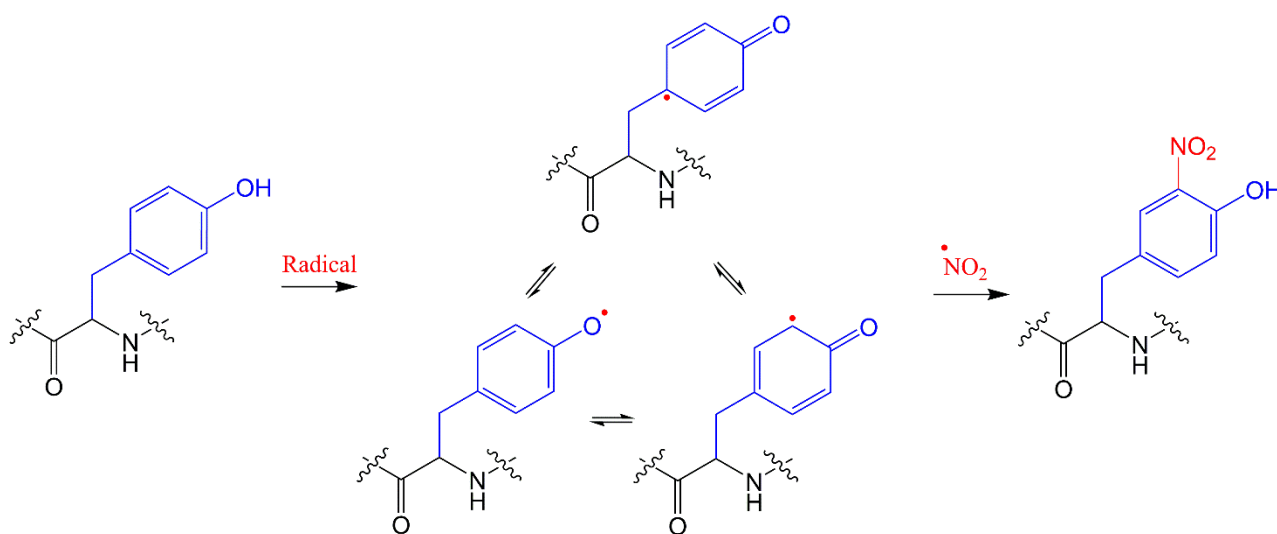


Figure 1. Radical nitration of Tyr residue yielding 3-nitrotyrosine (NTyr), with stabilization of the tyrosyl radical intermediate by delocalization of the radical.

Allergen nitration is not limited to airborne proteins. Food industries use various methods and additives that might induce allergen nitration. Nitrite and nitrate salts, two common conservatives, (14) are the main molecules responsible for the presence of NTyr in food products. (15) As NTyr residue is a biomarker found in infections and inflammation, this modification is also naturally present in animal-based food. For example, dairy cows regularly suffer from mastitis, an inflammation of the udder tissues, caused by physical trauma or microorganisms-related infections. This condition results in an increase in nitric oxide concentration in milk (16) and formation of nitrated milk proteins. (17)

The impact of allergen nitration on the immune system is still poorly understood. Changes in structures and properties upon nitration of the allergen are responsible for variations in its uptake, digestibility, and allergenicity. According to recent studies, the *in vitro* nitration of proteins including β -lactoglobulin (β -LG), ovalbumin, and Bet v 1 increases their allergenicity but has various unpredicted effects on their digestibility and sensitization capability. (4,6,18–20) In other cases, the allergenicity of proteins is not modified upon nitration. (19) The identification of nitration sites could help the understanding and prediction of the potential consequences of protein nitration.

The investigation of nitrated proteins, in the cases of allergy or other diseases, requires a reliable procedure to prepare them *in vitro* as their natural concentrations are low. Four different nitrating agents have been commonly used in recent studies to chemically nitrate proteins: sodium nitrite in the presence of hydrogen peroxide, (15,21–24) tetranitromethane, (6,25–27) NONO-ate derivatives (21,28–30) and peroxyxynitrite. (6,29,31,32) These nitrating agents are usually directly added to a protein solution using various molar ratios and incubation times to induce the targeted level of nitration. In addition to these purely chemical methods, protein nitration by electrochemistry in a standard 3-electrode setup was also proposed (33–35). This method is less commonly used and no complete study was made to understand the reactions occurring at the electrodes. In all cases, in-depth characterization and precise knowledge of the reaction outcomes are still lacking.

Herein, the five cited nitration methods were investigated using α -lactalbumin (α -LA) as a model compound. The reaction time and the molar ratio of nitrating agent: Tyr residues were varied in the interest of finding the optimal conditions. The nitration and oxidation degree were determined using high-resolution mass spectrometry by direct injection of the proteins followed by deconvolution of obtained spectra. The optimal nitrating agents and conditions were applied to various commercial proteins and to natural extracts obtained from cow's milk and chicken egg. Finally, we identified the nitration sites for all allergenic proteins by bottom-up proteomics and then compared them to known epitopes in order to get a hint of their potential impact on allergenicity modulation.

4.2 Materials and methods

4.2.1 Chemicals and materials

Sodium nitrite, tetranitromethane (TNM), diethylamine NONOate diethylammonium, iron (II) chloride, iron (III) chloride, boric acid, potassium chloride, Tyr, NTyr, iodoacetamide, trifluoroacetic acid, α -LA, β -LG, κ -casein, β -casein, bovine serum albumin (BSA) and ovalbumin were purchased from Sigma-Aldrich (Buchs, Switzerland). Dichloromethane (DCM, $\geq 99.9\%$) and acetonitrile (ACN, Rotisolv HPLC) were obtained from Carl Roth GmbH (Karlsruhe, Germany). Hydrogen peroxide 60 %wt, manganese (IV) oxide and copper (II) chloride were purchased from Acros Organics (Basel, Switzerland). Sodium dihydrogen phosphate, sodium hydrogen phosphate, sodium chloride, ammonium acetate, ammonium bicarbonate, DL-dithiothreitol and formic acid were purchased from Fluka (Buchs, Switzerland). Methanol (hypergrade for LC-MS, LiChrosolv) and lysozyme from egg white were obtained from Merck (Darmstadt, Germany). Sodium hydroxide and hemin were purchased, respectively, from RectoLab SA (Servion, Switzerland) and Porphyrin Products Inc. (Logan, USA). Hydrochloric acid (32 %) was obtained from Fischer Chemicals AG (Zurich, Switzerland). Trypsin from bovine pancreas was acquired from AppliChem GmbH (Darmstadt, Germany) and nickel wire (2 mm, purity $\geq 99.98\%$) from Advent Research Materials Ltd (Oxford, England). The Ag/AgCl reference electrode was purchased from CH Instruments Inc. (Austin, USA).

Ultrapure water was obtained from a Milli-Q Integral Water Purification System (Merck-Millipore, Zug, Switzerland) and used in all experiments. An Agilent 8453 G1103A spectrophotometer (Agilent Technologies, Waldbronn, Germany) was used for all UV-Vis analyses with quartz cuvettes (200-2500 nm spectral range). A high-current potentiostat/galvanostat (Autolab PGSTAT302N) from Metrohm AG (Utrecht, Netherlands) was used for the electrochemical experiments. Protein quantification was carried out using a commercial BCA protein assay (Pierce, ThermoFisher Scientific, Rockford, USA).

4.2.2 Preparation and quantification of peroxy nitrite solution

The preparation of peroxy nitrite was carried out by modifying a previously developed method. (36) First, 200 mg of NaNO_2 were dissolved in 880 μL Milli-Q water in a small beaker. A second solution composed of 120 μL of hydrogen peroxide 60 % (w/v) and 150 μL HCl 32 % was prepared and added to the sodium nitrite solution to form peroxy nitrous acid. As the half-life of the product is really short (1 s) under the acidic conditions, a NaOH 3M solution is quickly added to

the solution. Manganese dioxide was added to the solution to catalyze the decomposition of unreacted hydrogen peroxide. After 20 minutes of H_2O_2 decomposition in an ice bath, the solution was filtered and stored at -20°C until use. The peroxynitrite concentration was determined by UV-Vis spectroscopy. 10 μL of the stock solution were diluted in 700 μL of NaOH 100 mM and the absorbance of the solution was measured at 302 nm (molar extinction coefficient $\epsilon = 1700 \text{ M}^{-1}\cdot\text{cm}^{-1}$ (37)). The ONOO^- concentration of the stock solution was found to be 14.7 mM.

4.2.3 Protein nitration using NONOate

As all other NONOate compounds, diethylamine NONOate diethylammonium decomposes readily at neutral and acidic pH. For this reason, a 0.2 M diethylamine NONOate solution was prepared in 0.01 M NaOH. Adequate volumes of the NONOate stock solution were added to 400 μL of protein solution (1 $\text{mg}\cdot\text{mL}^{-1}$ in 25 mM PBS, pH 7.4) in order to obtain molar ratios between the nitrating agent and the Tyr residues in the sample ranging from 0 to 30 (mol/mol). NaOH 0.01 M was added to samples in order to obtain similar volumes and pH for all the experiments. At the end of the reactions, the proteins were purified and analyzed as described below.

4.2.4 Protein nitration using sodium nitrite and hydrogen peroxide

A stock solution of 0.2 M NaNO_2 in 25 mM PBS buffer (pH 7.4) was prepared and added to 400 μL of protein solution (1 $\text{mg}\cdot\text{mL}^{-1}$ in the same PBS buffer) in order to obtain molar ratios between the nitrating agent and the Tyr residues in the sample ranging from 0 to 30 (mol/mol). PBS buffer was added to the samples to ensure a similar volume in all the experiments. Various catalysts were tested and were added to the reaction solution to a final concentration of 25 μM . Iron (II) chloride, iron (III) chloride and copper (II) chloride were prepared as 0.01 M solutions in PBS buffer. Due to its low solubility at neutral pH, hemin was solubilized in NaOH 0.01 M. Despite the relatively high pH, the hemin quantity used was above the solubility limit in the given conditions. After centrifugation and collection of the supernatant, the exact hemin concentration was measured by UV-Vis spectroscopy at 385 nm ($\epsilon = 58400 \text{ M}^{-1}\cdot\text{cm}^{-1}$ (38)). The final hemin concentration corresponds to 3 mM. Concentrated hydrogen peroxide was diluted to a final concentration of 0.2 M and added to all the experiments with a 1:1 (mol/mol) ratio to NaNO_2 . At the end of the reaction, the proteins were purified and analyzed as described below.

4.2.5 Protein nitration using tetranitromethane

Tetranitromethane (TNM) was diluted in methanol to obtain a concentration of 0.2 M. Adequate volumes of the TNM stock solution were added to 400 μL of protein solution (1 $\text{mg}\cdot\text{mL}^{-1}$ in 25 mM PBS, pH 7.4) in order to obtain molar ratios between the nitrating agent and the Tyr residues in the sample ranging from 0 to 30 (mol/mol). Pure methanol was added to the samples to yield similar volumes of methanol in all the experiments. At the end of the reaction, two successive extractions of the unreacted TNM were performed using 50 μL of DCM. The aqueous phases were collected and the proteins were purified and analyzed as described below.

4.2.6 Protein nitration using peroxyxynitrite

The solution of peroxyxynitrite was thawed on ice right before its use. Adequate volumes of the ONOO⁻ stock solution were added to 400 μL of protein solution (1 $\text{mg}\cdot\text{mL}^{-1}$ in 25 mM PBS, pH 7.4) in order to obtain molar ratios between the nitrating agent and the Tyr residues in the sample ranging from 0 to 30 (mol/mol). NaOH 1.5 M was added to samples in order to obtain similar volumes and pH for all the experiments. After the reaction, proteins were purified and analyzed as described below.

4.2.7 Protein nitration using a 3-electrode setup

The electrochemical protein nitration was adapted from elsewhere. (39) A 0.4 M solution of sodium nitrite was prepared in a 100 mM sodium borate buffer (pH 9.5) and was added to 500 μL of protein solution (1 $\text{mg}\cdot\text{mL}^{-1}$ in the same sodium borate buffer) to obtain the desired NaNO₂:Tyr molar ratio. Potassium chloride (0.4 M in borate buffer) was added finally to the solution to compensate the addition of sodium nitrite and maintain a constant ionic strength between the experiments. Finally, the solution was diluted with the buffer to a final volume of 1.2 mL and placed in a 5 mL beaker. Two roughly polished nickel wires were used as the working and counter electrodes, with surface of approximately 25 and 150 mm^2 , respectively. A commercial Ag/AgCl reference electrode was used for all reactions. The positions of the 3 electrodes and the distances between each other were maintain identical in all experiments by using a 3D-printed lid adapted to the beaker used for the reactions. A small magnetic stirrer was used to decrease the diffusion limitations. A constant potential of 850 mV was applied for various set times and the current variation was recorded.

4.2.8 Preparation and nitration of natural extracts

Skimmed milk, obtained from a local store, was separated in its whey and casein fractions by acidic precipitation as previously detailed in *Chapter 2*. Briefly, 1 mL of milk was defatted by centrifugation before acidification to pH 4.5 using ammonium acetate buffer (50 mM, pH 4.5) to precipitate the caseins. The recovered and washed pellet was dissolved in 1 mL ammonium bicarbonate buffer (25mM, pH 8.5) whereas the supernatant containing the whey proteins was collected and ready to use. Before nitration, the casein and whey fractions were diluted 10 and 2 times, respectively, using 25 mM PBS (pH 7.4).

Egg white, separated from the yolk of a fresh organic egg purchased in local store, was diluted in 10 mM PBS solution (pH 7.4) to yield a solution with a protein concentration of approximately 1 w/w %.

The exact protein concentration of all extracts was determined by BCA assay in order to calculate the amount of nitrating agents required. Based on the typical protein compositions and the number of Tyr residues in the most abundant proteins of bovine milk and hen eggs, the theoretical concentration of tyrosine residues in each extract was estimated. Concentrations of 242, 351 and 174 μM of tyrosine residue were obtained for a 1 $\text{mg}\cdot\text{mL}^{-1}$ solution of whey proteins, caseins and egg white proteins, respectively. Using these values, the nitration reactions were performed as described above.

4.2.9 Proteins purification and quantification

The purification of the proteins obtained after various nitration reactions was performed using Amicon Ultra-0.5 mL centrifugal filters (Merck, Darmstadt, Germany) with a molecular weight cut-off of 3 kDa. Each sample was concentrated to 50 μL and washed two times with 450 μL of pure water in order to remove the reactants and decrease the salts concentration before mass spectrometry analysis.

After the purification, the effective concentrations of all samples were determined by BCA assay. BSA was used as a standard with concentrations ranging from 0 to 2000 $\mu\text{g}\cdot\text{mL}^{-1}$.

4.2.10 Nitration degree determination by UV-Vis spectroscopy for α -LA as a model allergenic protein

First, 25 μ L of the nitrated α -LA sample were diluted in 175 μ L of sodium borate buffer (100 mM, pH 9.5) and the absorbance of the samples were measured at 422 nm. The concentration of NTyr (C_{NTyr}) in the sample was determined using an external calibration using pure NTyr in sodium borate buffer (100 mM, pH 9.5). Then, the nitration degree (ND) was easily calculated for a sample containing a single protein as the concentration of NTyr measured spectroscopically, divided by the total tyrosine concentration (native and nitrated) in the sample, obtained from the BCA assay:

$$ND [\%] = \frac{C_{NTyr}}{C_{Prot} \cdot \frac{Nbr_{Tyr}}{Prot}} \cdot 100$$

Determination of the ND for natural extracts or samples containing several proteins was performed by mass spectrometry as described below, as the present method may not be applied. It is interesting to note that the ND determination may also be carried out solely using UV-Vis spectroscopy as described by K. Selzle *et al.* (32)

4.2.11 Mass spectrometry analysis of nitrated α -LA and nitration degree determination

Nitrated α -LA analyses were performed on a Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA) set to Protein mode by direct infusion using a TriVersa NanoMate chip-based nanoelectrospray ionization (nanoESI) source (Advion Biosciences, Ithaca, USA). The samples were diluted in ESI solution (50 % ACN, 49 % H₂O and 1 % formic acid) to a final protein concentration of 5 μ M. Five to ten μ L of the α -LA samples were injected via the Nanomate chip by applying a 1.2 - 1.4 kV ionization voltage. The mass spectra were recorded for 2 to 5 minutes in positive mode with a 120'000 resolution at 400 m/z . In a limited number of cases, the resolution was set to 240'000, due to the complexity of the samples. The maximum ion injection time was set to 500 ms with an automatic gain control (AGC) target of 3E6 and averaging of 10 microscans.

The mass spectra recorded were deconvoluted using Xtract algorithm within Protein Deconvolution 4.0 (Thermo Fischer Scientific, San Jose, USA). Proton was set as the charge carrier, with a mass of 1.007267 Da. The fit factor and the remainder threshold were set to 92-96 % and 25 %, respectively, while the signal to noise threshold was fixed at 2. As the expected products

masses are close to each other, the overlapping option was enabled. The corresponding resolution at 400 m/z was set according to the spectrum recording parameters.

The nitration degree was calculated based on the abundances of the various deconvoluted peaks, assuming similar ionization efficiencies for all isoforms, *i.e.* proteoforms, of a protein. The nitration degree of a single peak i containing one or several nitration sites is defined as the ratio of nitrated tyrosine residues to the total number of tyrosine (native and nitrated) of this peak:

$$ND_i[\%] = \frac{Nbr_{NTyr}}{Nbr_{Tyr+NTyr}} \cdot 100 = \frac{Nbr_{NTyr}}{Nbr_{Tyr,total}} \cdot 100$$

Based on this definition, the overall nitration degree of a proteoform group is calculated by taking into account all the proteoforms i and their fractional abundances FA_i :

$$ND [\%] = \sum_i FA_i \frac{Nbr_{i,NTyr}}{Nbr_{i,Tyr,total}} \cdot 100$$

The oxidation degree was calculated similarly by taking into account all the proteoforms corresponding to a single or multiple oxidation sites.

4.2.12 Nitrated proteins and natural extracts digestion using trypsin

2 μL of the nitrated protein samples were diluted in 18 μL of ammonium bicarbonate buffer 25 mM (pH 8.2) and denatured at 95 °C for 10 minutes. Then, 100 mM of dithiothreitol (DTT) in the same ammonium bicarbonate buffer were added to the protein solutions to a final DTT concentration of 5 mM and the solutions were incubated at 56°C for 30 minutes. After letting the solutions to cool down, iodoacetamide (IAA, 100 mM, in ammonium bicarbonate buffer) was added to a final concentration of 10 mM and the alkylation reaction was performed in the dark for 30 minutes at room temperature. Trypsin (1 $\text{mg}\cdot\text{mL}^{-1}$) was finally added with a ratio 30:1 (w/w, protein:trypsin) and the mixture was incubated overnight at 37 °C under moderate stirring. The tryptic reaction was stopped by acidification using trifluoroacetic acid to a final acid concentration of 0.1% and pH < 4. Tryptic peptides were extracted and concentrated using ZipTip C₁₈ pipette tips (Merck Millipore, Darmstadt, Germany) following the manufacturer protocol.

4.2.13 Mass spectrometry analysis of tryptic digests and data processing

Tryptic peptides obtained after ZipTip C₁₈ purification were injected on a ZORBAX Eclipse Plus C₁₈ column (2.1 x 150 mm, 5 μm, Agilent, Waldbronn, Germany) using a Dionex Ultimate 3000 HPLC system (Thermo Fischer Scientific, San Jose, USA) coupled to Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA). The column oven was set to 30°C and a fixed flowrate of 0.25 mL·min⁻¹ was used. Eluents A and B consisted, respectively, in 99.9:0.1 % H₂O:FA and 99.9:0.1 % ACN:FA. The concentration of eluent B was kept at 5 % for the first 3 minutes as equilibration step, before increasing it to 35 % in 40 minutes as effective separation gradient. It was then increased to 80 % in 1 minute and kept at this concentration for 2 minutes as washing step. Finally, the eluent B was decreased to 5 % in 1 minute and maintained for 3 minutes to reequilibrate the column. A blank injection was performed between each sample analysis.

A standard full MS/dd-MS² method was used for the analysis of the tryptic peptides. The resolution was set at 60'000 (at 400 *m/z*) for the full MS scans, with an AGC target of 3E6 and maximum injection time of 100 ms. The full MS spectra were recorded from 300 to 1900 *m/z* in profile mode. The MS² spectra were recorded in centroid mode with Top5 method and the resolution 15'000 at 400 *m/z*. The maximum injection time was fixed at 50 ms with 4 microscans and an AGC target of 1E6. A 2 *m/z* isolation window was used for the parent ions and the normalized collision energy of higher-energy collisional dissociation (HCD) fragmentation was set at 27 with default charge 2. Only peptides with an assigned charge between 2 and 7 were allowed for selection and subsequent fragmentation.

The obtained data were analyzed using the open-source softwares SearchGUI (40) and PeptideShaker. (41) The protein databases used were obtained from the Universal Protein Resource (UniProt, release July 2018) and contained the complete reviewed bovine (*Bos taurus*) and chicken (*Gallus domesticus*) proteomes, common keratin contaminants and bovine trypsin. Carbamidomethylation of cysteine residue (+ 57.0215 Da) was set as a fixed modification, whereas acetylation of protein N-terminus (+ 42.0106 Da), deamidation of asparagine and glutamine (+ 0.9840 Da), oxidation of methionine (+ 15.9949 Da), nitration of tyrosine (+ 44.9851 Da), nitrosotyrosine (+ 28.9907 Da) and aminotyrosine (+ 15.0103 Da) were set as potential modifications. A maximum of 4 missed cleavages were set with a precursor *m/z* tolerance of 10 ppm and a frag-

ment tolerance of 0.04 Da. The peptide identifications were validated with a false discovery rate (FDR) of 1 %.

4.2.14 Calculation of solvent accessible surface area

VMD (version 1.9.3) (42) and the included QwikMD plugin (43) were used to calculate the solvent accessible surface area (SASA) of various proteins. The PDB files used for the simulation were obtained from the Protein Data Bank in Europe (PDBe, www.pdbe.org (44)). The probe radius for the SASA calculations was set to 1.4 Å.

4.3 Results and discussion

4.3.1 α -Lactalbumin as a model protein for nitration

α -LA is a major milk allergen and accounts for approximately 20 % w/w of the total bovine whey proteins. (45) This protein is composed of 123 amino acids (14.3 kDa) and has a globular tertiary structure, stabilized by four disulfide bonds (C₆-C₁₂₀, C₆₁-C₇₇, C₇₃-C₉₁, C₂₈-C₁₁₁). (46) As α -LA is commercially available and well-studied, it was used as a model protein for nitration using various nitrating agents.

This protein contains 4 Tyr residues (Y₁₈, Y₃₆, Y₅₀, Y₁₀₃), positioned at various places in the tertiary structure of the protein (Figure 2), all susceptible of being nitrated. The solvent accessible surface area (SASA) gives a good representation of the amino acid positions and their potential accessibility by solvents and chemicals. The calculated SASA for residues Y₁₈, Y₃₆, Y₅₀, Y₁₀₃ are 87.2, 13.5, 7.2 and 61.4 Å² respectively. The average and median residue SASA for the overall protein are 56.0 and 41.7 Å². Based on these values, Y₁₈ is expected to be the more susceptible tyrosine residues to be modified as it gets relatively easily in contact with solvents and other chemical molecules, whereas Y₅₀ is deeply hidden in the protein tertiary structure as shown in Figure 2.

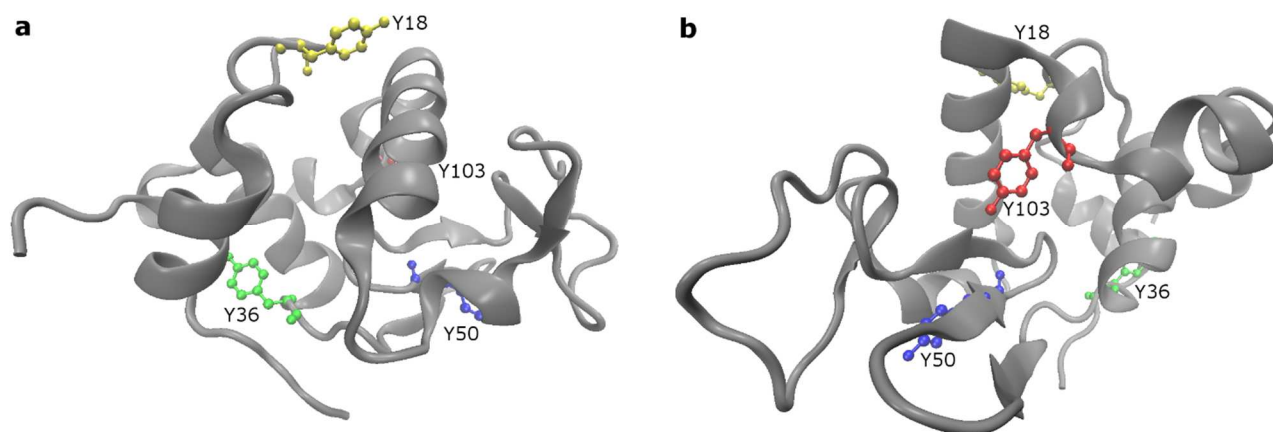


Figure 2. Representation of the tertiary structure of α -LA (1HFZ) obtained from the Protein Data Bank in Europe (44) from **(a)** front and **(b)** back. The tyrosine residues Y₁₈, Y₃₆, Y₅₀ and Y₁₀₃ are displayed in yellow, green, blue and red, respectively.

