

The definition of a representative control group was challenging. The risks attributed to thymectomy can be regarded as superimposed on risks related to the underlying cardiac defect. To distinguish the effects of thymectomy from the underlying heart defect, we used 2 different control groups. Individuals in the surgery control group underwent cardiac surgery before the age of 5 years, but the type of surgery did not involve thymectomy. Inherently the thymectomized individuals and the surgery controls do not share underlying cardiac defects, a fact that compromises comparability.

The nationwide Swedish registers enable follow-up with minimal losses.⁸ Nonetheless an ascertainment bias cannot be excluded because thymectomized persons are perhaps more likely than the general population to be managed at hospital clinics, with excellent register coverage, than by private caregivers where coverage is uncertain, or in primary health care without register coverage. However, the surgery control group is probably similar to thymectomized persons in this respect.

Early thymectomy leads to immunological changes reminiscent of those accompanied by aging.⁹ Possible clinical consequences of thymectomy do not necessarily become overt until later in life and therefore it is important to take into consideration the relatively young age and short follow-up time of most of the individuals included in this study. Their ages at the end of the study range from 1 to 37 years (mean, 14 years) and the mean follow-up time was 13 years.

This study detects risk differences for certain autoimmune diseases, cancer, infections, and asthma following early thymectomy. Because of the observational nature of this study, it was not possible to ascertain a causal mechanism. Even so, thymectomy must be regarded as a potential explanation for the findings, and we find it advisable during early cardiac surgery to avoid total thymectomy if possible.

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Personalized and rapid test for food-related allergy



To the Editor:

Skin prick tests and oral food challenges are the criterion standards for *in vivo* food allergy diagnoses.¹ To confirm the *in vivo* diagnosis, typically *in vitro* analyses of specific IgEs are performed, referred to as component-resolved diagnosis (CRD). Such tests are also useful in estimating the risk associated with particular allergen classes and allergy persistence,²⁻⁴ although they are able to detect IgEs only against known allergens, thus limiting the clinicians in the choice of potential sensitizers. To tackle this issue, we introduce in this study a new personalized allergy CRD test based on immunomagnetic separation with whole food extract application and mass spectrometric detection (Fig 1, A). Immunomagnetic separation is used to extract IgEs from the blood serum of an allergic patient using magnetic beads functionalized with antihuman IgE antibodies. Then, allergy CRD is performed by probing the binding of obtained IgEs/antihuman IgE antibodies immunocomplexes with individual protein solutions or natural extracts, corresponding to the targeted allergy. After elution of captured allergens and IgEs from the beads surface, we identify extracted proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS).

First, IgE extraction optimization was carried out with a 2 µg/mL solution of commercial control human IgEs, mimicking their concentration in a real sample, and then with a commercial blood serum from a patient with known allergy to milk. The obtained optimal conditions are detailed in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org. As a second step, we conducted the proposed milk allergy CRD on the same commercial blood serum using standard milk protein solutions: β-lactoglobulin (β-LG, A and B isoforms), BSA, and lactoferrin (LF). The collected MALDI-MS spectra (Fig 1, B and C) indicated that BSA-specific and LF-specific IgEs were present in the patient blood serum, causing his sensibility to these proteins. Meanwhile, no β-LG signal was detected at *m/z* 18,400 (Fig 1, D), pointing out the absence of β-LG-specific IgEs in patient blood serum.

Afterwards, whole ultra-heat treatment (UHT) milk fractions were directly used for CRD and yielded clear MALDI-MS signals