Evaluation of SASA is not enough to fully predict the modification localizations in a protein as the physicochemical properties of neighboring amino acids and the protein flexibility needs to be taken into account. For this reason, identification of the nitrotyrosine residues by techniques such as mass spectrometry or crystallography is required.

4.3.2 UV-Vis spectroscopy of 3-nitrotyrosine

Quantification of NTyr in solution is easily carried out by UV-Vis spectroscopy. NTyr absorbance is strongly dependent on the pH of the solution. (47,48) At basic pH the NTyr displays an intense band at 422 nm, which was used to determine nitrated tyrosine residues concentrations in samples based on an external calibration. In the chosen conditions (sodium borate buffer, pH 9.5), a good linearity was obtained between 1 and 500 μ M with a correlation coefficient R^2 of 0.999. At this pH, the extinction coefficient was estimated to be $2143.1 \pm 22.5 \text{ M}^{-1}\text{cm}^{-1}$ (Figure 3).

All nitrated samples in this work were brought to pH 9.5 using the same sodium borate buffer and this calibration curve was used to determine the nitration degree (ND) by UV-Vis spectroscopy.

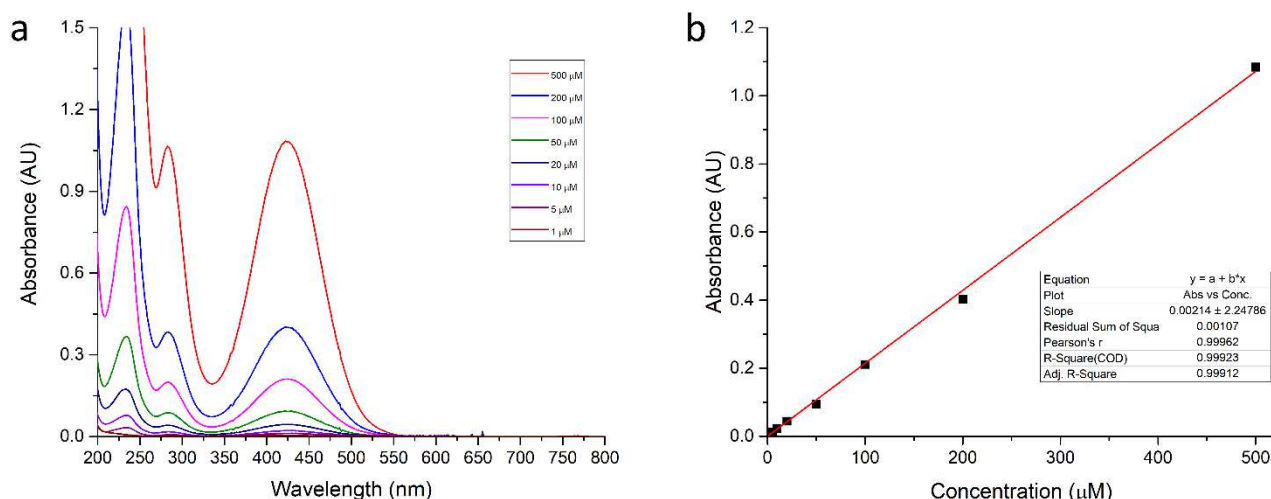


Figure 3. (a) UV-Vis spectra and (b) calibration curve for NTyr with concentrations ranging from 0 to 500 μM in sodium borate buffer 100 mM, pH 9.5.

4.3.3 Nitration of α-LA by NONOate

Nitration of Tyr residues may be carried out using chemicals containing a NONOate functional group. These molecules are usually stable at high pH and degrade at physiological or acidic pH to release nitric oxide (NO[•]) as shown in Figure 4. Previously, various NONOate containing molecules were used to nitrate protein, peptides and fatty acids. (21,28–30)

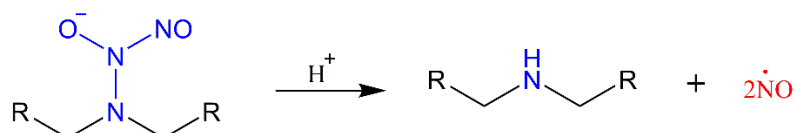


Figure 4. Degradation of NONOate group at acidic pH to a secondary amine and nitric oxide.

The various commercially available molecules containing NONOate groups all have different decomposition kinetics, depending on the molecule structure, reaction temperature, pH and nature of the buffer. In the present study, diethylamine NONOate diethylammonium (further referred only to NONOate) was used as a nitric oxide donor for the nitration of α-LA. According to the supplier, the half-life of this NO-donor is 16 minutes in phosphate buffer at 22°C and neutral pH. A 0.2 M NONOate solution in 10 mM NaOH was diluted in 25 mM PBS buffer, pH 7.4, to a final NONOate concentration of 250 μM and the degradation was followed by UV-Vis spectroscopy (Figure 5).

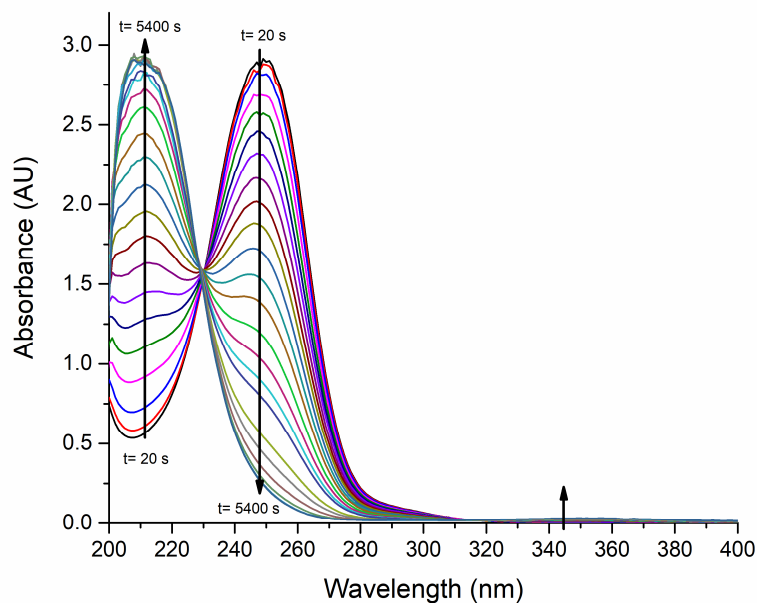


Figure 5. UV-Vis spectra of 250 μM NONOate degradation at 22°C. NONOate was prepared as a 0.2 M solution in 10 mM NaOH and diluted to its final concentration in 25 mM PBS buffer, pH 7.4.

NONOate displays a strong absorbance at 248 nm, decreasing over time following an exponential decay (Figure 6.a). The degradation reaction follows a first order kinetics ($R^2 = 0.99$) with a rate constant of approximately $7.93 \cdot 10^{-4} \text{ s}^{-1}$, resulting in a half-life of 874 s. Thus, the reaction time for the nitration of α -LA using NONOate was set to 30, 60 and 240 minutes. For the two shorter reaction times, the corresponding amount of unreacted NONOate represents approximately 24.0 and 5.8 % of initial concentration, whereas after 240 minutes of degradation, only 1 % of the initial NONOate remains. The constant release of nitric oxide in solution by NONOate is responsible for the nitration of Tyr residues. As NONOate degradation follows first order kinetics, the rate of nitric oxide release is extensive at the beginning of the reaction and decreases over time. It is worth mentioning that the NONOate degradation study was carried out in the absence of proteins, as each protein may influence slightly the reaction in a different way. Two degradation products are detected in the UV-Vis spectra of Figure 5. They absorb at 211 nm and 343 nm and show both, as expected, an exponential growth (Figure 6.b).

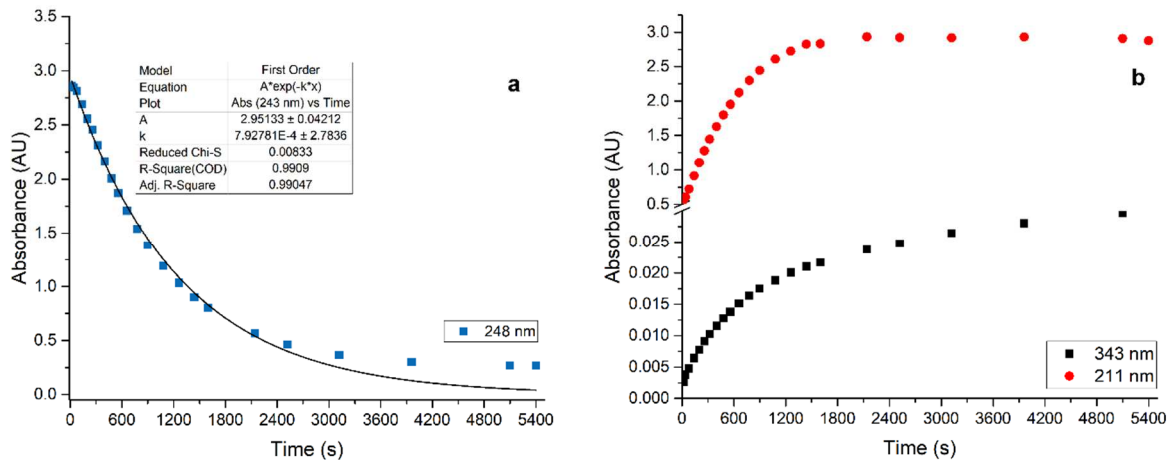


Figure 6. Absorbance measured at (a) 248 nm, (b) 343 and 211 nm for the degradation of 250 μM NONOate at 22°C in 25 mM PBS buffer.

α -LA was nitrated using NONOate with various ratios of NONOate:Tyr residues and reaction times, in order to identify the best conditions with respect to the nitration degree of the protein. With a NONOate:Tyr residues molar ratio fixed at 30:1, the ND of α -LA reached 1.05 % after 30 minutes of reaction (Figure 7.a, red line). After 1h of reaction, approximately 94 % of the NONOate is degraded and the ND of α -LA was measured at 1.35 %. Meanwhile, increasing the reaction time to 4 hours, corresponding to approximately 17 NONOate half-lives, resulted in a ND of 2.51 %. These results indicate that the nitric oxide release is faster than the nitration of Tyr residues, as the ND continues to progress significantly after almost all the NONOate has been degraded.

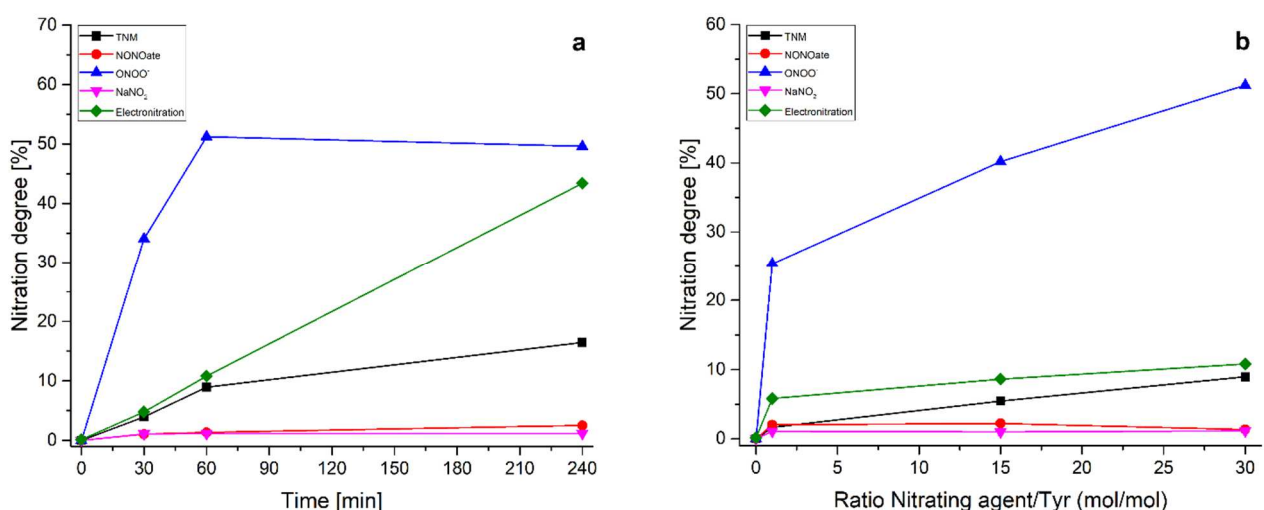


Figure 7. NDs of α -LA measured by UV-Vis spectroscopy after reaction with various nitrating agents (a) for 0, 30, 60 and 240 minutes and a ratio of nitrating agent:Tyr residues of 30:1 (mol/mol) and (b) with ratios of nitrating agent:Tyr residue ranging from 0:1 to 30:1 (mol/mol) for 1 hour. Iron (II) chloride was used as the catalyst for the NaNO₂/H₂O₂ nitration procedure. The ND was calculated as detailed in the *Materials and Methods* section 4.2.11.

The nitration of α -LA was then carried out for 1 h with various molar ratio of NONOate to Tyr residues (Figure 7.b, red line). Ratios of 1:1, 15:1 and 30:1 yielded *ND* of 2.01, 2.22 and 1.35 % respectively. With the higher ratio tested, the formation of small nitric oxide bubbles was observed on the walls of the test tubes. As the liberation of NO^\cdot by the NONOate is faster than its consumption by the nitration reaction, the bubbles grow during the reaction. This phenomenon explains the low influence of the NONOate: Tyr residues ratio on the *ND* values; the nitration reaction is limited by the solubility of nitric oxide in the chosen conditions and the relative rate constants of the NO^\cdot production and consumption.

4.3.4 Nitration of α -LA by sodium nitrite and hydrogen peroxide

One possible pathway for the *in vivo* nitration of proteins is the reaction of nitrite anions, resulting from the normal NO metabolism, with peroxidases. (49–52) Peroxidases, such as the horseradish peroxidase, are heme-containing proteins that utilize mainly hydrogen peroxide to oxidize various compounds. (53,54) Free heme, iron atoms, amyloid beta/heme complex and copper atoms are also shown to catalyze protein nitration using hydrogen peroxide and nitrite. (21,54,55). Thus, in the present study, α -LA was also nitrated using hydrogen peroxide and sodium nitrite in the presence of a catalyst. Iron (II) chloride (FeCl_2), iron (III) chloride (FeCl_3), copper (II) chloride (CuCl_2) and hemin (a protoporphyrin IX complex) were used as catalysts with a final concentration of 25 μM . The nitration reaction was performed with a 30:30:1 molar ratio of H_2O_2 : NaNO_2 :Tyr residues for 1 h at room temperature. The nitration degree obtained using FeCl_3 , FeCl_2 and CuCl_2 were very similar; 1.90, 1.14 and 1.84 %, respectively (Figure 8). Meanwhile, it was not possible to determine the *ND* obtained with hemin as the catalyst. Despite the use of centrifugation filtering devices with a 3kDa molecular weight cut-off, hemin was always detected in the purified samples. As this compound absorbs strongly at 385 nm with a molar extinction coefficient of 58400 $\text{M}^{-1}\text{cm}^{-1}$ (38), this band superposes itself partially with the NTyr band, hindering the analysis. Extensive centrifugal purification and cold-acetone precipitation of the proteins were unable to remove traces of hemin in the samples. For this reason, it is believed that α -LA may form a relatively stable complex with hemin.

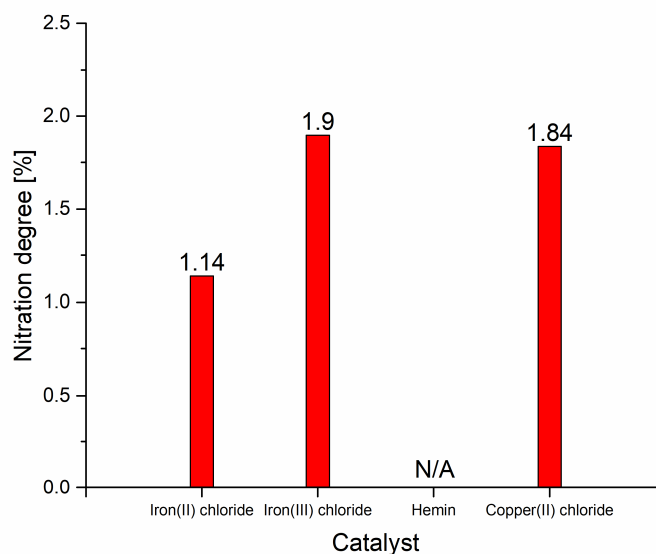


Figure 8. *NDs* of α-LA by sodium nitrite and hydrogen peroxide using FeCl₂, FeCl₃, CuCl₂ and hemin as catalysts, measured by UV-Vis spectroscopy. The final catalyst concentration was set to 25 μM.

FeCl₂ was finally chosen to nitrate α-LA with reaction times ranging from 30 to 240 minutes (Figure 7.a, pink line) and with various NaNO₂:Tyr residues ratios (Figure 7.b, pink line). Under all those conditions, the *ND* obtained remained low and ranged from 0.99 to 1.15 % only.

4.3.5 Nitration of α-LA by tetranitromethane

Tetranitromethane (TNM) has been extensively used since the 1960s for the nitration of various biological molecules, including proteins. (18–20,25,26,32,47,56–58) TNM is a highly toxic compound presenting strong organic oxidizer properties. As TNM has extremely low water solubility, it is usually prepared in methanol or ethanol before adding it to the protein solution. It decomposes itself to release NO₂[·] radicals responsible for the protein nitration. (59) The conditions employed for TNM-mediated nitration are usually considered gentle although they yield a high *ND*.

As expected, the *ND* measured by UV-Vis spectroscopy after nitration of α-LA by TNM were higher than for the nitration mediated by NaNO₂/H₂O₂ or NONOate. TNM nitration of α-LA, with a TNM:Tyr residues molar ratio of 30:1, resulted in a *ND* of 3.94 and 8.95 % after a 30 min and 1 h reactions, respectively. Increasing the reaction time to 4 h yielded a 16.46 % *ND* (Figure 7.a, black line). However, a gel-like precipitate started to form at the 3-hour mark. The precipitate was removed by centrifugation before protein purification and analysis. In fact, TNM is known to induce a relatively high number of side reactions that could lead to polymerization of proteins

and peptides caused by inter- and intra-molecular cross-linking, as already reported previously. (60–62) Protein polymerization is a multi-parameters process, depending on the concentration of the protein, the secondary and tertiary structure, and the ratio of TNM:Tyr residues. Decreasing the TNM:Tyr molar ratio from 30:1 to 15:1 and 1:1 resulted in a linear decrease of the *ND* with obtained values of 8.95, 5.45, 1.64 % for a 1 h reaction (Figure 7.b, black line), while no precipitation of α -LA was detected in these experiments.

TNM nitration displays relatively high *ND* explaining its widespread use. However, oligomerization, polymerization and other side-reactions are detrimental and limit the TNM applicability depending on the study goal. As the above results are obtained from direct UV-Vis spectroscopy of the purified samples, possibly containing soluble α -LA oligo/polymers, the obtained *ND* may be shifted from the true *ND* value of α -LA monomers. For high TNM:Tyr residue ratios and/or for long reaction times, the separation of protein monomer from other soluble oligomers/polymers is required. Recently, F. Liu *et al.* developed a SEC-HPLC-DAD method for the simultaneous detection of protein oligomers and their *ND*. (27) In the present study, the oligomers were not separated but mass spectrometry was used in complement to UV-Vis spectroscopy to determinate the *ND* as discussed below.

4.3.6 Nitration of α -LA by peroxyntirite

In vivo protein nitration may be induced also by peroxyntirite (ONOO^-), formed in physiological conditions by superoxide anions (O_2^-) and nitric oxide radicals. (63,64) Peroxyntirite is a strong oxidant, more reactive than his parent molecules, and is highly unstable with a half-life of 10 to 20 ms in biological conditions. (65) Peroxyntirite is protonated at physiological pH to yield peroxyntirous acid (ONOOH , pKa 6.5). The O-O peroxy bond, weakened in the ONOOH molecule, is easily cleaved by homolysis to yield NO_2^- and $\cdot\text{OH}$ radicals. (66) The complete ONOO^- pathway for the nitration is displayed in Figure 9.

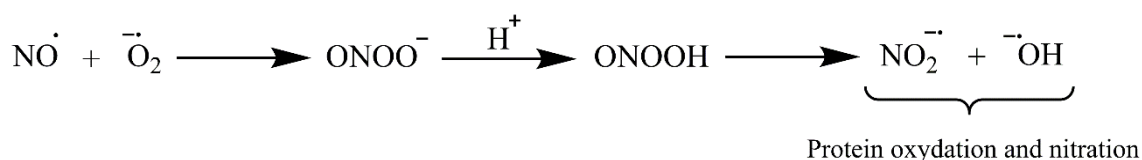


Figure 9. Pathway for the liberation of nitrogen dioxide and hydroxyl radicals from nitrogen oxide and superoxide anions under physiological conditions.

As peroxyxynitrite is highly unstable, it was stored, directly after its synthesis, at -20°C in a concentrated sodium hydroxide solution (see *Materials and Methods* section 4.2.2). After being thawed on ice, it was used to nitrate α -LA under the same conditions as the other nitrating agents.

For all ONOO^- :Tyr residues ratios tested, the *ND* was significantly higher than for other nitration methods with values of 25.35, 40.19 and 51.23 % for 1:1, 15:1 and 30:1 molar ratios, respectively (Figure 7.b, blue line). Consumption of ONOO^- is relatively fast and the appearance of a yellow color, corresponding to NTyr, is readily visible to the naked eye, even for the 1:1 molar ratio. Reacting α -LA with peroxyxynitrite (30:1 molar ratio ONOO^- :Tyr residues) for 30 minutes yielded a *ND* of 34.03 %, whereas increasing the reaction time to 240 minutes resulted in a 49.60 % *ND* (Figure 7.a, blue line). Meanwhile, under all the chosen conditions, extensive oxidation reactions lead to polymerization and precipitation of the protein, as it was observed for some TNM nitration experiments. Centrifugation was used to separate the precipitate from the supernatant. As it is widely known, peroxyxynitrite is responsible for the nitration of tyrosine residues and extensive oxidative degradation. (29,32) Addition of radical scavengers, such as tocopherols or glutathione, may limit greatly the oxidative damages. Nevertheless, their structures, responsible for their reactive oxygen species capture properties, are also known to interact strongly with nitric oxide and its radical derivatives. (67–69) For this reason, decreasing the oxidative damage to proteins by using radical scavengers while maintaining high *ND* is complicated if not impossible and, hence, was not implemented in the current work. Despite obtaining a higher *ND*, the oxidative damages induced during the nitration process by peroxyxynitrite limit its use for structural studies aiming to produce nitrated-only allergenic proteins.

4.3.7 Nitration of α -LA by electrochemical nitration

Electrochemical modification of proteins is a selective process that may occur at methionine, tryptophan and tyrosine residue depending on the design of the reactions and the applied potentials. (33,70) Electrochemical nitration was successfully carried out with a relatively high selectivity for various proteins such as hen egg white lysozyme (34,39,71) and equine skeletal myoglobin. (68) The main advantages of electrochemical nitration over conventional chemical reactions are the high reaction control offered by the applied potential and the theoretical absence of side-products, unwanted oxidation and unreacted chemical residues. Moreover, the nitration reaction is easily stopped by switching off the applied potential. (67)

Herein, α -LA was electro-nitrated using sodium nitrite in a standard 3-electrode setup with nickel wires (2 mm diameter) used as the working and counter electrodes. The applied potential was set to 850 mV vs Ag/AgCl reference electrode, as it typically corresponds to the oxidation reaction measured in the cyclic voltammetry experiments for solutions composed of α -LA and sodium nitrite. During the nitration reaction, the current evolution was recorded and ranged between 0.04 and 0.25 mA. A typical cyclic voltammogram and chronoamperogram for a system containing α -LA and sodium nitrite are displayed in Figure 10. The cyclic voltammogram shows an irreversible reaction with oxidation and reduction reactions at 0.85 and 0.55 V, respectively (Figure 10.a). Depending on the respective α -LA and sodium nitrite concentrations, a steady state reaction is obtained after 5 to 10 minutes, corresponding to a stable current as illustrated in Figure 10.b. Appearance of a yellow coloration of the solution, corresponding to the nitration of Tyr residues, is easily observed by the naked eye.

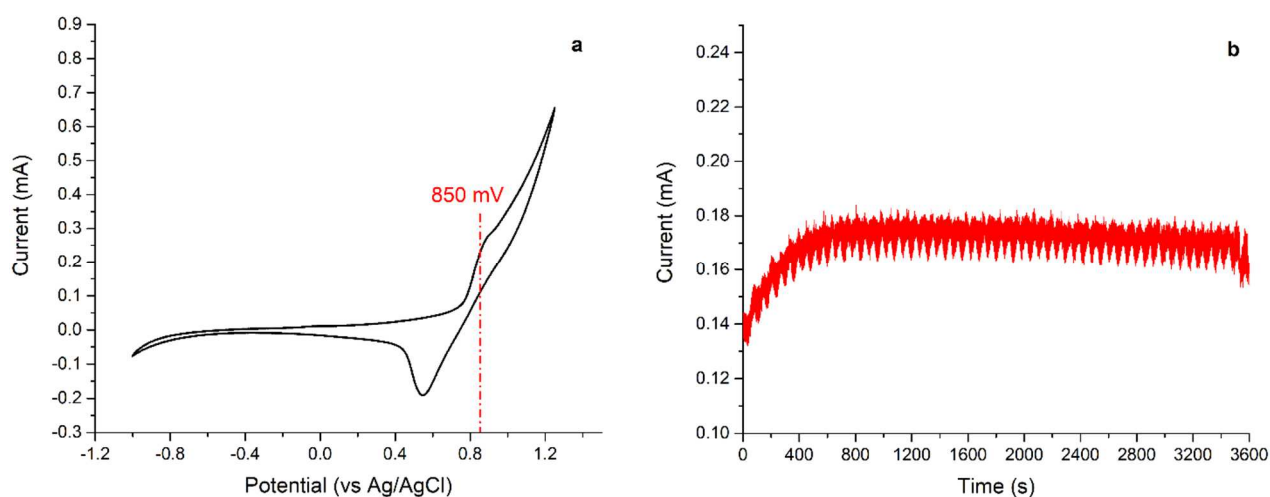


Figure 10. (a) Cyclic voltammogram (CV) obtained for a solution of sodium nitrite and α -LA with molar ratio of 30:1. The CV was measured between -1 and 1.25 V (vs Ag/AgCl) with step potential of 2.5 mV and scan rate of 0.1 V/s. **(b)** Chronoamperogram recorded for the 1-hour electrochemical nitration of α -LA by sodium nitrite with the same molar ratio. The current was measured with a 1-second interval time and with an 850 mV applied voltage.

For a 1 h reaction, the *ND* obtained for NaNO_2 :Tyr residues ratios of 1:1, 15:1 and 30:1 were 5.83, 8.61 and 10.79 % respectively (Figure 7.b, green line). Increasing further the ratios to 1000:1 and 10000:1 resulted only in a slight increase in the *ND*, to 16.94 and 27.13 %, respectively (data not shown). For α -LA electrochemical nitration, increasing the reaction time appeared more beneficial compared to increasing the nitrating agent concentration. For a 30:1 NaNO_2 :Tyr residues molar ratio, a reaction time of 240 min yielded a 43.73 % *ND* (Figure 7.a, green line). Moreo-

ver, the *ND* obtained by electrochemical nitration displays a good linearity with respect to the reaction time with a R^2 of 0.999. It is interesting to note that despite the high *ND* and/or concentration of the nitrating agent, no protein precipitation was observed for this nitration reaction. This highlights the expected selectivity of the electrochemical process.

4.3.8 Analysis of nitrated α -LA proteoforms by mass spectrometry

The direct use of UV-Vis spectroscopy is limited to the analysis of nitrated proteins. The chemical nitration reactions are known to induce extensive oxidation and cross-linking of proteins. Extra steps, such as size-exclusion chromatography, are required to separate the nitrated monomers from the large variety of produced oligomers. In addition, the identification and quantification of the oxidized proteins is not feasible, as they do not absorb strongly in the UV/visible range or are not distinguishable from their native state.

Mass spectrometry (MS) is a technique of choice for the detection and precise identification of intact proteins, offering high sensitivity and selectivity. MS is often hyphenated to separation techniques such as liquid chromatography or capillary electrophoresis for the analysis of protein samples. The recent developments and improvements of high-resolution (HR) mass spectrometers of Orbitrap family allow analyzing simple samples of relatively small proteins (molecular weight < 40 kDa) by direct infusion. For large proteins, this technique may be limited among other reasons by the current maximum resolution available on HRMS instruments. In the present study, the nitrated samples of model protein contain mainly native α -LA of 14 kDa molecular weight and various natural or synthetic proteoforms, each with a small mass shift. The Q Exactive HF Orbitrap mass spectrometer resolution is high enough to resolve isotopically all proteoforms from each other, allowing Xtract deconvolution algorithm to calculate the exact masses of all proteoforms present in the samples. Therefore, nitrated samples of α -LA were analyzed by direct infusion nanoESI-HRMS without a prior separation step. The complex spectra produced were then deconvoluted to obtain the molecular weight of the various products present in the solution. Based on their exact masses, it was possible to identify directly and quickly the proteoforms obtained by nitration. Assuming that the PTMs induced by nitration have no or little impact on the ionization of the proteoforms, the *ND* can be estimated by using the fractional abundances of the detected species as described in *Materials and Methods* section 4.2.11.

The analysis of nitrated α -LA samples revealed a large number of possible modifications introduced on a native protein during nitration process. All the identified proteoforms are listed in Table 1. Reported monoisotopic masses were obtained by averaging the monoisotopic mass values detected for each proteoform from several experiments. It is important to note that the deconvolution process sometimes results in mass error of +1 or +2 Da. As the Xtract deconvolution algorithm does not always identify correctly the monoisotopic peaks of the less abundant proteoforms, the output masses may be shifted upwards.

Table 1. All identified α -LA proteoforms obtained after nitration with various nitrating agents.

Measured monoisotopic mass [Da]	Mass shift [Da]	Theoretical mass shift [Da]	Modification	Composition ^(a)
14168.72	N/A	N/A	Native α -Lactalbumin	N/A
14184.75	16.03	15.99	Oxidation	O
14190.76	22.04	21.98	Sodium adduct	H(-1) Na
14200.76	32.03	31.99	Double oxidation	O(2)
14206.74	38.02	37.95	Calcium adduct	H(-2) Ca
14213.72	45.00	44.99	Nitration	H(-1) N O(2)
14222.71	53.99	53.94	Oxidation + Calcium adduct	O H(-2) Ca
14229.72	61.00	60.98	Oxidation + Nitration	H(-1) N O(3)
14246.3	77.58	76.98	Oxidation (2x) + Nitration	H(-1) N O(4)
14258.69	89.97	89.97	Nitration (2x)	H(-2) N(2) O(4)
14266.71	97.99	97.99	Oxidation of a S-S bond to two sulfonic acids	H(2) O(6)
14282.71	113.98	113.98	Oxidation + Oxidation of a S-S bond to two sulfonic acids	H(2) O(7)
14297.71	128.98	129.98	Oxidation (2x) + Oxidation of a S-S bond to two sulfonic acids	H(2) O(8)
14311.69	142.96	142.97	Nitration + Oxidation of a S-S bond to two sulfonic acids	H(1) O(8) N
14313.69	144.97	145.97	Oxidation (3x) + Oxidation of a S-S bond to two sulfonic acids	H(2) O(9)
14327.72	159.00	158.97	Nitration + Oxidation + Oxidation of a S-S bond to two sulfonic acids	H(1) O(9) N
14344.66	175.94	174.96	Nitration + Oxidation (2x) + Oxidation of a S-S bond to two sulfonic acids	H(1) O(10) N
14356.68	187.96	187.96	Nitration (2x) + Oxidation of a S-S bond to two sulfonic acids	O(10) N(2)
14364.67	195.95	195.97	Oxidation of a S-S bond to two sulfonic acids (2x)	H(4) O(12)
14372.65	203.93	203.95	Nitration (2x) + Oxidation + Oxidation of a S-S bond to two sulfonic acids	O(11) N(2)
14380.99	212.26	211.97	Oxidation + Oxidation of a S-S bond to two sulfonic acids (2x)	H(4) O(13)
14389.63	220.90	219.95	Nitration (2x) + Oxidation (2x) + Oxidation of a S-S bond to two sulfonic acids	H(1) O(12) N(2)
14395.64	226.92	241.93	Oxidation (2x) + Oxidation of a S-S bond to two	H(4) O(14)

			sulfonic acids (2x)	
14401.65	232.93	232.94	Nitration (3x) + Oxidation of a S-S bond to two sulfonic acids	H(-1) O(12) N(3)
14409.66	240.94	240.96	Nitration + Oxidation of a S-S bond to two sulfonic acids (2)	H(3) O(14) N
14417.66	248.94	248.94	Nitration (3x) + Oxidation + Oxidation of a S-S bond to two sulfonic acids	H(-1) O(13) N(3)
14426.63	257.90	256.95	Nitration + Oxidation + Oxidation of a S-S bond to two sulfonic acids (2x)	H(3) O(15) N
14462.72	294.00	293.96	Oxidation of a S-S bond to two sulfonic acids (3x)	H(6) O(18)
14479.64	310.92	309.95	Oxidation + Oxidation of a S-S bond to two sulfonic acids (3x)	H(6) O(19)
14492.82	324.10	324.11	Lactosylation	C(12) H(20) O(10)
14508.77	340.05	340.10	Oxidation + Lactosylation	C(12) H(20) O(11)

(a) Atomic composition of the modifications with respect to the native α -LA.

The observed monoisotopic mass of native α -LA was 14168.72 Da. In untreated samples, various modifications, including oxidation, sulfonation of cysteine residues, lactosylation and combinations of these PTMs were already detected. Examples of obtained mass and deconvolution spectra are shown in Figure 11.

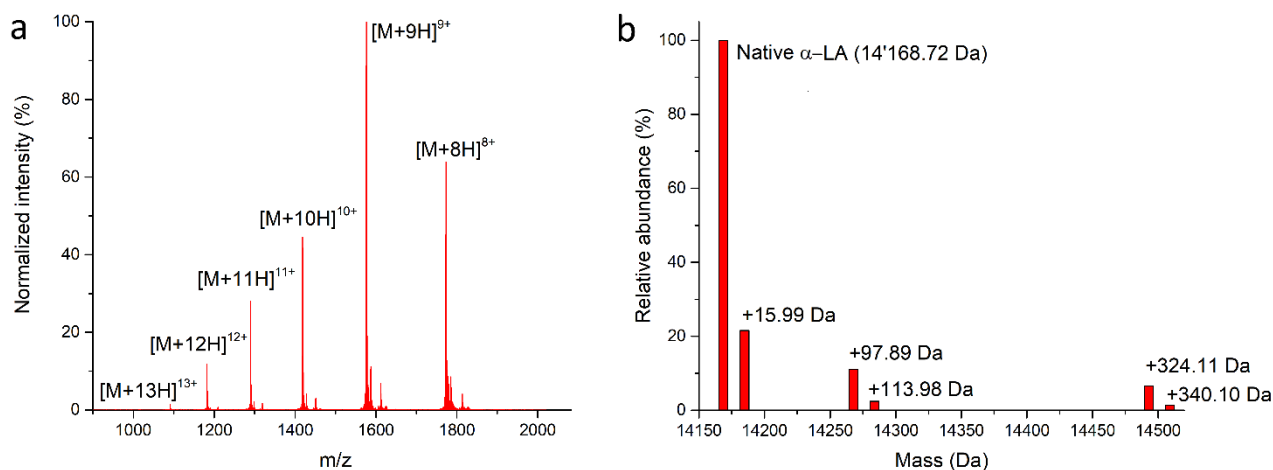


Figure 11. (a) nanoESI-HRMS spectrum of α -LA (5 μ M in ESI solution) and **(b)** the corresponding deconvoluted spectrum. The MS spectrum was measured by direct infusion using HRMS instrument with a resolution of 120'000 at 400 m/z , after purification of the samples using a centrifugation filtering devices with a 3kDa molecular weight cut-off. The deconvolution was carried out using the Xtract algorithm within Protein Deconvolution 4.0 with parameters as listed in *Materials and Methods* section 4.2.11. All identified α -LA proteoforms are listed in Table 1.

Oxidation is a common PTMs corresponding to an increased mass of +15.99 Da and is mainly observed on histidine, methionine, tryptophan and tyrosine residues. (72) Cysteine resi-

dues may also undergo oxidation to form sulfenic, sulfinic and sulfonic acids with characteristic mass shifts, as shown in Figure 12. In α -LA, all the cysteine residues form disulfide bonds and only simultaneous oxidation of two cysteine residues may be detected. In the untreated samples, a proteoform with a mass shift of +97.99 Da was clearly detected, corresponding to the breaking and further oxidation of a disulfide bond to two sulfonic acids, each corresponding to a +48.99 Da shift. Cysteine was not detected in the form of sulfenic or sulfinic acids in the native or oxidized/nitrated samples. Under the relatively harsh reaction conditions applied in this work, it is plausible that once a disulfide bridge is broken, the cysteines are readily and completely oxidized. Lactosylation was observed as well in untreated α -LA solutions with a 324.11 Da increase in mass. It is a common PTM of milk proteins, induced by the thermal or high-pressure treatment of milk. (73,74) Sodium and calcium adducts were also detected in all the samples with a respective mass of 14190.76 Da (+21.98 Da) and 14206.74 Da (+37.95 Da).

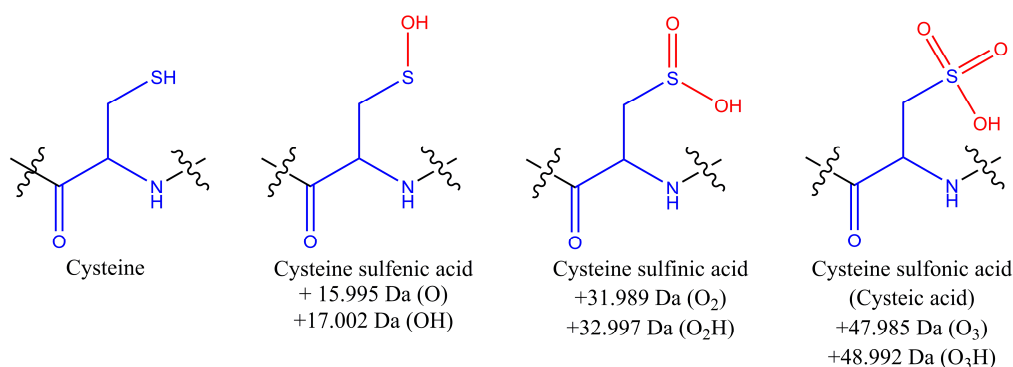


Figure 12. Cysteine residue and its oxidative derivatives, with mass shifts in Da and compositions with respect to reduced cysteine (free thiol) and oxidized cysteine (disulfide).

In the different nitrated samples, nitration was detected either as the only modification or in combinations with various oxidations. Based on nanoESI-HRMS analysis, the chemical treatment of α -LA with TNM, peroxyxynitrite and $\text{NaNO}_2/\text{H}_2\text{O}_2$ yielded *ND* ranging from 1.74 to 6.31 % for the different tested nitrating agent:Tyr residues molar ratios (Figure 13.b). Similar values were obtained for the electrochemical nitration of the protein. These results differ from those obtained by UV-Vis spectroscopy. *ND* values for TNM and electrochemical treatment are lower when measured by MS, whereas NONOate and $\text{NaNO}_2/\text{H}_2\text{O}_2$ treatment display higher *ND* values in comparison with UV-Vis data. Remaining nitrating agents and/or presence of soluble protein adducts in

the purified samples may explain the observed deviations for the UV-Vis results, thus making MS an important tool for correct *ND* evaluation.

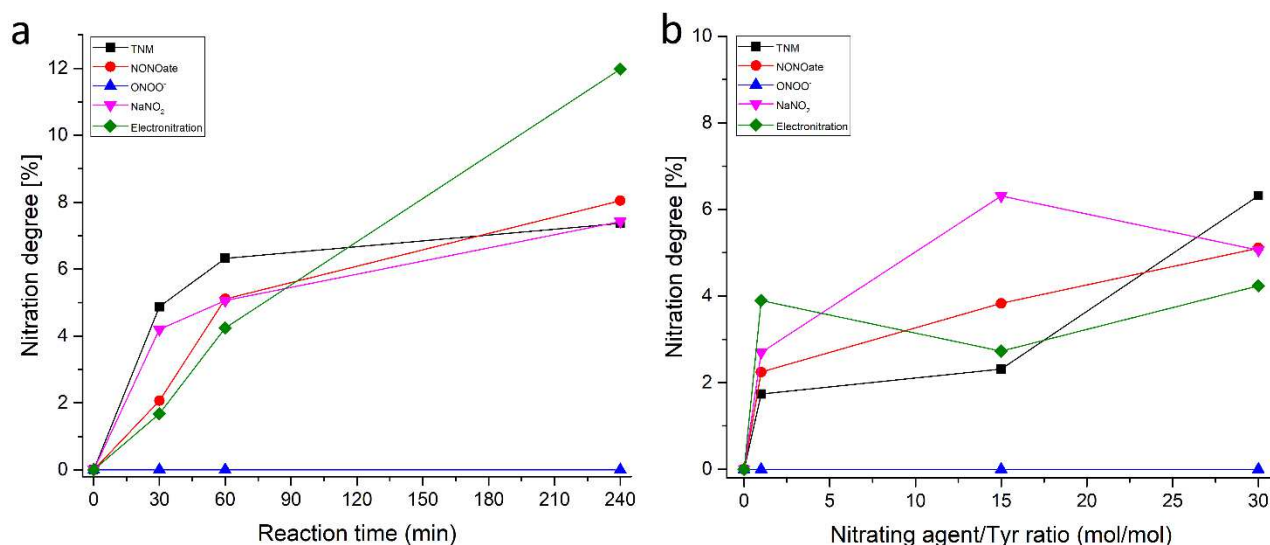


Figure 13. *NDs* of α -LA measured by nanoESI-HRMS after reaction with different nitrating agents for (a) reaction times from 0 to 240 minutes with a fixed molar ratio of 30:1 various and (b) with nitrating agent:Tyr residues molar ratios ranging from 0:1 to 30:1 with a reaction time of 1 h. Iron (II) chloride was used as the catalyst for the $\text{NaNO}_2/\text{H}_2\text{O}_2$ nitration procedure. The *NDs* were calculated as detailed in the *Materials and Methods* section 4.2.11.

The variation of reaction time for nitration with TNM displays a similar effect on the α -LA *NDs* as for nitration with $\text{NaNO}_2/\text{H}_2\text{O}_2/\text{FeCl}_2$ and NONOate (Figure 13.a, black, pink and red lines, respectively). The *ND* values for these nitrating reagents rose quickly to 2-5 % after 30 minutes of reaction, while the further *ND* increase between 60 and 240 min was far more modest. It resulted in the *NDs* ranging between 7.36 and 8.05 % after 240 min of reaction. As expected from UV-Vis data, electrochemical nitration demonstrates a different behavior compared to the other nitrating agents (Figure 13a, green line). The *ND* increases linearly ($R^2 = 0.98$) with the reaction time, corresponding to a steady-state reaction at the electrode. A maximum of 11.98 % was obtained for electro chemical nitration after a 4-hour reaction.

Based on nanoESI-HRMS results, the four catalysts used for the $\text{NaNO}_2/\text{H}_2\text{O}_2$ nitrating procedure resulted in *ND* ranging from 3.23 to 5.06 % (Figure 14, black color). Using UV-Vis spectroscopy, the *ND* for hemin was not measured due to the interference of hemin probably forming a hemin/ α -LA complex. The deconvolution of MS spectra of nitrated products obtained from hemin-mediated nitration of α -LA displayed no such complex and the *ND* was calculated at 3.35 %. It

could be possible that α -LA and hemin form a non-covalent complex that dissociates in the MS source during ionization process.

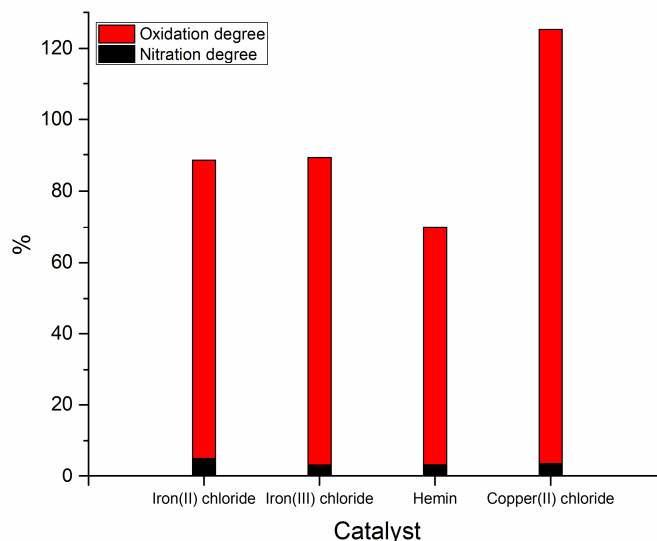


Figure 14. NDs and ODs of α -LA measured by nanoESI-HRMS after reaction with $\text{NaNO}_2/\text{H}_2\text{O}_2$ with various catalysts. The molar ratios of $\text{H}_2\text{O}_2:\text{NaNO}_2:\text{Tyr}$ residues was set at 30:30:1 with a reaction time of 1 h. The catalysts were added to yield a final concentration of 25 μM . The ND and OD were calculated as detailed in the *Materials and Methods* section 4.2.11.

As was already mentioned above, oxidation is a known modification induced during the chemical nitration of proteins, however its degree cannot be evaluated by UV-Vis spectroscopy. In contrast, ESI-HRMS allows the identification and relative quantification of the oxidative sub-products of nitration. For all samples, the oxidation degree (OD) was calculated by taking into account the relative abundances of all species exhibiting at least one oxidation, as described in the *Materials and Methods* section 4.2.11.

As expected, nitration using sodium nitrite and hydrogen peroxide induces the highest ODs (Figures 14, red color, and 15.a and b, pink line) due to the uncontrolled Fenton-like reaction. In this case, the metal center of the catalyst interacts with hydrogen peroxide to release hydroxyl radicals, responsible for the protein oxidation and for the production of nitrite and tyrosine radicals, which combine themselves to form NTyr. Oxidation was found to be relatively limited with hemin as a catalyst (OD = 66.71 %), whereas the tested metal salts increased greatly the OD with values as high as 121.55 % for copper (II) chloride (Figure 14, red color). With a $\text{NaNO}_2/\text{H}_2\text{O}_2/\text{Tyr}$ residues molar ratio of 30:30:1 and iron (II) chloride as a catalyst, oxidation of α -LA is extensive

with *OD* values between 85 and 98 %, even for a short reaction time of 30 min (Figure 15.b, pink line). Decreasing this ratio to 1:1:1 resulted still in a 4-fold increase in *OD* compared to the blank samples with 56.21 vs 13.79 %, respectively, as shown in Figure 15.a, pink line.

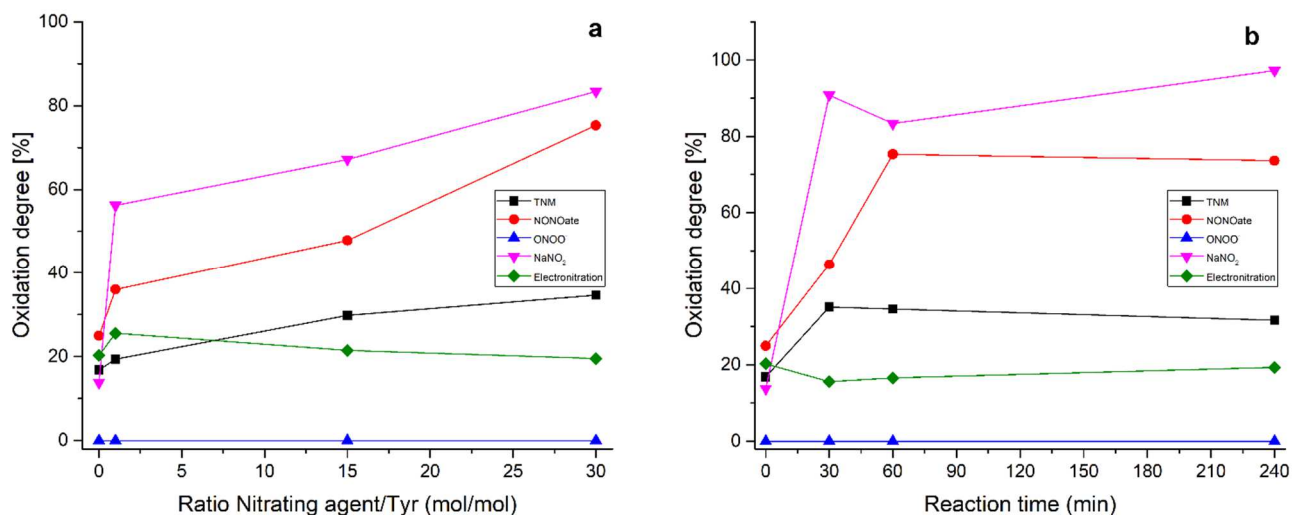


Figure 15. *ODs* of α -LA measured by nanoESI-HRMS after reaction with different nitrating agents for various (a) molar ratios of nitrating agent:Tyr ranging from 0:1 to 30:1 with a reaction time of 1 h and (b) reaction times from 0 to 240 minutes with a fixed molar ratio of 30:1. Iron (II) chloride was used as the catalyst for the $\text{NaNO}_2/\text{H}_2\text{O}_2$ nitration procedure. The *OD* was calculated as detailed in the *Materials and Methods* section 4.2.11.

As shown in figure 4, the degradation of NONOate results in the direct release of nitric oxide (NO^\cdot) in solution. This radical is a powerful oxidant and give rise to various reactive nitrogen species (RNS) responsible for protein oxidation and nitration. (75) Under the various reaction time and reagents tested, the *OD* determined by HRMS was considerable (36 to 75 %, Figure 15, a and b, red lines) but remained smaller than with the $\text{NaNO}_2/\text{H}_2\text{O}_2$ system. This difference could be also observed from the corresponding MS spectra as displayed on Figure 16.a and b. For both nitration systems, 6 charges states ranging from $[\text{M}+7\text{H}]^{7+}$ to $[\text{M}+12\text{H}]^{12+}$ were detected and isotopically resolved. The overall average charge state of all the deconvoluted spectra was 8.0 with a 0.3 standard deviation.

Meanwhile, TNM is considered a mildest alternative for nitration among tested chemical reagents, as it releases directly nitrogen dioxide radicals (NO_2^\cdot). Indeed, this reagent provided quite clean MS spectrum after nitration, as shown in Figure 16.c. With TNM:Tyr residues molar ratio of 30:1, the *OD* value was relatively stable for all reaction times tested, with values between 31 and 35 % (Figure 15.b, black line). Decreasing this ratio to 15:1 and 1:1 decreased the *OD* to

29.81 and 19.36 %, respectively (Figure 15.a, black line). These results show that the nitrogen dioxide radicals produced from TNM not only react with tyrosine residue but also with dissolved O₂ and various amino acids residues, still leading to some oxidative damages.