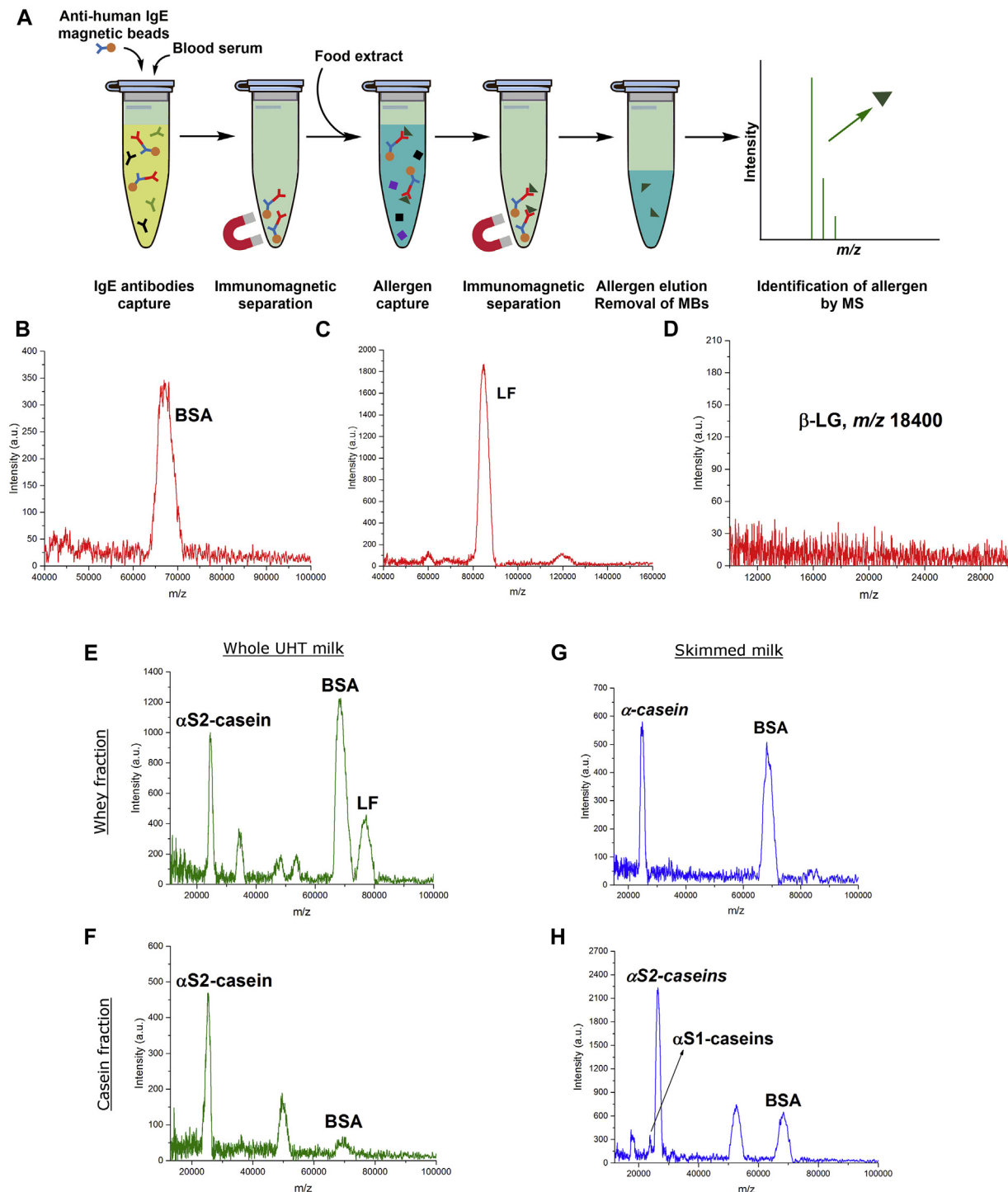


FIG 1. Developed allergy CRD procedure (A). MALDI-MS spectra obtained during the milk allergy CRD of the commercial blood serum using solution of BSA (B), LF (C), β -LG (D), whey (E) fraction of whole UHT milk, casein fraction of whole UHT milk (F), whey fraction of skimmed milk powder (G), and casein fraction of skimmed milk powder (H). MBs, Magnetic beads.

of BSA, LF, and α S2-casein (Fig 1, E and F), revealing the patient sensibility to these proteins. Extraction of casein traces from whey fraction, as well as BSA from casein fraction, highlighted the overall high sensitivity of developed CRD. CRD performance with skimmed milk fractions additionally identified α S1-casein

as a potential sensitizer (Fig 1, G and H). Furthermore, we successfully conducted negative control diagnoses using pure and BSA-fortified soymilk (see Fig E1 in this article's Online Repository at www.jacionline.org). The data presented are in a good accordance with each other, confirming the results

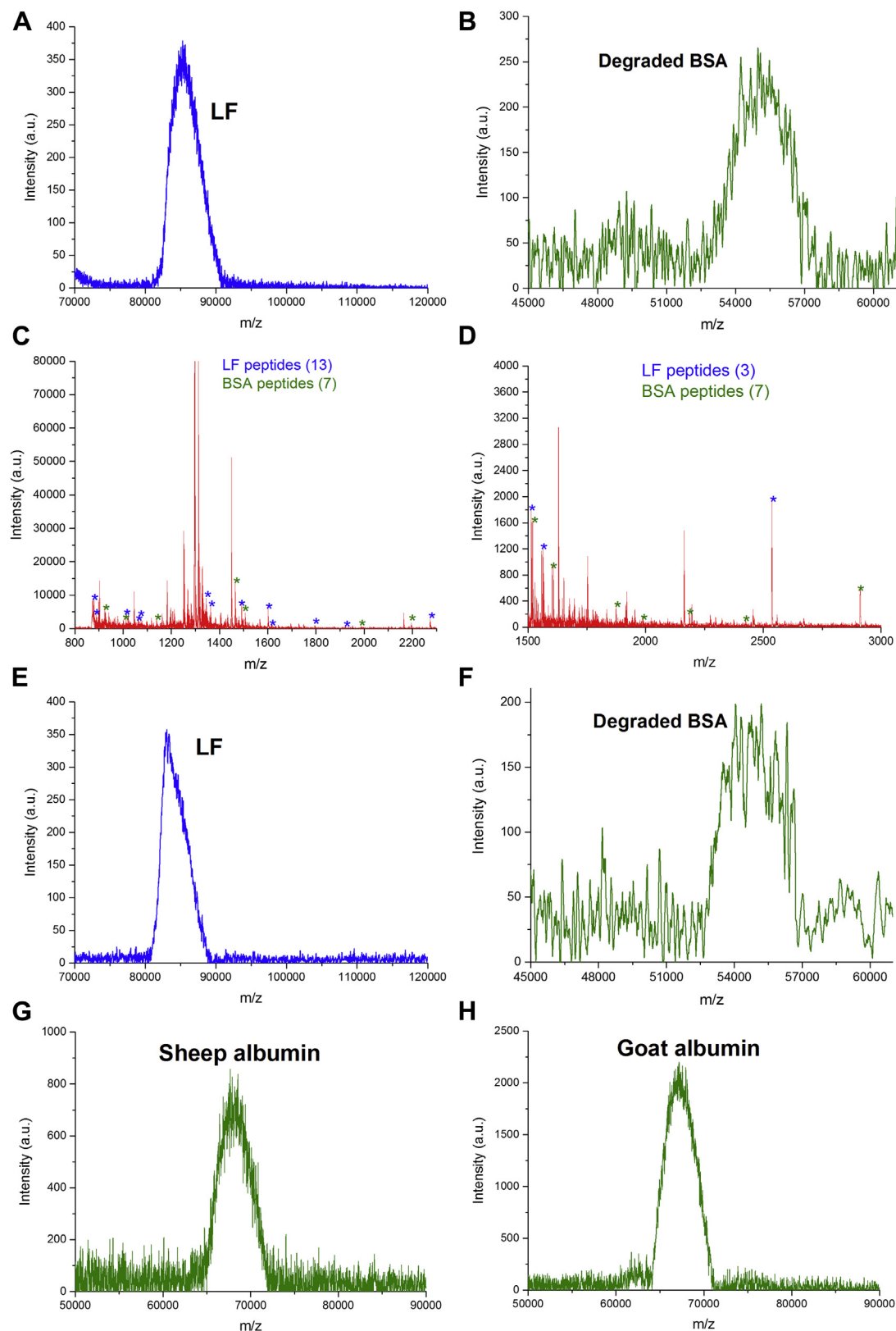


FIG 2. MALDI-MS spectra obtained during the milk allergy CRD of the clinical blood serum using allergenic whey fraction (**A** and **B**). The diagnosis was confirmed by PMF (**C** and **D**) and by using pure solution of LF (**E**) and BSA (**F**). MALDI-MS spectra corresponding to the cross-reactivity tests using the whey fractions of sheep (**G**) and goat (**H**) milk.

obtained with individual protein solutions. As in the previously published work,⁵ we showed that the chosen patient is potentially allergic to BSA, LF, and α S1- and α S2-caseins.