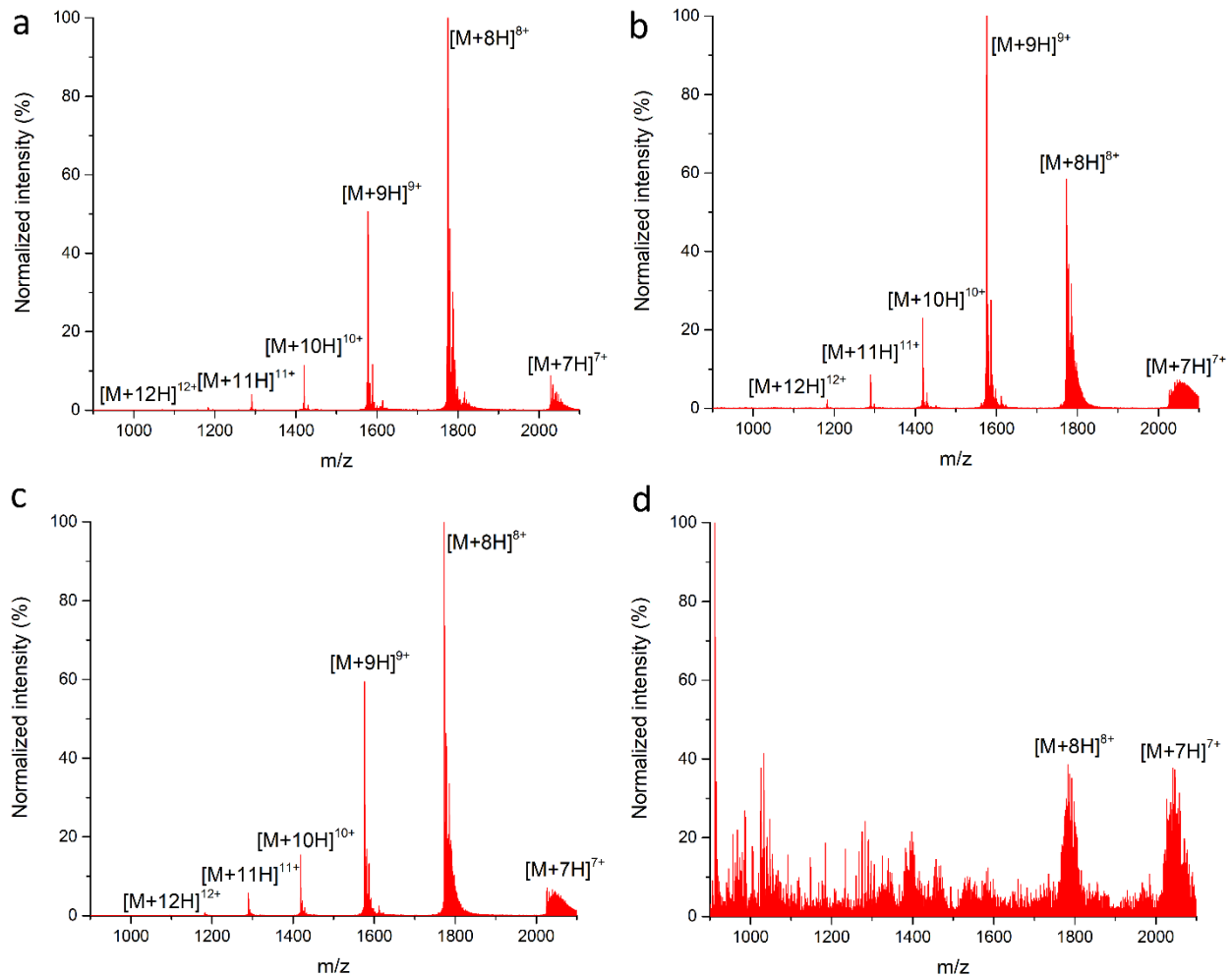


Figure 16. nanoESI-HRMS spectra of α -LA (5 μ M in ESI solution) after reaction with (a) NaNO₂ with iron (II) chloride, (b) NONOate, (c) TNM and (d) peroxynitrite with the detected charge states of α -LA nitration products. All reactions were performed with molar ratios of nitrating agent/Tyr residues of 30:1 for 1 hour. The MS spectra were measured by direct infusion using HRMS instrument with a resolution of 120'000 at 400 m/z, after purification of the samples using a centrifugation filtering device with a 3kDa molecular weight cut-off.

As was already mentioned in the section 4.3.6, nitration mediated by peroxynitrite resulted in extensive oxidative polymerization and partial sample losses. The soluble products were collected and analysed by nanoESI-HRMS as the other samples. The obtained MS spectra display a large number of degradation products as shown in Figure 16.d compared to other nitrating reagents tested (Figure 16.a-c) under the same conditions (nitrating agent:Tyr residues molar ratio 30:1, 1h reaction). Disregarding applied conditions, the software Xtract algorithm was not able to

deconvoluted MS spectra of peroxyxynitrite-mediated nitration reactions and the ND/OD values were therefore not determined. In addition, the very low MS signal intensity obtained demonstrated the overall loss of product due to the oxidative polymerization.

Electrochemical nitration resulted in the lowest detected OD values (Figure 14.a and b, green line) compared to the other nitration methods. In fact, the amount of oxidized products present in the initial sample does not increase during the electrochemical nitration process. The OD values were ranging from 16 to 21 % under all conditions tested, being in the same range as for the native α -LA samples. Resulting MS spectra were also very similar to native α -LA samples with distinct peaks of nitrated protein (Figure 17). These results demonstrate the high selectivity of electrochemical nitration obtained by applying the correct potential to the system of 850 mV. Higher potentials (≥ 1 V) might result in oxidation of other amino acids residues and oxygen evolution at the electrode.

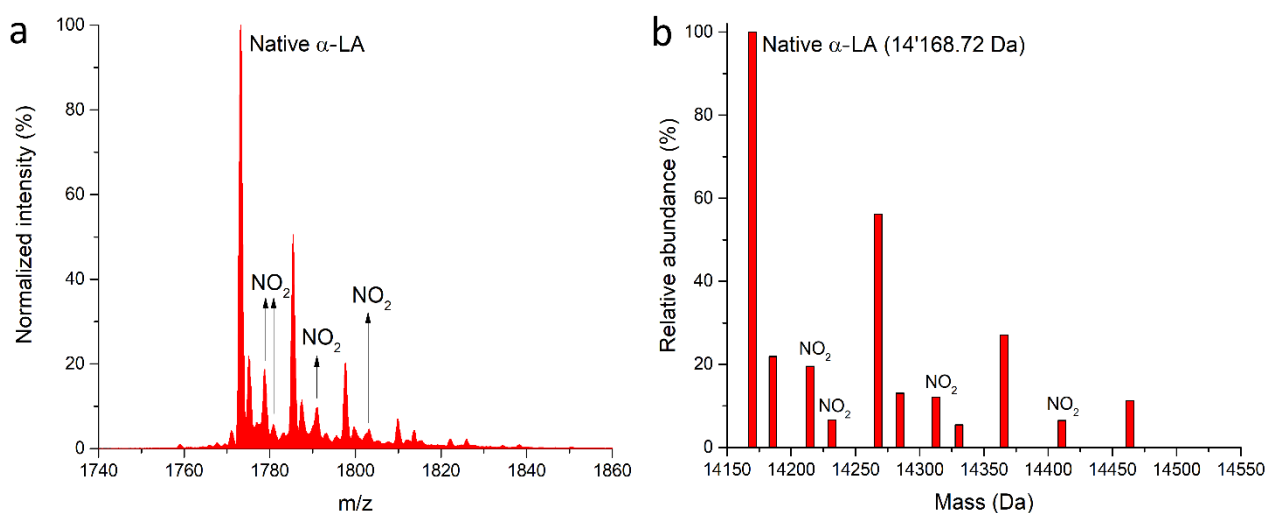


Figure 17. (a) nanoESI-HRMS spectrum of α -LA (5 μ M in ESI solution) showing the $[M+8H]^{8+}$ proteoforms and (b) the corresponding deconvoluted spectrum after electrochemical nitration. The reaction was performed with molar ratios of nitrating agent:Tyr residues of 30:1 for 1 hour with a 850 mV applied potential. The HRMS spectrum was measured by direct infusion using HRMS instrument with a resolution of 120'000 at 400 m/z , after purification of the samples using a centrifugation filtering devices with a 3kDa molecular weight cut-off. The deconvolution was carried out using the Xtract algorithm within Protein Deconvolution 4.0 with parameters as listed in *Materials and Methods* section 4.2.11. The nitrated proteoforms are labeled and all identified α -LA proteoforms are listed in Table 1.

Overall, protein nitration was found to be best performed by TNM and the electrochemical nitration procedure. They both present high ND and no or limited unwanted oxidation, compared to the other nitrating agents, thus they were chosen for the nitration of more complex protein samples as discussed below.

4.3.9 Nitration of milk proteins and milk natural extracts

After choosing the most suitable nitrating reagents on the example of α -Lac, various allergenic proteins and their extracts were nitrated with TNM and by electrochemical nitration. Then, obtained reaction mixtures were analyzed by bottom-up proteomic approach in order to identify the nitrated tyrosine residues and compare them to the known epitopes of these proteins. Such precise localization and identification of nitrated tyrosine residues in an allergen is helpful to understand its structure and can help to predict its chemical and allergenic properties.

First, pure milk proteins and milk fractions were nitrated using TNM, with a 30:1 molar ratio of nitrating agent:Tyr residue and a 1-hour reaction time. After purification and digestion of the resulting proteins, a standard bottom-up proteomic (BUP) approach was carried out. Detailed tables containing the complete BUP results including protein sequence coverage and detected native or nitrated Tyr residues, for each tested allergenic proteins and extracts, containing also non-allergenic proteins, are present in Appendix Tables I and II. It is important to note that the initial methionine and the potential signaling peptides were not taken into account in the numbering of the amino acid residues in this work.

For pure milk allergens, the high protein sequence coverages obtained in the BUP analysis allowed the identification of all the theoretically known Tyr residues in their native or nitrated forms. α -LA, β -lactoglobulin (β -LG), bovine serum albumin (BSA), κ -casein and β -casein were identified with sequence coverages of 100, 100, 94.5, 84.2 and 71.9 %, respectively (Appendix, Table I). As the purity of the commercial allergens is not always guaranteed by the manufacturer, various proteins were also nitrated and consequently detected alongside the target proteins. As these proteins are present in trace amounts in the commercial samples, not all of their Tyr residues were detected. For the milk protein extracts, as expected, protein sequence coverages in the casein and whey fractions were lower than for pure proteins due to the complexity of the sample matrices (Appendix, Table II). However, the use of natural extracts opens the possibility to detect the nitration sites in all proteins composing the corresponding milk fraction, including for example lactadherin and lactoperoxidase for casein fraction, providing valuable full picture of nitration effect on milk and its components. All identified nitration sites of the known milk allergens within milk fractions are summarized below in Table 2.

Table 2. Milk allergens and their Tyr residues detected after milk fractions nitration by TNM^(a) and identification of the nitration sites by BUP approach.

Protein	UniProt N°	Total Tyr	Detected NTyr ^(b)	Detected Tyr ^(b)
α-LA	P00711	4	3: Y18, Y36, Y103	4: Y18, Y36, Y50, Y103
β-LG	P02754	4	4: Y20, Y42, Y99, Y102	4: Y20, Y42, Y99, Y102
BSA	P02759	20	19 : Y30, Y84, Y137, Y147, Y149, Y155, Y156, Y160, Y262, Y318, Y331, Y333, Y340, Y352, Y369, Y400, Y410, Y451, Y496	14 : Y30, Y84, Y139, Y147, Y262, Y318, Y331, Y333, Y340, Y352, Y369, Y400, Y451, Y496
Lactoferrin	P24627	21	3: Y166, Y657, Y665	6: Y166, Y189, Y433, Y657, Y660, Y665
β-Casein	P02666	4	4: Y60, Y114, Y180, Y193	3: Y60, Y114, Y193
αS1-Casein	P02662	10	10 : Y91, Y94, Y104, Y144, Y146, Y154, Y159, Y165, Y166, Y173	10 : Y91, Y94, Y104, Y144, Y146, Y154, Y159, Y165, Y166, Y173
αS2-Casein	P02663	12	7: Y78, Y89, Y95, Y98, Y100, Y179, Y203	7: Y89, Y95, Y98, Y100, Y179, Y203 Y206
κ-Casein	P02668	9	9: Y25, Y30, Y35, Y38, Y42, Y43, Y58, Y60, Y61	9: Y25, Y30, Y35, Y38, Y42, Y43, Y58, Y60, Y61

(a) TNM/Tyr ratio of 30 (mol/mol) and reaction time of 1h

(b) Tyrosine residues not listed in detected NTyr/Tyr columns were not detected in the experiments

As can be seen from Table 2, milk fractions reactions with TNM resulted in extensive nitration of Tyr residues. For the major milk allergens, all tyrosine residues were detected except for α-S2 casein and lactoferrin, potentially due to their lower concentrations in natural extracts. In most of the cases, TNM was able to nitrate almost all Tyr residues, even the ones deeply buried in the tertiary structure of the proteins. For example, all Tyr residues of β-LG (Y₂₀, Y₄₂, Y₉₉ and Y₁₀₂) were detected with and without nitro groups. The SASA of Y₁₀₂ was calculated to be 2.1 Å² and is therefore almost inaccessible to solvent and chemicals compared to residues Y₂₀, Y₄₂ and Y₉₉ with respective SASA of 150.9, 48.2 and 47.2 Å². For BSA, on one hand only the tyrosine residue Y₁₃₉ was not detected as a nitrated species and has a calculated SASA of 0 Å². On the other hand, Y₁₄₉, Y₃₃₁ and Y₃₅₂ have also a SASA of 0 Å² but were, nevertheless, detected in their native and nitrated forms. Moreover, for this protein not all Tyr were detected in their native form, while nitrated one was clearly present. For α-LA, nitration of Y₅₀ was not detected despite the calculated SASA for this residue (7.2 Å²) potentially due to the loss of some peptides during BUP analysis, a quite common limitation of this approach. These results show the high TNM efficiency for the nitration of proteins and its poor Tyr selectivity. Measurements of SASA may provide useful information but is

not enough to predict the position of nitrated residues in a given protein especially with such powerful nitrating reagent. In addition, such calculations require available and complete 3D structures with decent resolutions. Despite numerous studies on milk allergens, not all allergens have been completely characterized. The results for all the SASA calculations for known milk allergens having an available 3D structure on the PDBe servers (44) are displayed in Appendix Table III.

The same milk proteins and extracts were also nitrated electrochemically with a NaNO_2/Tyr molar ratio for 30:1 for 4 hours. The detected allergens and nitration sites are summarized in Table 3, whereas the detailed analyses results are detailed in Appendix Tables IV and V.

Table 3. Milk allergens and their Tyr residues detected after milk fractions electrochemical nitration ^(a) and identification of the nitration sites by BUP approach.

Protein	UniProt N°	Number of tyrosine	Detected NTyr ^(b)	Detected Tyr ^(b)
α -LA	P00711	4	1: Y18	2: Y18, Y103
β -LG	P02754	4	2: Y42, Y99	4: Y20, Y42, Y99, Y102
BSA	P02759	20	4: Y84, Y160, Y262, Y340	6: Y84, Y262, Y340, Y352, Y400, Y496
Lactoferrin	P24627	21	0	3: Y523, Y534, Y526
β -Casein	P02666	4	3: Y114, Y180, Y193	1: Y193
α S1-Casein	P02662	10	4: Y91, Y94, Y104, Y173	7: Y91, Y94, Y144, Y146, Y154, Y159, Y166
α S2-Casein	P02663	12	4: Y78, Y89, Y179, Y203	4: Y78, Y89, Y100, Y179
κ -Casein	P02668	9	1: Y61	1: Y61

(a) NaNO_2/Tyr ratio of 30 (mol/mol) and reaction time of 4h

(b) Tyrosine residues not listed in detected NTyr/Tyr columns were not detected in the experiments

The sequence coverages obtained for these experiments were relatively low compared to the previous ones using TNM as a nitrating agent. Individual protein digestion yielded sequence coverage as low as 21.4 % for electrochemically nitrated β -casein, whereas values ranging between 60 and 100 % would be expected for pure protein solutions. While normally for natural extracts, lower sequence coverages and decreased numbers of detected Tyr residues compared to pure protein solutions, are expected due to the complexity of the samples, in case of electrochemical nitration the situation was reversed (Appendix Tables IV and V). In both cases, the limited number of detected peptides did not always allow the identification and characterization of all

tyrosine residues. Several repetitions of the digestion procedures with similar and modified parameters did not result in any results improvements. The search parameters for the BUP data analyses were also modified to include: the oxidations of methionine, cysteine, lysine, and proline residues; the nitration of tyrosine, tryptophan, cysteine, and methionine residues; the reduction of all potential nitro groups to nitroso or amine groups. Despite the extended list of allowed modifications, no better sequence coverages were obtained for the digestion of electrochemically nitrated milk proteins and extracts. Unpredicted influence of reaction conditions of electrochemical nitration on protein loss or digestibility, an undetected residue modification or the large number of nitro groups present on the proteins may be responsible for the limited digestion and poor peptide detection observed. Further investigation of this issue with digestion efficiency after electrochemical nitration is required but lies out of the scope of the current work.

β -LG is the only milk allergen for which all the tyrosine residues have been identified in this case (Table 3). All of the four Tyr residues (Y₂₀, Y₄₂, Y₉₉ and Y₁₀₂) were detected in their native form, whereas only Y₄₂ and Y₉₉ were also found as nitrated species. Despite the fact that Y₂₀ is more exposed in the β -LG tertiary structure compared to the other tyrosine residues, it was not detected in a nitrated form. The conditions required for the electrochemical nitration are usually considered as mild compared to other nitrating agents and more selective what could explain the absence of nitrated form of Y₂₀, if simple target peptide loss during BUP experiment is ruled out. All of the nitrated tyrosine residues detected in these samples were also present in the TNM-mediated nitration experiments.

4.3.10 Comparison of detected nitration sites in milk proteins with previously reported data

The nitration of milk proteins was the aim in various previous studies. Bovine α -LA was nitrated by Denton W. and Ebner K. in order to study the effect of tyrosine modification on the lactose synthetase activity of α -LA. (76) They performed the nitration of α -LA with a 64 molar excess of TNM and showed that the number of nitrated tyrosine increases with time. After 250 minutes, the four tyrosine residues were modified but only two of them were nitrated. Formation of polymers was also observed in their experiments, as expected for such high TNM amount compared to the protein. Klee and Klee showed already in 1972 that 3 of the four tyrosyl residues are nitrated by TNM in 20 to 30 minutes with lower molar ratios. (77) Due to the technologies at that time, no identification of the nitration sites was carried out. In another study, Prieels *et al.* examined the

nitration of human α -LA and observed a first nitration occurring at Y₁₀₃, followed by Y₁₈ at higher TNM concentrations (24 and 32 molar ratio TNM:Tyr residue). (56) Bovine and human α -LA are relatively similar with a 74 % identity calculated using the SIM tool (78) of the ExPASy server. (79) The positions of the four Tyr residues are conserved and the direct neighboring amino acids are identical except for Y₁₀₃ for which an aspartic acid residue in human protein is replaced by an asparagine in bovine one. Nevertheless, due to the difference between bovine and human α -LA sequences resulting in variations of the tertiary structure and the chemical environment of the Tyr residues, a direct comparison of the human and bovine proteins should be done carefully. In our experiments, only Y₁₈ was nitrated after the electrochemical procedure (Table 2), whereas Y₁₈, Y₃₆ and Y₁₀₃ were all susceptible of nitration when exposed to TNM (Table 3) similar to Prieels *et al.* results.

Amoresano *et al.* recently developed a new approach for the identification of nitrated proteins in complex sample mixtures. (80) They detected the presence of nitrated β -LG in TNM-treated milk using mass spectrometry and identified Y₄₂ and Y₉₉ as the two NTyr present in the proteins. Electrochemical nitration of β -LG (Table 3) yielded the same nitrated residues, whereas TNM-mediated nitration (Table 2) showed that all Tyr residues were susceptible of being nitrated under the chosen conditions. Interestingly, Diesner *et al.* showed that the TNM-nitration of β -LG has a marked impact on anaphylactic reaction compared to native β -LG. (19) However, they did not identify the position of the nitration in the sequence of this allergen.

BSA is often used as model protein for nitration studies (47,81) as it is well characterized and relatively inexpensive compared to other proteins. Nevertheless, identification of the nitration sites requires expensive instruments and is not often carried out. Walcher *et al.* showed that at least 16 (Y₃₀, Y₁₃₇, Y₁₄₇, Y₁₄₉, Y₁₅₆, Y₁₆₀, Y₂₆₂, Y₃₁₈, Y₃₃₁, Y₃₃₃, Y₃₅₂, Y₃₆₉, Y₄₀₀, Y₄₁₀, Y₄₅₁, Y₄₉₆) of the 20 tyrosine residues were nitrated by TNM, whereas only 3 are modified after exposition to nitrogen dioxide and ozone. (82) Zhang *et al.* carried out the nitration of BSA by TNM and located 17 of the Tyr residues present in the proteins. (26) Among them, ten NTyr residues (Y₁₃₇, Y₁₄₇, Y₁₄₉, Y₁₅₆, Y₂₆₂, Y₃₃₃, Y₃₄₀, Y₄₀₀, Y₄₅₁, Y₄₉₆) were identified. In each study, the Tyr residues coverage was not complete and some of them were not detected. In the present study, tryptic peptides containing all the tyrosine residues were identified and three more additional NTyr (Y₈₄, Y₁₅₅, Y₁₆₂) were detected bringing the number of nitration sites to 19 (Table 2). Differences with previous works in the nitra-

tion sites detected are explained by the variations in the nitration methods and performed MS analyses.

It is interesting to note that BSA possesses a region rich in Tyr residues, with 6 of them present in the sequence region AA 137-160. In current work, various peptides were detected for this region with different combinations of native and nitrated Tyr residues. The peptide RHPYFYAPELLYYANKY containing 5 Tyr residues was detected by BUP analysis with varying possible nitration positions, one among them being completely nitrated as shown in corresponding MS/MS spectrum in Figure 18. In the present case, proximity of already nitrated residues does not block the successive nitration of neighboring positions. It was previously suggested that a negatively charged amino acids (glutamate, E, and aspartate, D) placed in the Tyr residue vicinity or preceding it may promote the nitration. (83,84) Hence, it is possible that the nitro group, globally neutral but bearing a negative charge delocalized on both oxygen atoms, influences the nitration in the same way as glutamate and aspartate. In addition to the Tyr position in the primary structure, it was suggested that the secondary and tertiary structure have more impact on the nitration efficiency. Tyr residues present in loops and away from disulfide bond are expected to be easier to nitrate. (26,83,84) The peptide RHPYFYAPELLYYANKY is present in a loop and is readily nitrated, despite having small SASA values ranging from 0 to 26 Å². This example shows that prediction of nitration sites is not an easy task as it depends on multiple parameters.

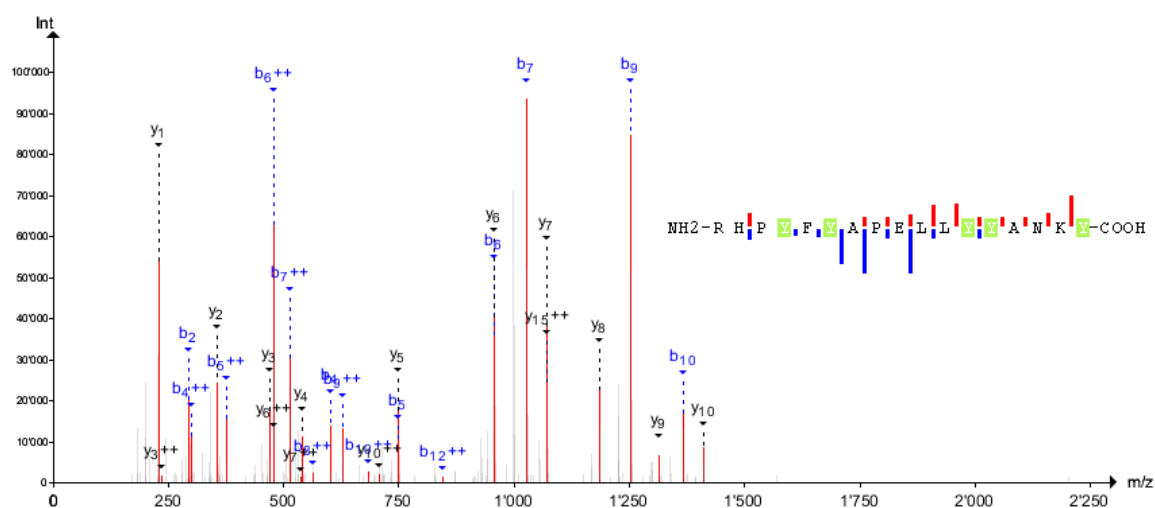


Figure 18. MS/MS spectrum of the peptide RHPYFYAPELLYYANKY obtained during the BUP analysis of pure BSA after a 1-hour nitration using TNM with a molar ratio of TNM:Tyr residues of 30:1. The parent ion was detected at m/z 811.68 with a +3 charge and m/z 609.01 with a + 4 charge. B and Y ions are displayed, respectively, by the blue and red lines in the peptide sequence representation whereas nitrated tyrosine residues are highlighted in light green.

To the best of our knowledge, nitration sites in lactoferrin and α S1-, α S2- and κ -casein were never identified previously. A few studies reported the nitration of these proteins without identifying the modification sites, as it was not relevant to the study or not easily feasible at the time. (85–88) Thus, nitration sites discovered here (Table 2 and 3) are reported for the first time for these allergens. For β -casein, Amoresano *et al.* reported Y₁₈₀ as a nitration site, (80) also detected within current study. However, they did not specify if the other tyrosine residues of β -casein were not detected at all or were identified in their native forms only.

4.3.11 Nitration of egg proteins and egg white extract

Pure egg proteins and an extract from hen egg white were submitted to the same nitration reactions and BUP analyses as described above for milk proteins and milk fractions. The detected nitration sites for TNM-mediated reaction are summarized in Table 4 whereas the detailed results are present in Appendix Tables VI and VII.

Lysozyme C was well characterized with sequence coverage of 87.76 % (Appendix Table VI). The three present Tyr residues (Y₂₀, Y₂₃ and Y₅₃) were all detected in their native forms as well as nitrated ones. For ovomucoid, containing 6 Tyr residues, only the residue Y₇₃ was not detected. Among all the other Tyr residues, only Y₁₆₁ was not susceptible of being nitrated. Compared to the other proteins, ovoinhibitor displayed a large number of Tyr residues observed only in nitrated forms and not detected in their native states (Y₈, Y₄₆, Y₁₁₁, Y₃₅₄, Y₃₆₅, Y₄₁₃). While for ovalbumin, only four Tyr residues (Y₂₉, Y₉₇, Y₁₁₁, Y₁₁₇) were detected out of the ten ones present in the protein sequence. Moreover, all these residues were identified only in their nitrated form. Similar situation was also observed for ovotransferrin with only one Tyr residue identified among 21 theoretical ones and only in its nitrated form, however in this case mainly due to its low abundance in the egg white extract.

Table 4. Egg proteins, pure or from egg white extracts, and their Tyr residues detected after nitration by TNM^(a) and identification of the nitration sites by BUP approach.

Protein	Uniprot N°	Total Tyr	Detected NTyr ^(b)	Detected Tyr ^(b)
Lysozyme C	P00698	3	3: Y20, Y23, Y53	3: Y20, Y23, Y53
Ovomucoid	P01005	6	4: Y37, Y46, Y102, Y141	5: Y37, Y46, Y102, Y141, Y161
Ovoinhibitor	P10184	19	11: Y8, Y46, Y111, Y139, Y168, Y205, Y336, Y354, Y365, Y413, Y424	8: Y37, Y102, Y139, Y168, Y205, Y234, Y336, Y424
Riboflavin-binding protein	P02752	9	3: Y3, Y53, Y89	2: Y3, Y107
Ovotransferrin	P02789	21	1: Y483	0
Ovalbumin	P01012	10	4: Y29, Y97, Y111, Y117	0

(a) TNM/Tyr ratio of 30 (mol/mol) and reaction time of 1h

(b) Tyrosine residues not listed in detected NTyr/Tyr columns were not detected in the experiments

The same egg proteins and extracts were nitrated electrochemically using a 30:1 molar ratio of NaNO₂:Tyr residue and a 4-hour reaction time. Identified nitration sites are displayed in Table 5 with detailed results listed in Appendix Tables VIII and IX. As previously obtained for the electrochemical nitration of milk proteins, low digestion efficiency was observed with coverage sequence ranging from 15 to 77 %. For example, only two and one Tyr residues were detected for ovalbumin and ovomucoid, respectively, and none of them as NTyr. Lysozyme C is the only electronitrated egg protein for which a complete Tyr residues mapping was obtained. On the three Tyr present in the protein, Y₂₃ and Y₂₀ were both nitrated and Y₂₀ was also identified in its native form.

Table 5. Egg proteins, pure or from egg white extracts, and their Tyr residues detected after nitration by electrochemistry^(a) and identification of the nitration sites by BUP approach.

Protein	Uniprot N°	Total Tyr	Detected NTyr ^(b)	Detected Tyr ^(b)
Lysozyme C	P00698	3	2: Y20, Y23	2: Y20, Y53
Ovomucoid	P01005	6	0	1: Y37
Ovotransferrin	P02789	21	3: Y71, Y92, Y580	4: Y71, Y431, Y430, Y521
Ovalbumin	P01012	10	0	2: Y29, Y42

(a) NaNO₂/Tyr ratio of 30 (mol/mol) and reaction time of 4h

(b) Tyrosine residues not listed in detected NTyr/Tyr column were not detected in the experiments

4.3.12 Comparison of detected nitration sites in egg proteins with previously reported data

As for milk proteins, a few studies focused on the nitration of egg proteins and allergens. Changes in structure and/or loss of activity upon nitration were in general the goal of these studies and the nitrated sites identification was not carried out in most of the cases. For example, Curry-McCoy *et al.* used peroxyxynitrite to nitrate lysozyme C in order to study its degradation properties and function upon modification. (89) They observed the formation of nitrated protein adducts resulting in a decrease of catalytic activity and an increase in degradation by proteasome. On the other hand, Vaz *et al.* used peroxyxynitrite and myeloperoxidase to nitrate lysozyme C *in vitro* and analyzed the tryptic hydrolysates to identify the modification sites. (90) Y₂₃ was shown to be modified to a higher extend than Y₂₀, whereas Y₅₃ was not nitrated. These results were similar to those obtained by Cooper *et al.* in a series of publications in which they carried out the nitration of lysozyme C electrochemically. (34,39,91) They identified Y₂₃ as the main nitration site, followed by Y₂₀. Triple nitration was not observed in their experiments. In the current work, TNM nitration of lysozyme C also led to the formation of nitrated Y₅₃ (Table 4) whereas electrochemical nitration yielded results in accordance with previously cited studies. The wider extend of nitration in TNM-mediated reactions may be credited to its higher and uncontrolled liberation of radicals compared to the electrochemical method. In addition, Y₂₀ and Y₂₃ are more readily accessible to solvents and chemicals compared to Y₅₃, confirmed by the calculated SASA of 62.3, 46.1 and 25.1 Å², respectively (Appendix Table III).

Ovomucoid is a common egg allergen and its nitration by TNM was performed in various studies. (19,92–95) In these articles, nitration was carried out to understand the structural and chemical properties of the protein, (93–95) to identify the IgE/IgG binding sites on an ovomucoid fragment, (92) and to study the allergenicity of the native and nitrated ovomucoid in mouse model. (19) The nitration of ovomucoid was showed to have no impact on its allergenicity. (19) No complete identification of the nitration sites was carried out to the best of our knowledge for this protein. Similarly, ovoinhibitor was never nitrated and no modification positions were identified.

Ovotransferrin is a relatively large protein (77.9 kDa) from the iron-binding glycoproteins family. This protein is able to bind reversibly two Fe³⁺ ions and two CO₃²⁻ anions, and is known for its antibacterial activity. (96) The identification and characterization of the iron-binding regions were carried out by modifying chemically the protein and measuring its Fe-binding activity. TNM-

mediated nitration of the protein allowed the determination of the Tyr residues involved in the iron ion stabilization. The first study on this subject was carried out by Tsao *et al.* (97) but was limited by the fact that the complete protein sequence was deduced from mRNA only later by Jeltsch and Chambon. (98) In 1982, J. Williams showed that all the 21 Tyr residues of iron-free ovotransferrin were potentially nitrated by TNM in the following conditions: 10-fold molar excess of TNM and 3 days of reaction at room temperature. The protection of ten Tyr residues (Y₄₂, Y₈₂, Y₉₂, Y₁₈₈, Y₁₉₁, Y₃₁₉, Y₄₁₅, Y₄₃₁, Y₅₂₁, Y₅₂₄) against nitration was observed due to their binding to the Fe³⁺ atom or conformation changes. (99) Recent studies confirmed the presence of Y₉₂, Y₁₉₁, Y₄₃₁ and Y₅₂₄ in the iron binding pockets of ovotransferrin. (96,100) In the present study, ovotransferrin was detected in egg white extract but was not nitrated as a pure protein. For this reason, the identification of all tyrosine residues was not possible. Among the detected Tyr residues, Y₇₁, Y₉₂, Y₄₈₃ and Y₅₈₀ were observed as nitrated residues after treatment with TNM or after electrochemical nitration in the presence of sodium nitrite (Table 4 and 5). As both apo and holo form of ovotransferrin are present in egg white, nitrated Y₉₂ was detected despite its presence in the list of protected residues.

Ovalbumin is the main constituent of egg white but its functions are unknown. It belongs to the serpins protein family but does not possess the inhibitory properties of standard serpins towards serine proteases. It is believed to act as a storage protein. (101,102) Gruijthuisen *et al.* nitrated ovalbumin using TNM and used the nitrated protein to determine its immunogenic and allergenic properties. An increase of allergenicity of TNM-nitrated ovalbumin in mice models was showed compared to the native protein. (4) A few years ago, Yang *et al.* used ovalbumin to study the kinetics of TNM-mediated nitration by HPLC coupled to a diode array detector of UV-Vis absorption. (47) In their recent work, Hochscheid *et al.* demonstrated the potential of nitrated ovalbumin to trigger allergic reaction in rodent models. (103) In these studies, the nitration procedures were very similar but no identification of the nitration sites was carried out. To the best of our knowledge, the only study aiming to identify ovalbumin nitration sites is the work of Zhang *et al.* (26) They identify five Tyr residues (Y₉₇, Y₁₁₁, Y₁₂₅, Y₂₁₂ and Y₂₈₁) in the native and nitrated states, two unnitrated ones (Y₁₀₆ and Y₁₁₇), whereas they were not able to detect Y₂₉, Y₄₂ and Y₂₉₁. As in their research, current experiments were not able to identify the complete set of tyrosine residues present in ovalbumin. Although, it was possible to identify both Y₂₉ and Y₄₂ and show that Y₂₉ may be nitrated by TNM, Y₄₂ was also found in its native form (Table 4). Y₁₁₇ was also amongst the list

of nitrated tyrosine residues. Variation in the nitration sites is mainly dependent on the nitration reaction, digestion and analysis parameters. Nevertheless, these results display the large nitration potential of ovalbumin. Additional experiments and optimization in the various methods are required to fully identify the nitration sites of ovalbumin, including Y₂₉₁ not detected in any of the studies.

4.3.13 Position of nitration sites with respect to known epitopes

The IgE epitope is defined as the specific antigenic determinant site on a given protein, recognized by the paratopes of IgE antibodies and contributing to its allergenicity. (104,105) Identification of the IgE epitopes structures is important for the understanding of allergies, the prediction of protein allergenicity, development of new accurate diagnosis strategies, immunotherapy, and the production of hypoallergenic foods. (106–109) Epitopes have been classified as linear (consecutive sequence of amino acids), conformational (discontinuous amino acids brought into close vicinity due to the protein tertiary structure) and hybrid. (107) The identification of all epitopes of a protein, called epitope mapping, is most often carried out using X-ray crystallography, nuclear magnetic resonance, hydrogen-deuterium exchange, mass spectrometry, peptide-based approaches (based on ELISA, for example) or selective amino acids mutagenesis. (106,110) As determination of conformational epitopes is technically more complicated, the majority of the known IgE epitopes are linear ones.

Thanks to the progress in genomics and proteomics, the epitope-related data amount produced has increased drastically. Various databases have been created in the last decades to collect, organize and present epitope data in searchable forms. (108,111–115) Since 2004, the Immune Epitope Database (IEDB, iedb.org) aims to collect experimental data on B-cell and T-cell epitopes in over 20'000 peer-reviewed publications and makes it available and easily searchable on their website. (115) This database regroups epitope data from human and other species with all the experimental contexts and parameters. It also has the advantage of taking into account the positive and negative assay results obtained in the literature and calculating the response frequency by taking the number of patients in all studies. As different studies used complex peptide libraries with various but overlapping peptide sequences, IEDB reports all data and statistics to every amino acid residue. Herein, IEDB was used to compare the identified nitration sites obtained in this work and found in the literature to the known epitopes.

For small, common and well-known allergens, such as α -LA (123 amino acids) or β -LG (162 amino acids), epitope mapping has been extensively carried out using a large number of overlapping peptides. As these small proteins are one of the major milk allergens, the complete sequence is potentially recognized by IgE antibodies. For α -LA, six studies have been able to identify 70 peptides (out of 91 tested) displaying IgE-binding potential. (116–121) Among these epitopes, only two conformational/discontinuous epitopes (IEDB Epitope ID 97142 and 97143) were reported. (117) As the complete α -LA sequence was found to be potentially recognized by IgE antibodies, the calculation of the response frequency (RF) performed at the amino acid level allows to identify part of the sequence frequently recognized by the majority of allergic patients. Figure 19 displays the data exported from the IEDB database for α -LA corresponding to 144 assays (positive and negative) including each between 1 and 26 tested patients. The RF value, corresponding to the lower 95 % confidence interval calculated on IEDB, allows identifying four main IgE-binding regions with RF values superior to 0.2: AA 1-24, AA 46-58, AA 73-78, and AA 87-108 (Figure 19). Among the four Tyr residues (Y_{18} , Y_{36} , Y_{50} and Y_{103}) present in α -LA, Y_{36} has the lowest RF value of 0.15, whereas the three others are in region more often recognized by allergic patients (RF 0.27-0.37). Nitration was localized on residues Y_{18} , Y_{36} , and Y_{103} in the present study (Table 2 and 3) whereas only Y_{18} and Y_{103} were previously reported in the literature for the human α -LA. (56) Nitration and oxidation occurring at these positions have potentially a strong effect on IgE-binding and allergenicity as there are in region recognized by IgE antibodies of a majority of patients. Recently, Ackaert *et al.* carried out the nitration of the major birch pollen allergen Bet v 1 and found an increase in its allergenicity compared to the native allergen. (18) In this study, they determined by crystallography and HPLC-MS that Y_5 , Y_{66} , Y_{81} , Y_{83} and Y_{150} are the principal nitration sites in Bet v 1 after treatment with TNM. Three of those nitration positions (Y_{81} , Y_{83} and Y_{150}) are included in known IgE epitopes, (122–126) illustrating the potential relation between position of the nitrated residues and allergenicity modulation.

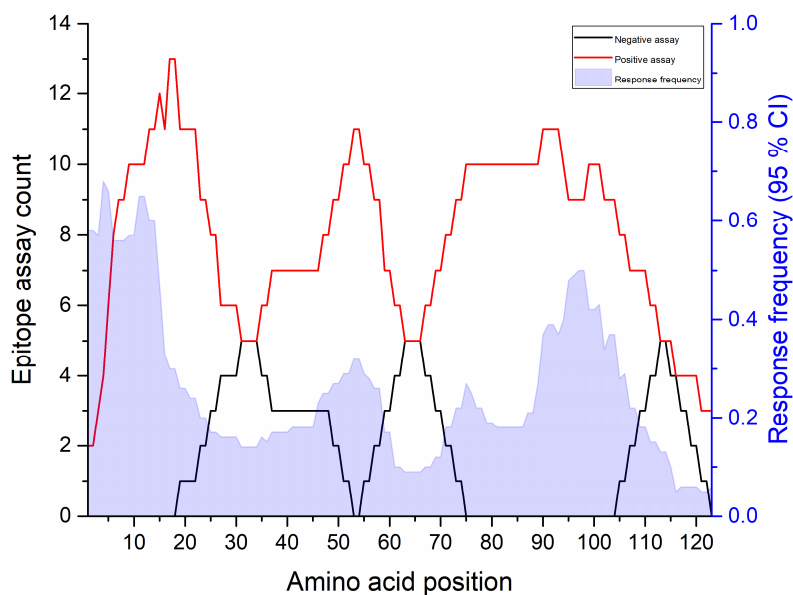


Figure 19. Number of IgE epitope assays carried out and calculated RF for each amino acid residues of α -LA. Data were exported from the IEDB servers (115) with the following search parameters: any epitopes structure, B cell assays, human host, allergic disease, no MHC restriction, limited to IgE epitopes.

Epitope data were also exported from the IEDB tools (115) for all available milk and egg white allergens (Appendix Figure I) as it was done above for α -LA in Figure 17. Positions of nitrated residues both identified in the present study and in the literature were compared to the known epitopes and the results are summarized in Table 6 and 7, for milk and egg allergens, respectively. In these tables, only the Tyr residues tested and reported in at least one study are included.

As was already mentioned above, the other small milk allergens, *i.e.* β -LG and the four caseins, have been also extensively studied and their complete sequence was tested for IgE epitopes determination (Appendix Figure I). Most of the identified NTyr residues are located in regions highly recognized by IgE antibodies with RF ranging from 0.20 to 0.84 (Table 6), with only β -LG having two Tyr residues (Y₂₀ and Y₄₂) with RF values below 0.2. In average, all the identified nitrated residues of these five proteins are present in epitope sequences, recognized by half of the allergic patients, thus, demonstrating the importance of understanding the role of nitration in allergenicity.

IgE epitopes of larger allergens, such as BSA and lactoferrin, have been less studied. It is mainly due to the size-related issues (harder handling/analysis and huge peptide libraries) and

their lower prevalence in cow's milk allergy. For example, BSA is the main allergen in beef allergy (127) but only 3.8 % of patients suffering from milk allergy are sensitive to it. (128) BSA epitopes have only been studied by Tanabe *et al.* using peripheral blood samples from beef allergic children. (129,130) They tested 20 peptides and 10 of them were found to be potential IgE epitopes, with two regions giving higher responses: AA 337-346 and AA 452-460. Y₃₄₀ is the only Tyr residue identified in the current work which is located in these two IgE determinant regions and was present in two tested peptides: HPEYAVSVLL and EYAV. According to the IEDB calculations, Y₃₄₀ has a response frequency of 0.83 based on 26 patients in 3 assays (Table 6). Introducing a chemical modification on such small epitope as EYAV changes drastically its properties and may strongly alter its IgE recognition and allergenicity. Meanwhile, lactoferrin epitopes have never been identified to the best of our knowledge.

Table 6. Identified nitration sites in milk allergens and their presence in IgE epitopes

Protein	UniProt N°	Nitration site (a)	Nitration site in literature (ref)	Number of negative assay (b)	Number of positive assay (b)	RF (b) (lower bound of 95 % CI)
α-LA	P00711	Y18	Y18 (56) (c)	0	13	0.3
		Y36	-	4	6	0.15
		Y103	Y103 (56) (c)	0	9	0.39
β-LG	P02754	Y20	-	10	12	0.16
		Y42	Y42 (80)	15	13	0.11
		Y99	Y99 (80)	15	11	0.25
		Y102	-	11	11	0.3
BSA	P02759	Y137	Y137 (26)	1	0	0
		Y155	-	1	0	0
		Y156	Y156 (26,82)	1	0	0
		Y160	Y160 (82)	1	0	0
		Y318	Y318 (82)	1	0	0
		Y340	Y340 (26)	0	3	0.83
		Y369	-	0	1	0.04
β-casein	P02666	Y60	-	3	13	0.5
		Y114	-	4	17	0.39
		Y180	Y180 (80)	3	10	0.68
		Y193	-	2	13	0.43
αS1-casein	P02662	Y91	-	4	24	0.34
		Y94	-	5	24	0.39
		Y104	-	7	13	0.21
		Y144	-	6	17	0.44
		Y146	-	4	20	0.46
		Y154	-	4	19	0.32
		Y159	-	4	19	0.34
		Y165	-	2	20	0.38

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		Y166	-	4	21	0.34
		Y173	-	2	25	0.42
αS2-casein	P02663	Y78	-	6	6	0.23
		Y89	-	3	10	0.64
		Y95	-	1	12	0.63
		Y98	-	1	11	0.51
		Y100	-	2	10	0.42
		Y179	-	7	7	0.67
		Y203	-	4	2	0.22
		κ-casein	P02668	Y25	-	4
Y30	-			4	12	0.74
Y35	-			4	16	0.73
Y38	-			4	14	0.73
Y42	-			4	12	0.64
Y43	-			4	12	0.61
Y58	-			1	14	0.83
Y60	-			0	14	0.84
Y61	-			0	15	0.83

- (a) Nitration sites identified after TNM-treatment or electrochemical nitration
 (b) Data obtained from IEDB servers (115)
 (c) Nitration sites identified in human α-LA

The four allergens identified in egg white are ovomucoid, ovalbumin, ovotransferrin and lysozyme C, whereas egg yolk contains different allergens including chicken serum albumin. (105,131,132) According to IEDB, only ovomucoid and ovalbumin have known IgE epitopes (Table 7 and Appendix Figure I.g-h). As for small milk allergens, peptide libraries corresponding to the complete ovomucoid sequence have been produced and used to identify the epitopes. More than one hundred epitopes have been determined in 6 different studies, (133–138) resulting in a total coverage of the protein sequence despite the low overall RF (0.03 to 0.31, Appendix Figure I.g). For this protein, two amino acid regions display a slightly higher prevalence for IgE binding capacity than the rest of the protein: AA 1-21 and AA 86-107. In the present study, among the four detected NTyr residues of ovomucoid, Y₁₀₂ is the only one present in those higher IgE-binding regions and may have a greater impact on allergenicity than other Tyr residues.

Table 7. Identified nitration sites in egg white allergens and their presence in IgE epitopes

Protein	UniProt N°	Nitration site ^(a)	Nitration site in literature (ref)	Number of negative assay ^(b)	Number of positive assay ^(b)	Response frequency ^(b) (lower bound of 95 % CI)
Ovomucoid	P01005	Y37	-	0	15	0.11
		Y46	-	1	16	0.10
		Y102	-	1	15	0.20
		Y141	-	2	12	0.07
Ovalbumin	P01012	Y29	-	7	0	0
		Y97	Y97 (26)	3	1	0.01
		Y111	Y111 (26)	6	0	0
		Y117	-	6	0	0
		-	Y125 (26)	6	0	0
		-	Y212 (26)	6	0	0
		-	Y281 (26)	6	0	0

(a) Nitration sites identified after TNM-treatment or electrochemical nitration

(b) Data obtained from IEDB servers (115)

Ovalbumin epitopes have been identified in four different studies according to the IEDB data. (139–142) The publications of Esayed *et al.* were focused only on part of the protein sequence: the N-terminus decapeptide, (139) AA 11-70 (141) and AA 323-339 (140). Mine and Rupa were the first to carry out the complete epitope mapping of ovalbumin using arrays of overlapping synthesized peptides. Despite a large numbers of negative assays, they identified five main regions of IgE epitope binding (AA 38-49, 95-102, 191-200, 243-248, 251-260) using the pooled blood sera of 18 different patients. (142) Those regions contain two Tyr residues (Y₄₂, Y₉₇), and only Y₉₇ was detected in a nitrated form in this work and in a previously published study. (26) More recently, Benedé *et al.* have identified the IgE epitopes of ovalbumin after digestion process with human and stimulated gastroduodenal fluids. (143) This study is not yet available on the IEDB data. In their work, they obtained 8 peptides, containing each approximately 10 amino acids, displaying an IgE binding potential. The higher RF was obtained for the peptide AA 370-385, to which 80 % of the tested blood sera from allergic patients were reacting. However, none of those 8 peptides contains a Tyr residue.

Considering ovotransferrin and lysozyme C, it is estimated that approximately 35 % of hen's egg allergic patient are allergic to one of these allergens. (144–146) To the best of our knowledge, ovotransferrin epitopes remain to be identified, despite the availability of its sequence and 3D-structure, (147) whereas lysozyme C IgE epitopes have been studied only once but those

data are not yet available on the IEDB. Jiménez-Saiz *et al.* produced peptides from lysozyme C after a simulated gastrointestinal digestion and identified their properties, such as their IgE binding potential. (148) Three peptide fragments were shown to be IgE epitopes: AA 11-27, 57-83 and 108-122. The first two Tyr residues of lysozyme C (Y₂₀ and Y₂₃) are susceptible of nitration, as shown in this work (Tables 4 and 5) and in the literature (34,39,90,91) and are also part of the first IgE epitope. Hence, mono- and di-nitration of the IgE epitope AA 11-27 may result in drastic allergenicity changes.

All in all, the position of nitration sites within various milk and egg white allergens has been compared to their known epitopes. Based on the available literature on allergen epitopes, no general patterns or amino acid sequences have been shown to have a higher IgE-binding potential. For small and common allergens, almost the complete protein sequence may be recognized by IgE antibodies. It is nevertheless possible to distinguish various regions of the protein sequences having higher RF values. For example, the central region of κ -casein (AA 82-94) displays lower IgE affinity (*i.e.* lower RF values) compared to the rest of the protein (AA 1-81 and 95-169) as shown in Appendix Figure I.f.

Tyr residues are present in several egg and milk allergen epitopes but they have not been observed to be more represented than other amino acids. On the one hand, the nitration of Tyr residues present in known epitope regions may result in a hindering of the IgE antibodies binding. On the other hand, the modification of non-IgE-recognized Tyr residues may result in partial unfolding or structural changes in the tertiary allergen structure resulting in the exposition of new epitopes. Performing the epitope mapping of native and nitrated allergens may yield important data regarding the effect of nitration on protein allergenicity. Complete studies using techniques such as X-ray crystallography or quantifying the affinity of IgE antibodies to nitrated proteins are required to understand fully the nitration impact. Nevertheless, the effect of allergen nitration is expected to change from one protein to another and vary for each patient due to the multitude of possible IgE paratopes.