The proposed CRD procedure can potentially suffer from the restrictions of MALDI-MS detection in linear mode over a large mass range, because resolution decreases quickly with the mass range increase,⁶ resulting in peak broadening and poor discrimination between the molecules with close m/z values. Therefore, peptide mass fingerprinting (PMF) with MALDI-MS detection in reflectron mode was implemented for allergen identification. Extracted allergenic proteins were digested with trypsin immobilized into macroporous ordered siliceous foam, ensuring fast (<1 hour) proteolysis with low level of trypsin autolysis.⁷ In single CRD experiment with whey fraction of whole UHT milk, patient's sensibility to LF, BSA, and α S1- and α S2-caseins was confirmed by detecting 9, 11, 6, and 9 corresponding peptides, respectively (see Table E1 and Fig E2 in this article's Online Repository at www.jacionline.org).

In addition to these tests, the developed method was successfully applied to 5 supplementary blood sera: 1 patient with a proven milk allergy, 3 with an egg white allergy, and 1 neither allergic to milk nor egg used as a negative control. In the case of egg allergy, the PMF procedure has been slightly modified to take into account the trypsin inhibitor effect of ovomucoid and the resistance to trypsin proteolysis of lysozyme. Information on these patients and detailed CRD results are present in this article's Online Repository at www.jacionline.org.

As a proof-of-concept, we applied the proposed allergy test to determine the sensitization profile of a clinical patient suffering from protein contact dermatitis, when exposed to the cow's whey fraction. This diagnosis was confirmed by skin prick test with corresponding whey fraction, while ImmunoCAP and ImmunoCAP ISAC (Phadia/ThermoScientific, Sweden) analyses performed in clinical settings with common milk allergens yielded inconclusive results. A detailed clinical case record is present in this article's Online Repository at www.jacionline.org. The Immunology and Allergology Department of Institut Central des Hôpitaux (Sion, Switzerland) provided both the patient's blood serum and whey fraction to perform the developed allergy CRD.

CRD performance with allergenic whey fraction revealed the presence of LF-specific IgEs in the patient blood serum due to LF peak observation in the MALDI-MS spectrum (Fig 2, A). A second weak peak was also detected with m/z around 55,000 (Fig 2, B), corresponding possibly to partially degraded BSA. To identify with certainty the extracted allergens, we carried out PMF (Fig 2, C and D), identifying in total 16 and 12 unique peptides for LF and BSA, respectively (see Table E2 in this article's Online Repository at www.jacionline.org). In addition, we validated these results and reconfirmed the identity of the signal at m/z 55,000 by realizing the diagnosis with pure LF and BSA solutions (Fig 2, C and D). Importantly, both partially degraded BSA and LF were identified as sensitizers for this patient due to direct application of allergenic whey fraction. Meanwhile, standard *in vitro* clinical test for BSA sensitivity failed potentially because of structural variations (like posttranslational modifications or partial protein degradation) between commercial proteins, used in the test, and the ones, present in allergenic whey fraction.

Moreover, to complete allergy diagnosis, we also performed the cross-reactivity studies by developed CRD. Application of

goat and sheep milk whey fractions indicated patient's sensitivity to the serum albumins of these 2 species (Fig 2, E and F), that is, potential cross-reactivity between goat, sheep, and bovine albumins, caused by their high sequence homologies (>90%). Cosensitization to these allergens also should not be excluded because it is hard to differentiate it from cross-reactivity.

Described clinical sample analysis clearly demonstrates the diagnostic potential of developed immunomagnetic separation with MALDI-MS detection for personalized allergy CRD, especially when commercial *in vitro* test results are inconclusive. The proposed technique provides an efficient and rapid allergy test with both types of allergen sources, individual allergen solutions and allergenic food extracts. Although herein we applied it mainly to cow's milk allergy diagnosis, the described procedure can be easily extended to any kind of food allergy with minor modifications, as it was shown for egg allergy. Our method ensures 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. We believe that the designed CRD approach can overcome issues of standard diagnostic methods at the molecular level, because it is not limited to a standard allergens list, allows precise allergen identification, and facilitates cross-reactivity studies.

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Online Repository: Personalized and rapid test for food-related allergy

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Supplementary Methods

Chemicals 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 sulfoxide (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 Bioreclamation LLC (Westbury, NY, USA). All the milk proteins used, polysorbate surfactant Tween 20, 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), soy milk, 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 serum of a clinical patient with possible milk allergy and the bovine whey fraction, to which he was sensitive, were kindly provided by the Hospital of Sion (Switzerland).