4.4 Conclusions

Nitration of milk and egg white allergens has been studied using various chemical reactions and the nitration sites have been identified by nanoESI-HRMS in order to access the information about potential influence of nitration on allergenic properties of tested proteins. α -LA was

firstly used to compare the different *in vitro* nitrating methods available: NONOate, peroxyxynitrite, TNM, $\text{NaNO}_2/\text{H}_2\text{O}_2$ in the presence of a catalyst, and the electrochemical nitration in presence of NaNO_2 . Heterogeneous nitration using ozone and nitric oxide was not used in the present work. After the nitration and purification of α -LA, the nitration degree (*ND*) was determined for each experiment using UV-Vis spectroscopy, whereas nanoESI-HRMS was used to calculate both the nitration and the oxidation (*OD*). Under the chosen reaction conditions, TNM and the electrochemical nitration were found to yield a reasonable *ND* value with limited side reactions such as oxidation and cross-linking. Peroxyxynitrite treatment of α -LA resulted in a high level of nitration but induced extensive oxidative damages and polymerizations resulting in sample losses. NONOate and $\text{NaNO}_2/\text{H}_2\text{O}_2$ yielded low amounts of nitrated products while producing non-negligible side oxidations. The catalyst nature had low impact on the results in the nitration using sodium nitrite and hydrogen peroxide.

Based on these results, TNM and the electrochemical reaction were used to carry out the nitration of milk and egg allergens, as either pure solutions or natural extracts. After protein purification, the nitrated allergens were analyzed using a bottom-up proteomic (BUP) approach. TNM-mediated nitration was found to produce more nitrated sites compared to the electrochemical method. The obtained results for both nitration procedures are in partial agreement with previous studies; similar nitration sites were identified in some case, whereas new NTyr residues were discovered due to the different reaction conditions and analysis parameters. Rationalization and prediction of the modification sites is not straightforward as the nitration reaction involves the production and stabilization of various radicals. The calculation of solvent accessible surface area (SASA) was performed in order to give an idea of the potential modification sites but the obtained values do not always correspond to the experimentally identified Tyr residues. This may be explained by the partial unfolding under the chosen conditions, the flexibility of the proteins and the influence of neighboring amino acids. Additionally, the complete 3D structure of the protein is required in order to correctly perform the SASA calculations.

As allergen nitration is a suspected cause of the increase in prevalence of allergic diseases observed during the last decades, the literature was searched in order to compile the list of known milk and egg allergen epitopes and compare them to the identified nitration sites. It was found that Tyr residues are not more represented in epitopes than other amino acids and no general

sequence pattern was identified. Although, once Tyr residues identified as potential targets for nitration make part of the recognized epitope, their modification into NTyr can modulate positively or negatively the IgE antibodies binding and the proteins allergenicity. Thus, the characterization of various nitrating agents, confirmation of already known nitration sites and discovery of the new ones, conducted in the present work, bring useful information and novel insights to the problem of correlation between protein nitration and allergenicity. Additional studies using epitope mapping of native and nitrated allergens, X-ray crystallography of antibody-antigen complexes, and immune cell activation tests are required to understand completely the impact of nitration on allergenic properties of the proteins.

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4.6 Appendix

Table I. Detected milk proteins and their identified nitration sites after nitration of pure proteins by TNM (1h, molar ratio TNM/Tyr 30:1).

Allergen tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
α -LA	α -LA	100.00	Y18, Y36, Y103	Y18, Y36, Y50, Y103	
β -LG	β -LG	100.00	Y20, Y42, Y99, Y102	Y20, Y42, Y99, Y102	
	BSA	10.79	Y333, Y400		All others
β -Casein	β -Casein	71.92	Y60, Y114, Y180, Y193	Y114, Y193	
	α S1-Casein	66.00	Y91, Y94, Y144, Y146, Y154, Y159, Y165, Y166, Y173	Y91, Y94, Y144, Y146	Y104
	α S2-Casein	34.14	Y78, Y89, Y98, Y100, Y179	Y89, Y100, Y179	Y20, Y52, Y95, Y171, Y184, Y203, Y206
	Kappa-Casein	42.08	Y61		Y25, Y30, Y35, Y38, Y42, Y43, Y58, Y60
	β -LG	56.46	Y42, Y99	Y102	Y20
	α -LA	7.19			Y18, Y36, Y50, Y103
BSA	BSA	94.54	Y30, Y84, Y137, Y147, Y149, Y155, Y156, Y160, Y262, Y318, Y331, Y340, Y352, Y369, Y400, Y410, Y451, Y496	Y30, Y84, Y139, Y147, Y262, Y318, Y331, Y333, Y340, Y352, Y369, Y400, Y451, Y496	
	Transthyretin	52.55	Y104, Y113	Y69, Y104, Y113	Y77, Y105
	Complement C3	1.59	Y705		All others
K-Casein	K-Casein	84.17	Y25, Y30, Y35, Y38, Y42, Y43, Y58, Y60, Y61	Y25	
	α S1-Casein	74.00	Y91, Y94, Y104, Y144, Y146, Y154, Y159, Y165, Y166, Y173	Y91, Y94, Y146	
	β -LG	83.27	Y20, Y42, Y99, Y102	Y42	
	α S2-Casein	52.42	Y78, Y89, Y100, Y179, Y203	Y89	Y20, Y52, Y95, Y98, Y171, Y184, Y206
	Polymeric immunoglobulin receptor	17.58	Y122, Y164, Y169, Y228, Y390	Y200	All others
	Lactotransferrin	12.76	Y166		All others
	Complement C3	3.29	Y219, Y220	Y1536	All others
	BSA	13.01	Y340, Y451	Y496	All others
	α -LA	28.74			Y18, Y36, Y50, Y103

In vitro allergen nitration:
comparison of various nitrating agents

K-Casein	Lactadherin	9.76	Y226		All others
	Lipoprotein lipase	9.08			All others
	Beta-2-microglobulin	28.75			Y10, Y22, Y26, Y28, Y62, Y77
	Lactoperoxidase	5.45			All others
	Angiogenin-1	17.17			All others

Table II. Detected milk proteins and their identified nitration sites after nitration of milk fractions by TNM (1h, molar ratio TNM/Tyr 30:1).

Allergen tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
Casein fraction	α S1-Casein	75.87	Y91, Y94, Y104, Y144, Y146, Y154, Y159, Y165, Y166, Y173	Y91, Y94, Y104, Y144, Y146, Y154, Y159, Y165, Y166, Y173	
	β -Casein	74.78	Y60, Y114, Y180, Y193	Y60, Y1114, Y193	
	β -LG	86.33	Y20, Y42, Y99, Y102	Y20, Y42, Y99, Y102	
	K-Casein	55.53	Y25, Y30, Y35, Y38, Y42, Y60, Y61	Y25, Y30, Y35, Y38, Y42, Y43, Y58, Y60, Y61	
	α S2-Casein	42.64	Y89, Y95, Y98, Y100, Y179	Y89, Y95, Y98, Y100, Y179	Y20, Y52, Y78, Y171, Y184, Y203, Y206
	BSA	40.76	Y262, Y331, Y333, Y400, Y451, Y496	Y30, Y331, Y333, Y340, Y352, Y400, Y451, Y496	Y84, Y137, Y139, Y147, Y149, Y155, Y156, Y160, Y318, Y369, Y410
	Lactotransferrin	26.69	Y166, Y657, Y665	Y166, Y189, Y433, Y657, Y660, Y665	Y72, Y82, Y92, Y93, Y125, Y192, Y227, Y319, Y324, Y342, Y398, Y400, Y523, Y524, Y526
	Polymeric immunoglobulin receptor	18.40	Y228	Y122, Y228	All others
	Xanthine dehydrogenase/oxidase	9.39	Y598	Y28, Y688	All others
	Lactadherin	31.24	Y226	Y74, Y226	Y28, Y82, Y143, Y175, Y183, Y273, Y274, Y287, Y288, Y337, Y341, Y345, Y355
	Complement C3	3.05		Y610	All other
	α -LA	52.93			Y18, Y36, Y50, Y103
	Butyrophilin subfamily 1 member A1	25.93			Y106

In vitro allergen nitration:
comparison of various nitrating agents

	Lactoperoxidase	19.87			Y38, Y68, Y102, Y149, Y189, Y210, Y243, Y310, Y329, Y345, Y348, Y382, Y466, Y501, Y589
Whey fraction	β -Casein	70.49	Y60, Y114, Y193	Y60, Y114, Y193	Y180
	α S1-Casein	54.01	Y91, Y94, Y144, Y173	Y91, Y94, Y144, Y173	Y104, Y146, Y154, Y159, Y165, Y166
	α -LA	59.89		Y18	Y36, Y50, Y103
	α S2-Casein	31.26		Y179, Y203, Y206	Y20, Y52, Y78, Y89, Y95, Y98, Y100, Y171, Y184
	β -LG	57.56		Y42, Y99	Y20, Y102
	Osteopontin	18.64		Y20	Y142
	Glycosylation-dependent cell adhesion molecule 1	35.80			All others
	Butyrophilin subfamily 1 member A1	12.57			All others
	Polymeric immunoglobulin receptor	3.79		Y607	All others
	BSA	3.60			Y30, Y84, Y137, Y147, Y149, Y155, Y156, Y160, Y262, Y318, Y331, Y340, Y352, Y369, Y400, Y410, Y451, Y496
	Alpha-1-acid glycoprotein	9.70			All others

Table III. Calculated SASA for various milk and egg allergens ^(a)

Protein	Uniprot N°	PDBe	Total SASA (Å ²)	Average residue SASA (Å ²)	Tyr position	SASA (Å ²)	Tyr position	SASA (Å ²)
α-LA	P00711	1hfz	6832.547	56.0	18	87.3	50	7.2
					36	13.5	103	61.4
β-LA	P02754	1b8e	8501.335	55.9	20	150.9	99	47.2
					42	48.2	102	2.1
BSA	P02769	4f5s	28541.24	38.1	30	9.4	318	2.4
					84	18.2	331	0.0
					137	8.8	333	6.4
					139	0.0	340	20.4
					147	8.8	352	0.0
					149	0.0	369	4.3
					155	9.1	400	69.0
					156	9.1	410	13.7
					160	26.3	451	43.4
262	96.3	496	48.3					
Lactoferrin	P24627	1blf	29156.34	42.6	72	57.6	342	88.6
					82	9.7	398	7.2
					92	0.0	400	9.3
					93	8.7	433	2.9
					135	96.4	523	2.9
					166	52.3	524	47.1
					189	10.1	526	8.2
					192	0.0	657	36.3
					227	8.6	660	38.6
319	19.6	665	9.7					
Lysozyme C	P00698	193l	6657.44	51.6	20	62.3	23	46.1
					53	25.1		

(a) Calculations were performed with VMD (42) and QwickMD (43)

Table IV. Detected milk proteins and their identified nitration sites after electrochemical nitration of pure milk proteins using sodium nitrite (4h, molar ratio NaNO₂/Tyr 30:1).

Protein tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
β-Casein	β-Casein	21.43	Y114, Y180, Y193		Y60
	κ-Casein	18.95			Y25, Y30, Y35, Y38, Y42, Y43, Y58, Y61
	αS2-Casein	18.02			All others
	β-lactoglobulin	14.61	Y99		All others
	αS1-Casein	5.14			All others
	BSA	3.13			All others
β-Lactoglobulin	β-lactoglobulin	53.37	Y42, Y99	Y42, Y99, Y102	Y20
BSA	BSA	49.26	Y84, Y160, Y262, Y340	Y84, Y262, Y340, Y352, Y400, Y496	All others
κ-Casein	κ-Casein	42.63	Y61	Y61	All others
	αS2-Casein	32.43	Y78, Y89, Y179, Y203	Y89	All others
	β-lactoglobulin	13.48	Y99	Y99, Y20	All others
	Lactotransferrin	8.05		Y523, Y534, Y526	All others
	αS1-Casein	8.88			All others
	BSA	3.13			All others
	β-2-microglobulin	18.64			All others
α-Lactalbumin	α-Lactalbumin	30.99	Y18	Y18, Y103	All others

Table V. Detected milk proteins and their identified nitration sites after electrochemical nitration of milk extracts using sodium nitrite (4h, molar ratio NaNO₂/Tyr 30:1).

Extract tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
Casein fraction	β-Casein	45.09	Y114, Y180, Y193	Y193	Y60
	αS2-Casein	27.48	Y78, Y179	Y78, Y89, Y100, Y179	All others
	β-lactoglobulin	35.96	Y42, Y99	Y42, Y99	All others
	κ-Casein	42.63	Y61	Y61	All others
	αS1-Casein	37.85	Y91, Y94, Y104, Y173	Y91, Y94, Y144, Y146, Y154, Y159, Y166	All others
	Xanthine dehydrogenase/oxidase	4.5			All others
	Butyrophilin subfamily 1 member A1	18.06		Y90, Y358	All others
	Lactotransferrin	6.21			All others
	BSA	10.05			All others
Whey fraction	β-lactoglobulin	41.01	Y99	Y99	All others
	BSA	12.85			All others
	αS2-Casein	12.61	Y203		All others
	α-lactalbumin	23.24			All others
	β-Casein	10.71			All others

Table VI. Detected egg proteins and their identified nitration sites after nitration of pure proteins by TNM (1h, molar ratio TNM/Tyr 30:1).

Extract tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
Ovalbumin	Ovomucoid	73.33	Y37, Y46, Y102, Y141	Y37, Y46, Y102, Y141, Y161	73
	Ovalbumin	37.05	Y29, Y97, Y111, Y117		Y42, Y106, Y125, Y212, Y281, Y291
Lysozyme	Lysozyme C	87.76	Y20, Y23, Y53	Y20, Y23, Y53	

Table VII. Detected egg proteins and their identified nitration sites after nitration of egg fractions by TNM (1h, molar ratio TNM/Tyr 30:1).

Extract tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
Egg white	Ovomucoid	72.86	Y37, Y46, Y141	Y37, Y46, Y102, Y141, Y161	Y73
	Ovoinhibitor	51.48	Y8, Y46, Y111, Y139, Y168, Y205, Y336, Y354, Y365, Y413, Y424	Y37, Y102, Y139, Y168, Y205, Y234, Y336, Y424	Y80, Y96, Y293, Y321, Y456
	Riboflavin-binding protein	30.25	Y3, Y53, Y89	Y3, Y107	Y27, Y36, Y75, Y141, Y145
	Ovotransferrin	14.47	Y483		All others
	Lysozyme C	43.54	Y23	Y53	Y20

Table VIII. Detected egg proteins and their identified nitration sites after electrochemical nitration of pure egg proteins using sodium nitrite (4h, molar ratio NaNO₂/Tyr 30:1).

Allergen tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
Lysozyme C	Lysozyme C	77.5	Y20, Y23	Y20, Y53	
Ovalbumin	Ovalbumin	40.67		Y29, Y42	All others

Table IX. Detected egg proteins and their identified nitration sites after electrochemical nitration of egg extract using sodium nitrite (4h, molar ratio NaNO₂/Tyr 30:1).

Allergen tested	Protein detected	Coverage (%)	Detected Nty	Detected Tyr	Undetected Tyr
Egg white	Ovalbumin	39.12		Y29	All others
	Ovotransferrin	23.97	Y71, Y92, Y580,	Y71, Y431, Y430, Y521	All others
	Ovalbumin-related protein Y	12.11			All others
	Ovomucoid	15.71		Y37	All others
	Ovostatin	1.97			All others
	Lysozyme C	13.61			All others

In vitro allergen nitration:
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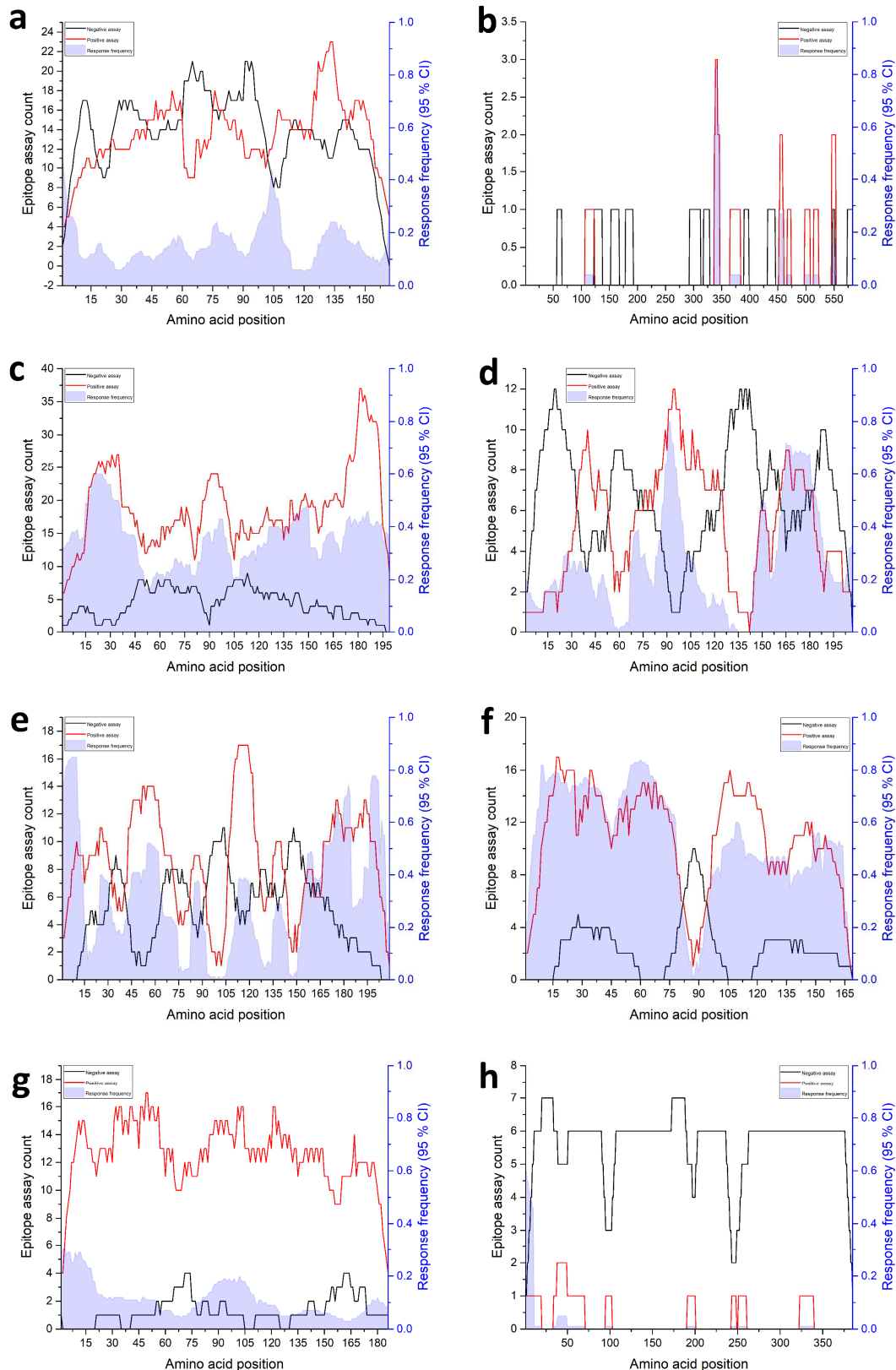


Figure I. Number of IgE epitope assays carried out and calculated RF for each amino acid residues of the following allergens: **(a)** β -LG, **(b)** BSA, **(c)** α S1-casein, **(d)** α S2-casein, **(e)** β -casein, **(f)** κ -casein, **(g)** ovomucoid, and **(h)** ovalbumin. Data were exported from the IEDB servers (115) with the following search parameters: any epitopes, B cell assays, human host, allergic disease, no MHC restriction, limited to IgE epitopes.

Chapter 5.

Quantification of peanut and hazelnut traces in food by bottom-up proteomics

5.1 Introduction

Peanut and tree nut allergies affect approximately 0.25 to 1.4 % of the population (1,2) and are responsible for 60 to 80 % of the fatalities caused by food-related anaphylaxis. (3,4) The minimum allergen amount inducing an allergic reaction varies for each type of nuts and for each patient. For peanuts, eliciting doses between 100 µg and 2 mg of proteins, corresponding to less than one hundredth of a peanut, have been reported. (5–8) For highly allergic patients, even small nut traces in food elicit potentially severe allergic reactions and anaphylaxis, resulting in the precautionary labelling of many food products by their manufacturers. The nut allergy management is based on strict avoidance and prompt treatment of the symptoms resulting from an exposure to culprit allergens with self-injected epinephrine. (9) Nevertheless, several cases of fatal anaphylaxis were reported after the ingestion of nut allergens hidden in normally innocuous food, such as sandwiches, pizza or candy. (10,11)

The presence of nut traces in food products is usually caused by their adventitious contamination during the processing of normally allergen-free food. The food industrial manufacturing is a multistep process and each production stage creates opportunities for the inclusion of food allergens in the final product. Cross-contaminations occur mainly during the handling and storage of raw material, the preparation of products on the same production lines or in the same factory, or because of the carry-over from previous processings. (12) In addition, changes in product formulation without modification of labels, incorrect and/or incomplete lists of ingredients, and the presence of unknown allergens in raw products are responsible for hidden traces of aller-

genic proteins in food. (12) The correct labelling of food product and accurate determination of allergen presence in food matrices are required for allergic patient's safety.

Along the years, various analytical methods have been developed to quantify allergens in food products, either as routine large-scale procedures in food industries or as state-of-the-art methods in laboratory researches. Enzyme-linked immunosorbent assays (ELISA) and polymerase chain reaction (PCR) are amongst the most popular and used methods for allergen quantification in industry. ELISA is designed for the fast screening of food products using the serum IgG antibodies (Abs) of immunized animals, able to bind to the allergens used for the immunization. The measurement of an enzyme activity linked to a secondary Abs, specific to the animal IgG-Abs, allows the identification and quantification of allergens in a sample. (13) ELISA is a robust method, reliable, sensitive, and highly specific thanks to the use of specially designed Abs. Nevertheless, this method is tedious and offers limited multiplexing options. (14) In addition to their widespread use in food industry, ELISA-based procedures are widely used and improved in research laboratories. For example, inductively coupled plasma-mass spectrometry (ICP-MS) has been combined with ELISA to increase its sensitivity and decrease matrix effects, allowing limit of detection (LOD) as low as $2 \mu\text{g}\cdot\text{g}^{-1}$ for peanut allergens in a food matrix. (15) The LOD for standard ELISA test varies greatly depending on the matrices, the target allergens and the specific method used (*i.e.* competitive or sandwich ELISA). For example in the case of cow's milk allergens detection, LODs ranging from 0.08 to $500 \mu\text{g}\cdot\text{L}^{-1}$ are commonly obtained for the analyses of infant formula and human milk, (16,17) juices, sorbets, and chocolate, (18) and cheese. (19)

The other large-scale methods of allergen identification used in industry are the PCR-based techniques. PCR is a powerful tool, based on the targeting and amplification of genes corresponding to the allergenic proteins of interest. With this method, LODs of less than $10 \text{mg}\cdot\text{kg}^{-1}$ have been reached for several allergens of milk, peanut, almond and soya. (20) Improvements of the basic PCR procedure, such as real-time PCR and PCR coupled to ELISA, are available and were used for example for fish, walnut, peanut, and hazelnut allergies. (21–24) PCR-based methods have the advantages of being fast, easily automated and multiplexed. By choosing suitable primers, cross-reactivity and false positive results are potentially avoided. (25) Nevertheless, PCR is limited by the stability of the target DNA during the various processing steps and by the food matrix purity. (26) Additionally, PCR detects the DNA strand coding for a given allergenic protein and

not the allergen itself. Therefore, a positive PCR test does not always guaranty the presence of the allergen in the food product. (27)

The recent developments and improvements of mass spectrometry (MS) have open the possibility of using this method for the accurate and sensitive allergen detection in food matrices. Application of proteomics strategies to identify and characterize allergens is sometimes referred to "*allergenomics*". (28,29) In most of the cases, the sample proteins are extracted and submitted to a bottom-up proteomic (BUP) approach to detect specific peptides marker, used to identify the corresponding proteins in the original food product. (30) Compared to methods based on DNA or Abs recognition, MS technology displays a higher reliability in the allergen identification with similar or better sensitivity. (30) In addition, the immunoassay results are limited by the possible modifications or hindering of epitopes occurring during standard food processing, whereas MS-based methods overcome these issues by identifying protein based on peptide biomarkers. (31) Moreover, MS offers the possibility to identify multiple allergens in single experiments and to determine features such as structural properties, post-translational modifications or glycosylation. Standard MS techniques do not allow a direct protein quantification, nevertheless, various quantification methods based on protein labelling with stable isotopes have been developed. (32,33) In the case of food allergens, label-free techniques are usually preferred. (31) Absolute protein quantification is based on the addition of the appropriate standard or by spiking the samples with the target protein/extract. Spectral counting, peptide ions intensities or area under the curve (AUC) are parameters extracted during the data processing, allowing the relative and/or absolute quantification of the corresponding peptides and proteins. (34,35) Label-free methods have been applied to quantify egg allergens in wine and bread, (36–39) milk allergens in wine, cookies and breakfast cereals, (36,40–43) and various nuts in biscuit and bread. (38,44–46) More information on MS-based techniques for protein trace quantification are provided in *Chapter 1* section 1.9.

In this chapter, the presence of peanut and hazelnut allergens in breakfast cereals, biscuits and chocolate products was determined by high-resolution mass spectrometry following a standard BUP approach. This was done in order to access the presence and absolute amount of allergens present in food products that theoretically should not contain any allergens, but are labeled by manufacturer as "may contain traces of allergens" within an ongoing clinical study "Assessing Tolerance to "May Contain Traces" Processed Foods in Tree Nuts or Peanut Allergic Children" (NCT03680066). More details about the study itself are provided below in *Results and dis-*

cussion section. A standard addition method was then used to quantify peanut and hazelnut proteins by BUP using an untargeted “*shotgun*” approach. For this method, the proteins of native and allergen-spiked samples were extracted under the same conditions and analyzed by BUP. Several peptides identified during the analyses are herein reported as satisfactory markers for the peanut and hazelnut quantifications based on the linearity observed for their ion intensities measured in MS¹ spectra.

5.2 Materials and methods

5.2.1 Chemical and materials

Acetone, iodoacetamide, trifluoroacetic acid and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Buchs, Switzerland). DL-dithiothreitol and formic acid (FA) were purchased from Fluka (Buchs, Switzerland). Sodium hydroxide was obtained from RectoLab SA (Servion, Switzerland). Hydrochloric acid (32 %) was obtained from Fischer Chemicals AG (Zurich, Switzerland), while n-hexane and acetonitrile (ACN, Rotisolv HPLC) were purchased from Carl Roth AG (Karlsruhe, Germany). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Acros Organics (Basel, Switzerland). Trypsin from bovine pancreas was acquired from AppliChem GmbH (Darmstadt, Germany). Organic and unsalted peanuts and hazelnuts were purchased in a local store.

Ultrapure water was obtained from a Milli-Q Integral Water Purification System (Merck-Millipore, Zug, Switzerland) and used in all experiments. An Agilent 8453 G1103A spectrophotometer (Agilent Technologies, Waldbronn, Germany) was used for all UV-Vis analyses with quartz cuvettes (200-2500 nm spectral range). Protein quantification was carried out using a commercial BCA protein assay (Pierce, ThermoFisher Scientific, Rockford, USA) with BSA as a standard.

5.2.2 Preparation of nut flours

Hazelnuts were blanched for 10 seconds in boiling water before putting them in cold water (4°C) for a couple of minutes in order to easily remove the skin/pellicle of the nuts, whereas peanuts were bought already peeled. Peanuts and hazelnuts were frozen in liquid nitrogen before grinding them in an electric kitchen grinder. The obtained powders/pastes were then defatted by adding n-hexane with a ratio of 1:5 (w/v) and the mixtures were stirred for 20 minutes at room temperature. The defatted flours were then filtered and dried overnight before storing them at -20°C until further use.

5.2.3 Nut protein extraction from food matrices and sample preparation

The food samples were frozen in liquid nitrogen before grinding them in an electric kitchen grinder. The protein extraction was carried out by incubating 1 g of the samples with 20 mL of 20 mM Tris-HCl buffer (pH 8.2) for 1 hour at room temperature and under vigorous shaking. The solutions were then submitted to a 20 minutes ultrasonic treatment using an ultrasonic processor VC505/500W (Sonics & Materials Inc, Newtown, USA). The amplitude was set to 20 % with 5 seconds of on/off pulses. The samples were then centrifuged before collecting and filtering the supernatant through a syringe filter (ChromaFil Xtra PVDF-45/25, Macherey-Nagel Ag, Oensingen, Switzerland). For each extract, 30 mL of cold acetone (-20°C) were added to 5 mL of the filtered supernatant and incubated overnight at -20°C in order to precipitate the proteins. After centrifugation, the protein pellets were washed two times with cold acetone and dissolved in 1 mL of the Tris-HCl buffer. A BCA assay was then used to determine the exact protein concentration in each sample. The proteins were then denatured at 95°C for 10 minutes before reducing the disulfide bonds at 56°C for 30 minutes using 200 mM of dithiothreitol (DTT) in the same Tris-HCl buffer, with a final DTT concentration of 5 mM. The alkylation of the cysteine residues was carried out in the dark for 30 minutes by adding iodoacetamide (IAA, 200 mM, in ammonium bicarbonate buffer) to a final concentration of 10 mM. Trypsin (1 mg·mL⁻¹) was finally added with a 30:1 ratio (w/w, protein:trypsin) and the solutions were incubated overnight at 37°C under moderate stirring. The tryptic reactions were stopped by acidification using trifluoroacetic acid to a final acid concentration of 0.1% and pH < 4. Tryptic peptides were extracted and concentrated using ZipTip C₁₈ pipette tips (Merck Millipore, Darmstadt, Germany) following the manufacturer protocol.

5.2.4 Mass spectrometry analysis of the food extracts by bottom-up proteomics

Tryptic peptides obtained after ZipTip C₁₈ purification were injected on a ZORBAX Eclipse Plus C₁₈ column (2.1 x 150 mm, 5 µm, Agilent, Waldbronn, Germany) using a Dionex Ultimate 3000 HPLC system (Thermo Fischer Scientific, San Jose, USA) coupled to Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA). The column oven was set to 30°C and a fixed flowrate of 0.25 mL·min⁻¹ was used. Eluents A and B consisted, respectively, in 99.9:0.1 % H₂O:FA and 99.9:0.1 % ACN:FA. The concentration of eluent B was kept at 3 % for the first 3 minutes as equilibration step, before increasing it to 35 % in 60 minutes as effective separation gradient. It was then increased to 80 % in 1 minute and kept at this concentration for 2 minutes as washing step. Finally, the eluent B was decreased to 3 % in 1 minute and maintained

for 3 minutes to reequilibrate the column. A blank injection was performed between each sample analysis.

A standard full MS/dd-MS² method was used for the analysis of the tryptic peptides. The resolution was set at 60'000 (at 400 *m/z*) for the full MS scans, with an automatic gain control (AGC) target of 3E6 and maximum injection time of 100 ms. The full MS spectra were recorded from 300 to 1900 *m/z* in profile mode. The MS² spectra were recorded in centroid mode with Top10 method and the resolution 15'000 at 400 *m/z*. The maximum injection time was fixed at 50 ms with 2 microscans and an AGC target of 1E5. A 2 *m/z* isolation window was used for the parent ions and the normalized collision energy (NCE) of higher-energy collisional dissociation (HCD) fragmentation was set at 27 with default charge 2. Only peptides with an assigned charge between 2 and 7 were allowed for precursor selection and subsequent fragmentation.

The obtained data were processed using the Trans-Proteomic Pipeline (TPP). (47) The protein database used was obtained from the Universal Protein Resource (UniProt, release February 2018) and contained the complete reviewed and unreviewed (both SwissProt and TrEMBL) proteomes of cacao, wheat, peanut and hazelnut. In addition, common keratin contaminants, bovine trypsin and the main milk and egg proteins were added to the database. Carbamidomethylation of cysteine residue (+ 57.0215 Da) was set as a fixed modification whereas acetylation of protein N-terminus and lysine (+ 42.0106 Da), deamidation of asparagine and glutamine (+ 0.9840 Da), and oxidation of methionine (+ 15.9949 Da) were set as potential modifications. A maximum of 4 missed cleavages were set with a precursor *m/z* tolerance of 10 ppm and a fragment tolerance of 0.04 Da. The peptide identifications were validated with a false discovery rate (FDR) of 1 %.

5.2.5 Sample spiking and quantification procedure

The sample spiking was performed by adding various amounts of peanut and hazelnut flours to the ground samples. The spiked samples were then thoroughly homogenized and extracted as described in *Materials and Methods* section 5.2.3. The sample C1 was spiked with 50, 100 and 20 ppm of peanut and hazelnut proteins, whereas 10 and 20 ppm spikes were used for the other food products. The peptides identified in the BUP analyses and demonstrating linear responses within the spiking ranges were used as quantification markers. For those peptides, the extracted ion chromatograms (XIC) were obtained in a post-acquisition step using Xcalibur 4.1 (Thermo Fisher Scientific, San Jose, USA) and their maximum intensities were used for the stand-

and addition calibration plots. A 10 % potential variation was assumed for the measured intensities and was represented as error bars in the standard addition plots. The linear regressions and subsequent quantifications were performed using Origin Pro 9 (OriginLab Corporation, Northampton, USA). The quantified protein concentrations are reported in ppm with error intervals calculated using the slope and intercept errors obtained for the linear regressions.

5.3 Results and discussion

5.3.1 Identification of peanut and hazelnut proteins in food products by bottom-up proteomics

The Immunology and Allergology division of the Geneva University Hospital (HUG, Switzerland) collected various normally innocuous food products suspected of causing allergic reactions after their ingestion by peanut and hazelnut allergic patients as part of an ongoing clinical study “Assessing Tolerance to “May Contain Traces” Processed Foods in Tree Nuts or Peanut Allergic Children”. Nut allergic patients aged between 2 and 18 years, with positive skin prick test, specific IgE Abs (> 0.35 kU/L) and oral food challenge (30 mg of protein or below) were recruited for this study. As the goal of this study is to evaluate whether children with clinical nut allergies could consume food products labeled “may contain traces of allergens”, it was necessary to estimate the peanut and hazelnut quantity in the food matrices caused positive allergic responses in the tested patients. In order to perform such analysis, the proteins extraction for all the provided samples was performed and first presence of allergens was assessed using BUP approach, followed by quantitation of observed nut traces using BUP approach with a standard addition method. These food products were two breakfast cereals (A1 and A2), two biscuits (B2 and B3) and four different chocolate products (C1, C2, C3 and C4). The samples are all commercial products and bear various allergy precautionary statements on their labels. A1 contains potentially peanut, whereas the presence of nuts is possible in A2, B2, C1, C2, C3, and C4. The biscuit B3 is the only product without a safety notice on its label.

The procedure for the protein extraction, detailed in *Material and Methods* section 5.2.3, was based on various extraction protocols previously published. (48–52) The procedure is relatively similar to the extraction of nut allergens previously used in Chapter 3. The PBS buffer is replaced by a Tris-HCl buffer with a pH of 8.2 and the extraction was assisted by the ultrasonic treatment of all samples. The use of ultrasounds helps the protein extraction on two levels. First, they reduce the size of food particles resulting in an increased surface available for protein extraction. In addi-

tion, the ultrasounds were shown to induce protein denaturation, favoring their extraction. (53–55) After their tryptic digestion, the protein extracts were analyzed by a BUP approach in order to screen the samples for the presence of peanut and hazelnut proteins. In addition to the nut proteins, the database used for the search included all the main milk and egg proteins and the complete proteomes of wheat (*Triticum aestivum*) and cacao (*Theobroma cacao*). Using a database including only the peanut and hazelnut proteomes resulted, as expected, in the failure of TPP identification models caused by the low number of hits. Including the proteomes of other species present in the food matrix was therefore required.

In a first step, proteins were extracted from the native samples and analyzed by BUP as described in *Materials and Methods* section 5.2.3-4. The protein concentrations obtained after the protein purification and resolubilisation steps ranged from 0.5 to 7.9 mg·mL⁻¹ (Table 1). The chocolates samples (C1-C4) showed the greater degree of protein extraction, whereas the amount of recovered proteins was limited for breakfast cereals (A1 and A2) and biscuits (B2 and B3). The number of identified proteins in the BUP analyses varies greatly between the samples as summarized in the Table 1. The higher numbers of identified proteins were obtained for samples B2 and B3 with 91 and 67 hits, respectively. A majority of these proteins originate from wheat flour, as it is a base ingredient in the biscuits. Meanwhile, only 10 to 20 proteins were identified for the chocolate products, despite the high protein concentrations measured with the BCA assays. For their part, the samples A1 and A2 display a poor protein identification. The chromatograms obtained during the BUP analyses of the eight samples (Appendix Figure I) reflect clearly the differences between the samples, with higher numbers of peaks arising from tryptic peptides separation for samples B2 and B3.

Table 1. Number of proteins identified in the first screening of the food extracts by a BUP approach and protein concentrations in the samples after purification and resolubilisation.

Sample	Total number of identified proteins	Number of identified proteins originating from:						Protein concentration (mg·mL ⁻¹)
		Wheat	Cow's milk	Cacao	Peanut	Hazelnut	Egg	
A1	3	1	1	0	1	0	0	0.5
A2	7	6	1	0	0	0	0	2.0
B2	91	77	6	0	6	1	0	1.8
B3	67	52	5	0	5	2	3	1.3
C1	20	3	8	2	4	2	1	5.0
C2	11	0	8	1	0	1	0	7.9
C3	10	1	3	2	3	0	0	4.2
C4	15	1	8	2	1	1	2	7.1

The first screening of the various food extracts showed the presence of peanut and/or hazelnut proteins in all of them except for the sample A2, as shown in Table 1. The peanut and hazelnut proteins identified during the BUP analyses are listed in Table 2. As expected for trace contaminations, the number of peptides and the sequence coverages are low for all identified nut proteins. Most of the detected proteins are not allergens. However, an isoform of *Cor a 9* (UniProt N° A0A0A0P7E3) was detected in samples B2 and C1, whereas *Ara h 9* (UniProt N° B6CEX8) was identified in sample B2. As the proteins are present only in traces, limiting the search to the known allergens would yield a truncated representation of the food samples contaminations. Indeed, the presence of non-allergenic nut proteins in the samples imply the potential presence of allergens even without their direct identification. For this reason, allergen traces quantification is usually reported in mg of the total allergenic extract per kg of the food matrix, corresponding to parts-per-million (ppm).

Table 2. Peanut and hazelnut proteins identified in the food samples by BUP analysis.

Sample	Protein (UniProt N°)	Origin	Probability ^(a)	Sequence coverage (%)	Independent spectra	Peptides
A1	A0A2H4V2F5	Peanut	0.92	1.6	1	LERISAPPLVILK
A2 ^(b)	-	-	-	-	-	-
B2	T2B9M0	Peanut	1.00	3.9	1	GILAADESTGTIGK
	A0A0A6ZDP1	Peanut	0.99	8.1	1	RVPTVDVSVVDLTVR
	Q6KBB0	Peanut	0.99	4.2	1	VAITQHASKP
	A0A0A0P7E3	Hazelnut	0.96	1.8	1	LNALPTNR
	A0A290GEJ8	Peanut	0.96	2.1	1	VLHLGLL
	B6CEX8	Peanut	0.96	8.7	1	QAACNCLK
	A0A109NX23	Peanut	0.93	0.9	1	IHAQTTVDAK

B3	A0A0A6ZDP1	Peanut	1.00	8.1	3	RVPTVDVSVVDLTVR / VPTVDVSVVDLTVR
	A0A1W5YMZ9	Hazelnut	0.97	0.5	1	IPLETKK
	E6Y6S2	Peanut	0.96	0.6	2	KGILEAHLSSCPNK
	G0Y6T2	Peanut	0.95	7.9	1	LINLLLK
	C7A7E6	Hazelnut	0.93	4.8	1	IALISQLR
	Q5I212	Peanut	0.93	8	1	E EKPEAELVATQLDKPEAK
	A0A290GEJ8	Peanut	0.92	2.1	1	VLHLGLL
C1	A0A0A0P7E3	Hazelnut	1.00	5.1	2	GLLLPQYSNAPELIYIER / WLQLSAER
	D8KXX2	Peanut	0.99	0.5	1	GVPELSK
	C7A7R2	Hazelnut	0.99	4.5	1	NPPLPWKR
	A0A290GI65	Peanut	0.98	2.5	1	HPNIVLLMGAVFQ
	B4UWB2	Peanut	0.95	3.4	1	FILPVLR
	G3GDS7	Peanut	0.95	2.4	1	LLFLPLSK
C2	C7A7R2	Hazelnut	0.97	4.5	1	NPPLPWKR
C3	A0A0A6ZDP1	Peanut	1.00	11.8	3	RVPTVDVSVVDLTVR / SSIFDAK / VPTVDVSVVDLTVR
	T2B9M0	Peanut	1.00	6.7	3	EGGVLPGIK / GILAADESTGTIGK / GILAA-DESTGTIGKR
	Q4JME6	Peanut	0.97	1.2	1	RLLPEQGTAE
C4	D8KXX2	Peanut	0.94	0.5	1	GVPELSK
	C7A7R2	Hazelnut	0.94	4.5	1	NPPLPWKR

(a) Protein probabilities obtained for the validation process in TPP with a 1 % FDR.

(b) No peanut or hazelnut proteins was identified in the A2 sample.

5.3.2 Identification of peanut and hazelnut peptide markers and quantification by standard addition

The peanut and hazelnut proteins in the different food products were quantified based on the peptide ion intensities measured in MS¹ spectra for native and spiked samples. As the peptide intensity is in general correlating with the its concentration in the sample, (35) spiking of the samples results in a linear increase in signal intensities for the peptides. As known quantities of peanut and hazelnut are added to the samples, it is possible to determine their initial concentration in the samples. In order to carry out successfully the quantification by standard addition, several conditions are necessary. Firstly, the peptides used for quantification are required to be detected easily both in the native and spiked samples. Due to the low working concentrations, even small variations in the extraction process or MS analysis result potentially in the loss of peptide markers. Secondly, the peptide markers should display a linear response in the analysis range. Lastly, the peptides need to be correctly identified and validated by TPP. The MS² spectra of all identified peanut/hazelnut peptides were verified by hand in order to reject the ones with bad

fragmentation and discard the systematic artifacts wrongly assigned to peptides. Based on these criteria, 2 and 4 peptide markers were found for hazelnut and peanut, respectively (Table 3).

Table 3. Peptide markers used for the quantification of peanut and hazelnut in various food matrices by BUP approach.

Peptide	Protein (UniProt N°)	Origin	Monoisotopic mass ^(a) (Da)	Monoisotopic <i>m/z</i> ^(a)	Charge state	Sample
GLLLPQYSNAPELIYIER	A0A0A0P7E3	Hazelnut	2088.1255	697.0491	+3	C1
RVPTVDVSVVDLTVR	A0A0A6ZDP1	Peanut	1653.9415	552.3211	+3	B3
NPPLPWKR	C7A7R2	Hazelnut	1006.5712	504.2929	+2	C2 / C4
VAITQHSKP	Q6KBB0	Peanut	979.5451	327.5223	+3	B2
LINLLLK ^(b)	GOY6T2	Peanut	826.5528	414.2837	+2	B3
VLHLGLL	A0A290GEJ8	Peanut	763.4956	382.7551	+2	B3

(a) Experimental values

(b) The peptide LINLLLK was identified with a deamidation of its asparagine

For samples A1, A2 and C3, no peptide markers were found and the quantification of the peanut and hazelnut traces was therefore not possible. Only a single peptide was detected in the initial screening of the sample A1, whereas several peptides were identified in the C3 extract (Table 2). Those peptides were not detected in all experiments, probably because their concentrations are close to the method LOD, and were therefore not selected as acceptable markers. For sample A2, the complete absence of identified peptides (Table 2) suggests either that no allergenic traces are present in the food matrix or that their concentrations are lower than the LOD. For all other samples (B2, B3, C1, C2 and C4), the peanut and hazelnut quantification was achieved using the peptide markers listed in Table 3 and is discussed below.

The hazelnut in chocolate C1 was quantified using the triply charged peptide GLLLPQYSNAPELIYIER. This peptide was also detected as a doubly charged ion. However, only the peptide bearing three charges was used for the hazelnut quantification as it displayed a better HCD fragmentation and a better linear response. A typical MS² spectrum of this peptide is shown in Figure 1.a, whereas the corresponding standard addition plot with spikes at 50, 100 and 200 ppm is displayed in Figure 1.b. This peptide demonstrated a good linearity within the spike range ($R^2 = 0.995$) and the hazelnut amount in the sample was found to be 111.9 ± 0.1 ppm. For this sample, the peptides WLQLSAER (hazelnut) and GVPELSK (peanut) were detected in all the native and spiked samples but were not behaving linearly.

For the biscuit B3, the quantification of peanut traces was carried using three different peptides. Both LINLLLK and RVPTVDVSVVDLTVR display relatively good fragmentation patterns with 9 and 11 product ions, respectively (Figure 1.c and 1.e). The peptide LINLLLK display a better linearity than RVPTVDVSVVDLTVR in the standard addition method with R^2 of 0.960 and 0.922 respectively (Figure 1.d and 1.f). Using those peptides, the calculated peanut concentration in sample B3 was found to be 5.8 ± 1.5 and 12.1 ± 0.3 ppm respectively. The third peptide marker found in this sample is the small peptide VLHLGG. Despite being poorly fragmented in the BUP analysis (Appendix Figure II.a), it was identified in all the B3 analyses and successfully validated by TPP with a 1 % FDR. The standard addition method, based on this peptide, yielded a relatively good linearity ($R^2 = 0.957$, Appendix Figure II.b) and a peanut concentration of 6.5 ± 0.8 ppm was obtained for the native sample. The calculated peanut concentrations in the sample differ depending on the peptides used. Small variations in the peptides properties and their respective interactions with the matrix may explain those fluctuations.

For sample B2, only the peptide marker VAITQHSPK was identified in all the samples. Its typical fragmentation spectra and the standard addition plot obtained with this peptide are displayed in Appendix Figure II.c-d. A relatively poor linearity ($R^2 = 0.922$) was obtained and a peanut concentration of 30.5 ± 3.2 ppm was calculated based on the standard addition data. The two peanut peptides GILAADESTGTIGK and RVPTVDVSVVDLTVR were identified in all B2 samples but displayed nonlinear response.

For samples C2 and C4, the same peptide (NPPLPWKR) was identified as a marker for hazelnut. However, the two sample matrices resulted in different behavior of the peptide. In sample C4, a relatively good linearity is obtained in the standard addition method ($R^2 = 0.947$), whereas this value decreases to 0.860 for sample C2 (Appendix Figure III.b and III.d). The hazelnut concentrations were estimated to be 23.5 ± 5.1 and 126.2 ± 13.4 ppm in samples C2 and C4, respectively. The results for all the food samples are compiled in Table 4.

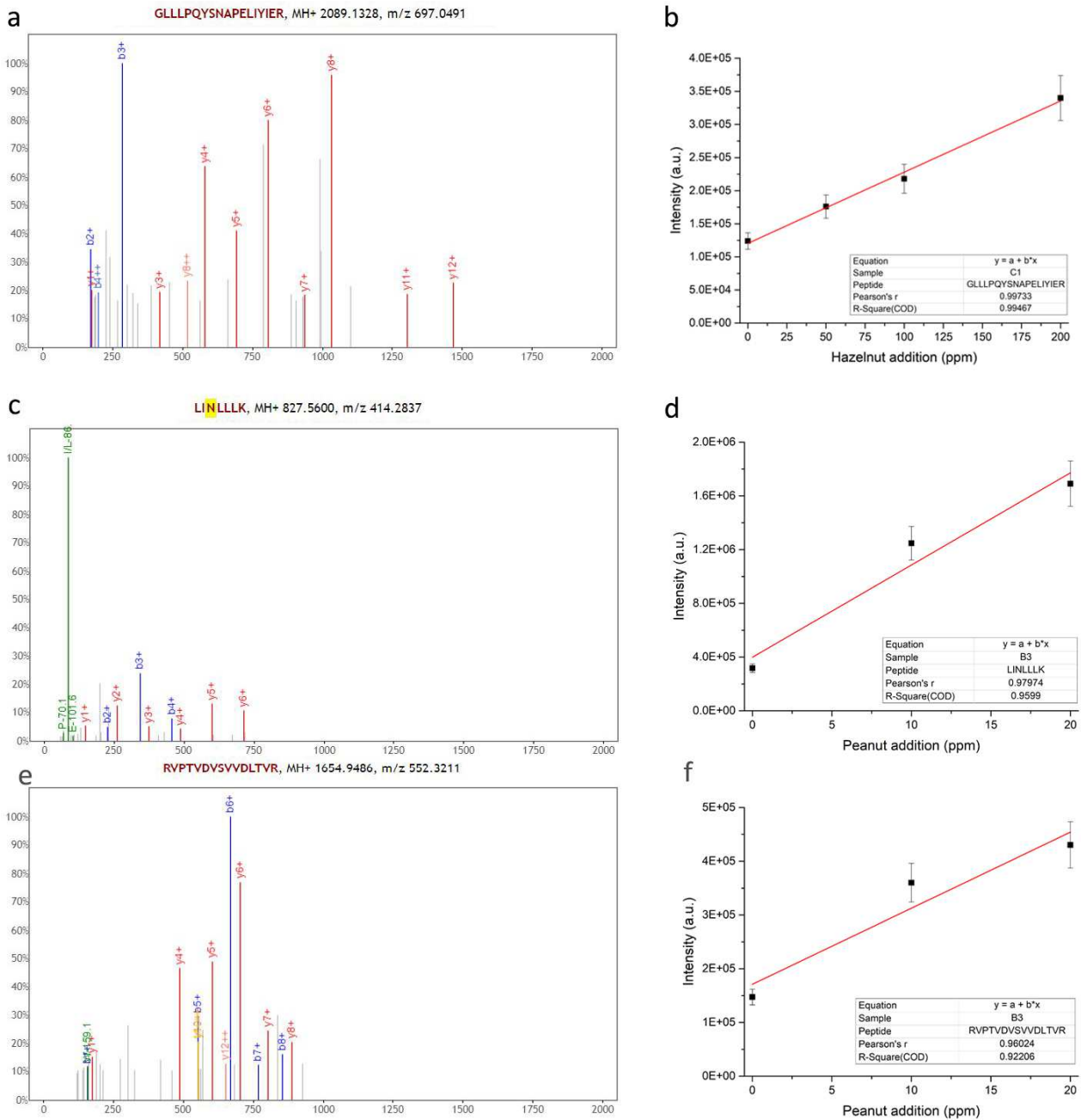


Figure 1. (a,c,e) MS² spectra of peptides GLLLPQYSNAPELIYIER (hazelnut), LINLLLK (peanut) and RVPTVDVSVVDLTVR (peanut) obtained during the BUP analyses and **(b,d,f)** the standard addition plots using these peptides markers for the quantification of peanut and hazelnut in the samples C1 and B3. The peptide LINLLLK was identified with a deamidation of its asparagine (highlighted in yellow in the sequence). The extraction and BUP parameters are as detailed in *Materials and Methods* section 5.3.2-4.

Table 4. Peanut and hazelnut concentrations obtained by BUP approach with a standard addition method for various breakfast cereals, biscuits, and chocolate products.

Sample	Peptide marker	Protein (UniProt N°)	Origin	R ²	PPM in sample
A1.01	--	--	--	--	--
A2.01	--	--	--	--	--
B2.01	VAITQHSKP	Q6KBB0	Peanut	0.922	30.5 ± 3.2
B3.01	RVPTVDVSVVDLT	A0A0A6ZDP1	Peanut	0.922	12.1 ± 0.3
	LINLLLK	G0Y6T2	Peanut	0.960	5.8 ± 1.5
	VLHLGLL	A0A290GEJ8	Peanut	0.957	6.5 ± 0.8
C1.01	GLLLPQYSNAPELIYIER	A0A0A0P7E3	Hazelnut	0.995	111.9 ± 0.1
C2.01	NPPLPWKR	C7A7R2	Hazelnut	0.860	23.5 ± 5.1
C3.01	--	--	--	--	--
C4.01	NPPLPWKR	C7A7R2	Hazelnut	0.947	126 ± 13.4

The accurate protein quantification by label-free MS methods requires the preparation and measurement of samples under similar and uniform conditions. (56) The variations in peptide intensities from one BUP procedure to the other are relatively challenging for the absolute analytes quantification. The fluctuations are caused mainly by unavoidable variations in the extraction procedures, by variations of the ionization efficiency overtime and by matrix effects. (57)

The improvement of the results is possible by increasing the number of spiked samples and by carrying out several repetitions for each sample. However, only a crude quantification of the allergen concentrations in the food products was required in this project in order to compare them with the allergy eliciting doses. For peanuts, a dose between 100 µg and 2 mg of peanut proteins is enough to induce allergic symptoms in patients. (5–8) In the case of hazelnut allergy, doses of 0.29 and 133.8 mg were found to induce allergic reactions in 5 and 10 % of patients, respectively. (58,59) Based on these numbers, 2 to 4 grams of chocolate C4 (hazelnut) or biscuit B2 (peanut) are already enough to elicit the first clinical symptoms for highly reactive patients. Those food product quantities are higher for the other samples, as they contain less nut traces. The absolute LOD and LOQ values were not determined experimentally as there are no commercially available biscuits, breakfast cereals or chocolate products certified to be nut allergens free in Switzerland. (60) However, the lowest peanut and hazelnut concentrations measured with the developed procedure of protein extraction and BUP analysis were 5.8 ± 1.5 and 23.5 ± 5.1 ppm, respectively. These values are in the same LOD/LOQ range obtained by Pilolli *et al.* for cookies using a relatively similar MS instrument (Q Exactive Plus Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific).

5.4 Conclusions

In this chapter, traces of peanut and hazelnut were identified and quantified in various industrial food products (breakfast cereals, biscuits and chocolate products) by a BUP procedure with a label-free quantification method based on peptide ion intensities in native and spiked samples. These food products were specially selected during ongoing clinical study as was described previously. Various peptide markers from peanut and hazelnut proteins were identified and used to quantify those allergenic sources in the food products. As there is actually no certified allergen-free products in Switzerland, the exact LOD/LOQ were not calculated in this work. However, the detected concentrations in the tested samples ranged from 6 to 30 ppm for peanut and 23 to 126 ppm for hazelnut. For two products, the presence of peanut and hazelnut was identified by detecting several peanut/hazelnut peptides but none of them was reliable markers for quantification. Only one sample displayed no detectable traces of peanut or hazelnut. Future works will include the importation of allergen-free certified products for the accurate LOD and LOQ determination and the further optimization of the methods. Additionally, the quantification will be performed based on spectral counting and AUC in order to possibly improve the results.

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5.6 Appendix

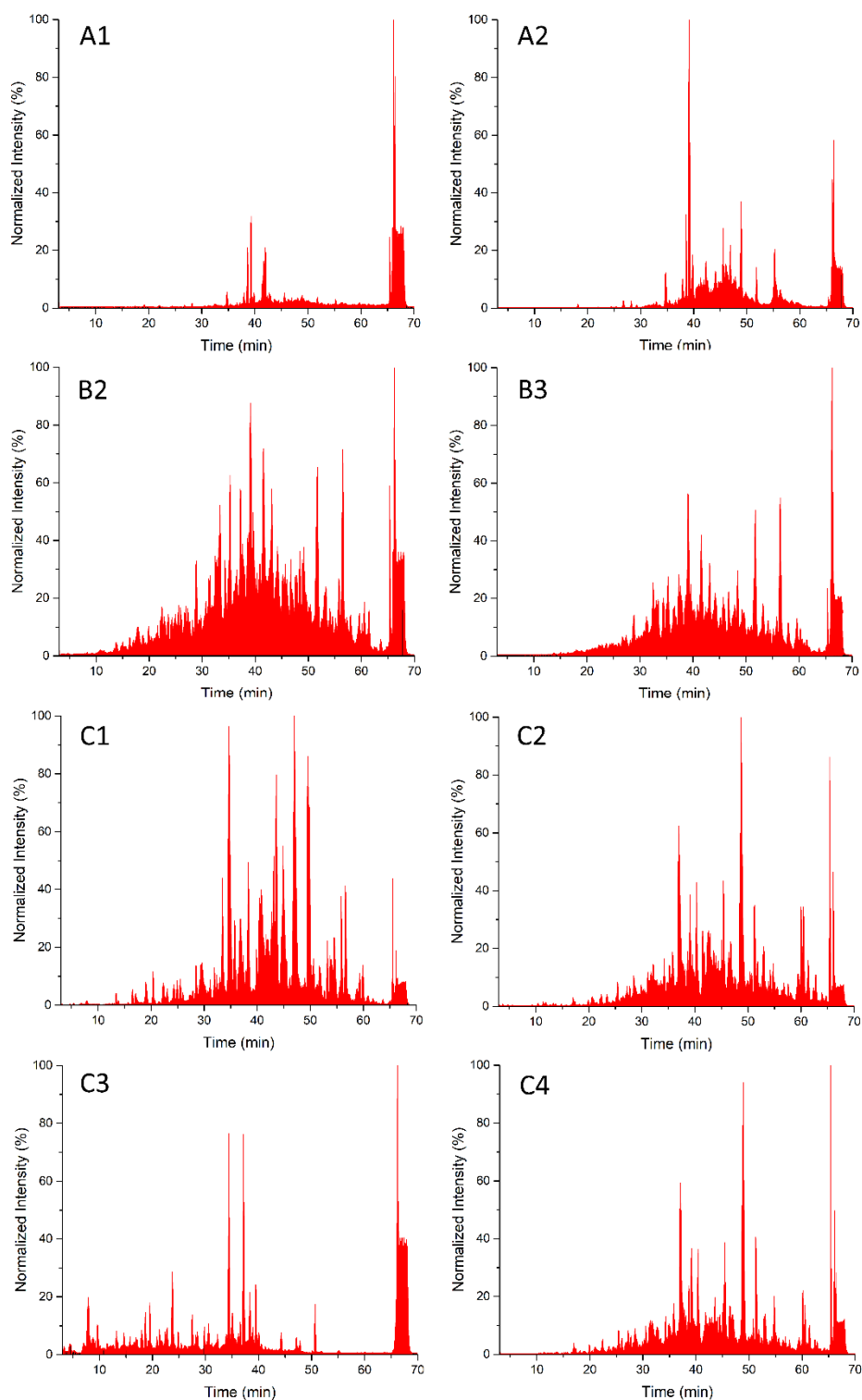


Figure I. Chromatograms obtained for the BUP analyses of breakfast cereals (A1, A2), biscuits (B2, B3) and chocolate products (C1-C4) on a HRMS instrument. The extraction and BUP parameters are as detailed in *Materials and Methods* section 5.3.2-4.

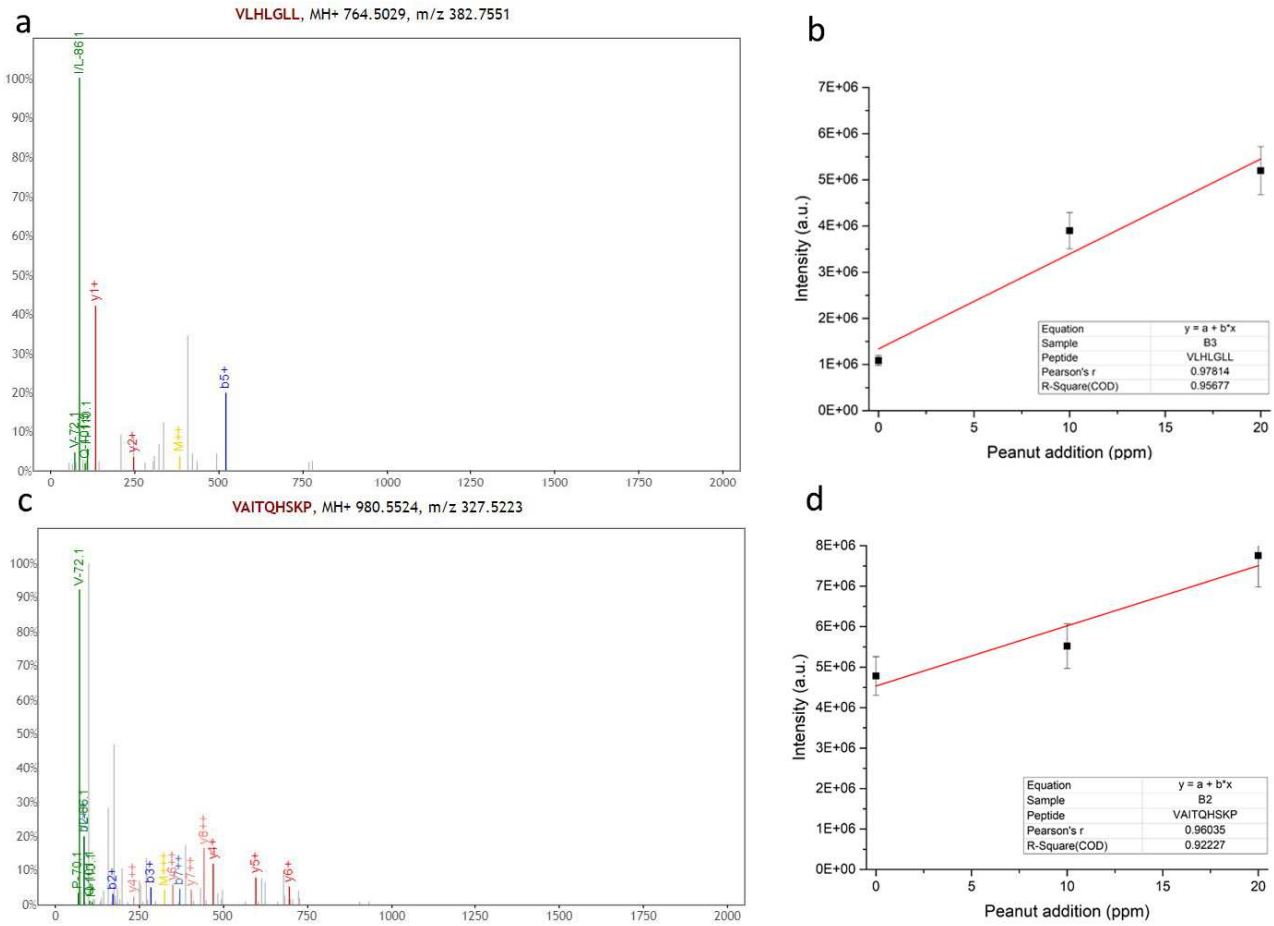


Figure II. (a,c) MS² spectra of peanut peptides VLHLGLL and VAITQHSKP obtained during the BUP analyses and **(b,d)** the standard addition calibration plots using these peptides markers for the quantitation of peanut and hazelnut in samples B3 and B2, respectively. The extraction and BUP parameters are as detailed in *Materials and Methods* section 5.3.2-4.

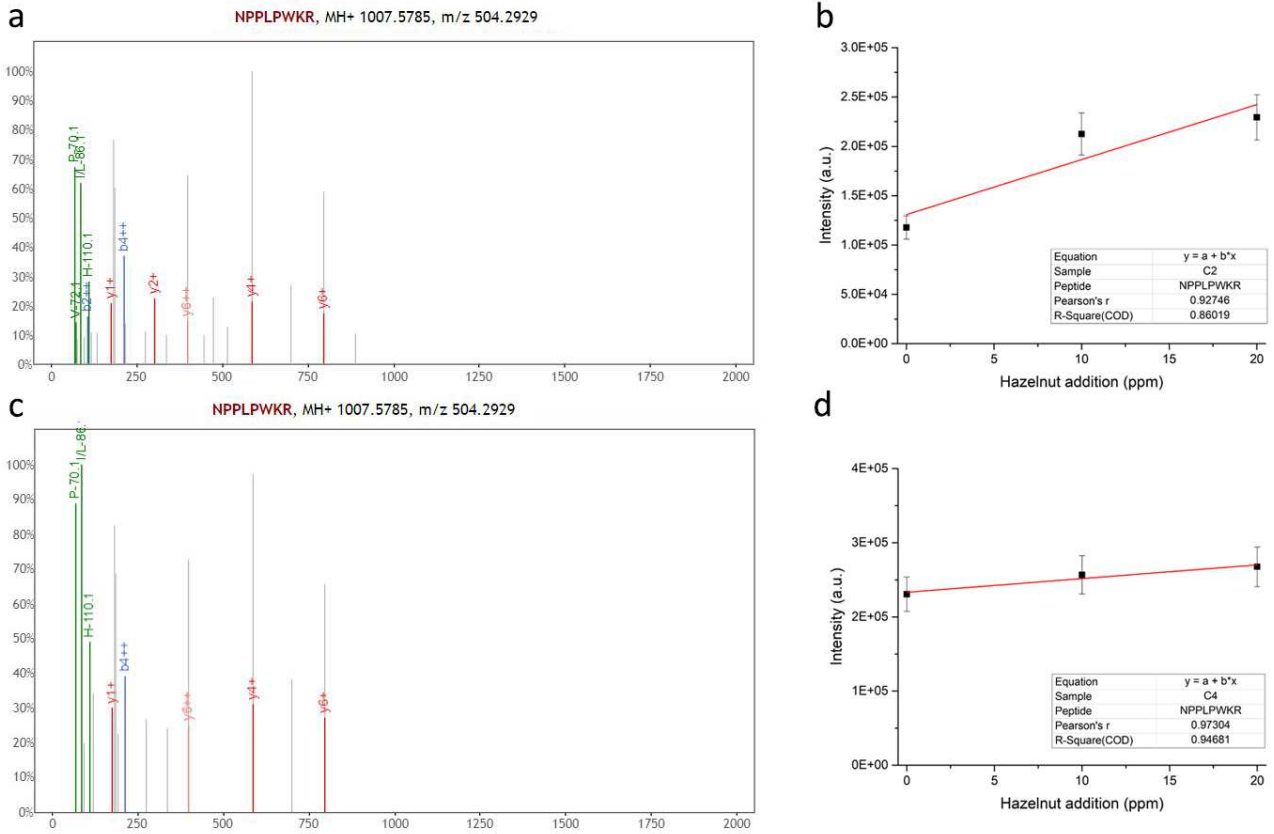


Figure III. (a,c) MS² spectra of the peptide NPPLPWKR (hazelnut) obtained during the BUP analyses and **(b,d)** the standard addition calibration plots using this peptide marker for the quantitation of peanut and hazelnut in samples C2 and C4, respectively. The extraction and BUP parameters are as detailed in *Materials and Methods* section 5.3.2-4.

Chapter 6.

General conclusions and perspectives

The first part of this thesis (Chapter 2) was devoted to the development of a fast and personalized allergy diagnosis based on IMS and MALDI-MS. The patient IgE antibodies were extracted from blood sera by magnetic beads coated with the appropriate capture antibodies. The immunocomplexes obtained on the magnetic beads were then probed with individual allergens or allergenic food extracts. After the elution of the captured sensitizers, MALDI-MS was used to identify them based on their intact masses or by peptide mass fingerprinting. The CRD procedure by IMS-MALDI-MS was successfully tested on several patients suffering from cow's milk and egg allergies. Additionally, two complex clinical cases of undetermined allergies were resolved using the developed procedure.

In Chapter 3, the personalized CRD procedure by IMS-MALDI-MS was modified and tested to diagnose peanut and tree nut allergies using patient blood sera collected for *ProNut* clinical study. In a first step, allergenic nut extracts were prepared in-house directly from the whole nuts *i.e.* almond, cashew nut, hazelnut, peanut, pecan nut, pistachio and walnut. BUP and TDP approaches were used to assess the content and quality of those extracts. The presence of the majority of the allergens in the extracts was confirmed by the BUP analyses, while partial or extensive protein degradation was observed in the TDP procedures. The homemade characterized nut extracts were employed to perform the CRD of nut allergies for 22 peanut-allergic patients by IMS-nanoESI-HRMS. On the individual allergen level, the diagnosis outcomes were in partial agreement with the standard commercial *in vitro* test (ImmunoCAP). Based on OFCs, ImmunoCAP and IMS-nanoESI-HRMS outcomes, important cross-reactivity and cross-sensitization information were obtained for several major peanut allergens. However, the reliable identification of cross-sensitizers was limited by the number of patients tested in the present work and the quality of the natural

extracts prepared in-house. Additional patients are therefore required to obtain a complete cross-reactivity/sensitization representation of peanut and tree nuts allergies.

Performing the allergy diagnosis by IMS-MALDI-MS or IMS-nanoESI-HRMS using natural extracts allows identifying in one fast experiment the complete sensitization profile of a patient. In addition, these methods open a possibility for identifying new, rare or modified allergens, whereas standard *in vitro* tests are limited to a given list of known allergens. Moreover, accurate epitope mapping and complete allergen characterization are achievable. Compared to the original IMS-MALDI-MS method, performing the diagnosis by IMS-nanoESI-HRMS increases the accuracy thanks to high-resolution achieved by the instrument and the use of a lock mass. Further improvements and optimizations of the two methods are planned to decrease the required experimental time and the sample consumption. Additionally, the IMS procedure will be automatized using a recently purchased pipetting robot (OT-1, Opentrons, New York, USA). This will increase the reproducibility and the quality of the procedure by reducing the errors and variations, caused by the human factor. Moreover, a large reduction in the overall time required for the IMS is expected and will result in decreased analysis costs.

The developed CRD methods are extremely versatile and not limited to food allergies. As a liquid sample is required for the IMS procedure, allergies to pollen, insect venom, mold and animal products (fur, dander and dust mites) are easily studied after dilution or extraction of the allergenic source of interest. IMS-nanoESI-HRMS will be used in the case of insect venom allergy. Hymenoptera allergies are effectively treated by immunotherapy for a majority of patients and the desensitization process is considered relatively safe. (1,2) However, severe anaphylactic reactions were observed during immunotherapy (3) or after the completion of the process. (4) Recently, the *Institut Central des Hôpitaux* (Sion, Switzerland) observed the case of a bee-allergic patient who suffered from an anaphylactic choc despite the successful completion of the immunotherapy. By using this patient blood serum, IMS-nanoESI-HRMS will allow comparing the native bee venom with the allergoids used for immunotherapy, resulting potentially in the characterization of new allergens or IgE epitopes.

In addition to allergenic diseases, the developed methods may be applied to other non-IgE-mediated pathologies. The lack of understanding and the complex diagnosis procedures make

autoimmune diseases the focus of numerous medical and bioanalytical studies. Autoimmune diseases are characterized by the irregular functioning of the immune system resulting in the generation of antibodies targeting the body tissues themselves. (5,6) The early detection and identification of blood-based autoantibodies are crucial in autoimmune diseases, as they may be present in blood serum before any clinical manifestation. (7) Additionally, the immune system has the ability to produce antibodies against tumor-associated antigens in the early stage of cancers. (8) As cancer is one of the leading causes of death, the identification of specific autoantibodies in various cancer types is the focal point of many studies. (9–12) However, the standard techniques for autoantibodies detection are mainly based on ELISA, immunofluorescence and protein microarrays, allowing the quantification of polyclonal antibodies but not the identification of specific monoclonal serum antibodies relevant for the diagnosis and prognosis of the disease. (13,14) Meanwhile, proteomic approaches have been used only recently for the identification of serum antibodies. (14,15) By coating the magnetic beads with the appropriate capture antibodies, IMS-nanoESI-MS may be applied to autoimmune diseases and cancer researches to extract, identify, and characterize the autoantibodies and their antigens following proteomic approaches.

In Chapter 4, the nitration of milk and egg white allergens has been studied using various chemical reactions and the nitration sites have been identified by nanoESI-HRMS following a BUP approach. Allergen nitration is one of the suspected causes of the allergy prevalence increase observed during the last decades. (16) However, the impact of allergen nitration on the immune system and IgE-binding potential is still poorly understood. The identification of nitration sites could help the understanding and prediction of the potential consequences caused by protein nitration. In further work, the allergenic response of nitrated proteins and extracts will be evaluated by IMS-nanoESI-HRMS and IMS-MALDI-MS. As shown in this chapter, oxidation is a standard side-reaction of protein nitration. The oxidized and nitrated proteoforms will thus be separated from each other and concentrated by liquid chromatography, capillary electrophoresis, OFFGEL electrophoresis or IMS using anti-3-nitrotyrosine antibodies, allowing the determination of the allergenicity impact of solely nitrated proteins and the identification of possible cumulative effects caused by the simultaneous oxidation of the products. Additionally, the epitope mapping of native and nitrated allergens will be carried out by IMS-nanoESI-HRMS. Both epitope excision and extraction procedures (17) will be performed to obtain a complete representation of the nitration impact on epitope

recognition by patient IgE antibodies. In addition to food allergens, it is planned to study the nitration of pollen allergens, such as the birch allergen *Bet v 1*.

In Chapter 5, traces of peanut and hazelnut were identified and quantified in various industrial food products (breakfast cereals, biscuits and chocolates) by BUP approaches with a label-free quantification method based on peptide ion intensities in native and spiked samples. As allergen-free certified food products are not currently available in Switzerland, the LOD and LOQ were not calculated. Further optimization of the methods and the importation of allergen-free certified products for the determination of the LOD/LOQ are planned.

Mass spectrometry techniques are becoming more popular for food quality controls as they offer a high reliability in allergen identification with similar or better sensitivity as the current tests based on ELISA. (18) As food processing modify the allergenicity of proteins, (19) the detection of allergens in a food by a standard BUP approach does not imply its potential binding to IgE antibodies. By using the blood sera of allergic patients, IMS-nanoESI-MS is able to identify accurately the presence of low abundant allergens in a food extract. This method may therefore be applied in food quality control by pooling together blood sera containing IgE antibodies against all the known allergens potentially present in the food product. IMS-nanoESI-MS would yield a true representation of the allergenicity potential of food products while maintaining accurate identification and high sensibility thanks to MS. Another application of IMS-nanoESI-HRMS will be the analysis of hypoallergenic food or allergoids, in which the allergenicity is intentionally reduced by physical or chemical processes. (20–26)

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Curriculum Vitae

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Technical skills and qualifications

Immunoassay	Allergy diagnosis
Liquid chromatography (LC, HPLC, UPLC)	Proteomics (Bottom-Up, Top-Down, Middle-Down/Up)
Mass spectrometry (MALDI-TOF-MS, ESI-MS, Orbitrap)	Protein modifications
Nuclear magnetic resonance (NMR)	Electrochemistry
Handling and analysis of antibodies	Sample preparation

Last/current Position

2015-2019 **PhD student at Laboratoire d'Électrochimie Physique et Analytique (LEPA), EPFL**
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Education and training

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 Master thesis : *"Immunomagnetic separation applied to IgE antibodies extraction and component resolved diagnosis of cow's milk allergy"*
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2010-2013 **Bachelor of Science – Chemistry, with an Erasmus exchange (Imperial College London, UK)**
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 Mathematics. Specific options: Biology / Chemistry. Complementary options: Physics
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Professional experience

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Relevant publications and contributions in conferences

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Journal of Allergy and Clinical Immunology, 2018, 141 (6), 2297-2300
- Paper **In vitro allergen nitration: comparison of various nitrating agents and identification of nitration sites by bottom-up proteomics**
M. Frossard, N. Gasilova, H. H. Girault
Under preparation, 2019
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Talk: *Personalized and rapid test for food-related allergy*
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Poster and talk: *Personalized and rapid food allergy test using natural allergenic extracts*
- Conference **Société Suisse d'Allergologie et d'Immunologie (SSAI) Congress 2017, St. Gallen (Switzerland)**
Poster: *Personalized and rapid food allergy test using natural allergenic extracts*
- Summer school / Conference **Mass Spectrometry in Biotechnology and Medicine (MSBM), 2016, Dubrovnik (Croatia)**
Poster and talk: *Rapid component-resolved diagnosis of cow's milk allergy by immunomagnetic separation with MALDI-MS detection*
- Conference **EPFL Integrative Food and Nutrition Centre, 2015, Lausanne (Switzerland)**
Poster: *Rapid component-resolved diagnosis of cow's milk allergy by immunomagnetic separation with MALDI-MS detection*

Awards

- 2017 **Best poster and oral presentation of the Innovative allergy diagnostic session**
European Academy of Allergy and Clinical Immunology (EAACI) Congress 2017 (Helsinki, Finland)
- 2015 **Syngenta Monthey Prize**, best results for the master cycle in the section of Chemistry and Chemical Engineering of EPFL
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