Functionalization of MBs

Functionalization of MBs was performed according to the manufacturer protocol (1). 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 resuspended in the storage buffer (10 mM PBS solution, 0.025% Tween 20, 0.02%

NaN₃, pH 7.4) to obtain a final concentration of 5 mg·mL⁻¹. All the MBs were used within a period of 3 weeks after their coating and were stored at 4°C.

Chemical cross-linking of IgE to MBs

The procedure of IgE cross-linking to the MBs was adapted from the DMS manufacturer protocol (2) to obtain an efficient cross-linking with minimal loss of antibodies activity. After the IgE 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 allergy) 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 CRD or stored in the storage buffer at 4°C.

Milk fraction and egg white protein solution preparation

A 20 mg·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.

Optimized immunomagnetic separation procedure for the milk allergy CRD

150 µg of anti-human IgE-antibodies-functionalized MBs were transferred into an Eppendorf tube and washed three times with the washing buffer. 100 µL of the blood serum from a milk 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 to MBs. The cross-linking procedure was adapted from the protocol of the chemical linker manufacturer¹⁹ and is detailed in Supplementary Methods.

The resulting MBs with the immunocomplexes, formed on their surface by anti-human IgE-antibodies and the patient IgE, were incubated with 200 μ L of individual milk protein solutions (200 μ g·mL⁻¹ concentration) or milk 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 were diluted three times 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 μ L of elution buffer (5 % acetic acid (HAc) or 1:3 (v/v) methanol/water mixture) to the MBs pellet and 1 μ L of the eluate was submitted to the MALDI-MS analysis. All CRD experiments were performed in triplicates.

Preparation of trypsin-loaded MOSF

1 mg of MOSF was added to 100 μ g 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 μ L of ammonium bicarbonate buffer, at a final working MOSF concentration of 5 mg·mL⁻¹.

Allergen digestion using trypsin loaded MOSF

For the captured milk allergens digestion, trypsin immobilization into MOSF was performed as described previously(3). Prior to the digestion, MBs bearing all immunocomplexes, formed during the allergy CRD, were added into 10 μ L of 25 mM ammonium bicarbonate buffer (pH 7.8), and directly denatured at 100°C for 5 min without an elution step. After the denaturation, MBs were removed from the reaction mixture. 1 μ L 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 μ L 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 55°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.

MALDI-MS analysis

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 50 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 14169.76 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 462 ppm. The obtained peptides mass spectra were processed using the FindMod tool from ExPASy (Expert Protein Analysis System) of the Swiss Institute of Bioinformatics (4). The mass tolerance for the similarity analyses was chosen as 0.5 Da with 3 allowed missed cleavages.

Control CRD experiments using soy milk and fortified soy milk

Soy milk, a beverage obtained from soy beans, is usually used as cow's milk substituent for people suffering from milk allergy. As soy proteins do not share similar amino acids 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(5). For these patients, rice-based or hydrolysed 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 soy milk (Fig. E1.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 μ L of soy milk diluted in 100 μ L of water (Fig. E1.b).

Meanwhile, fortifying soy milk with BSA to its final concentration of 200 μ g mL⁻¹ (Fig E1.c and insert) led to successful extraction and identification BSA during the CRD of milk allergy (Fig. E1.d) without detection of any soy proteins. These control tests confirmed the efficiency and selectivity of developed diagnostic technique and showed that the patient, whose blood serum was used, is not sensitive to soy proteins.

Identification of allergens extracted from whole UHT milk by peptide mass fingerprinting

After the allergy CRD using the UHT milk whey fraction, allergen digestion resulted in the MALDI-MS spectrum presented in Fig. E2. 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 (Online Repository Table I) 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(6,7), owing to an effective non-specific interaction suppression during developed CRD. Other unidentified signals could come from patient IgE, anti-human IgE-antibodies from the MBs coating and typical human keratin contaminants.

The peptide mass fingerprinting 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.

Application of the developed CRD method to additional blood sera with a proven allergy

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 in *Supplementary Methods*. Application of the whey fraction revealed a low intensity signal in the MALDI-MS spectrum (Fig. E3, a) corresponding to lactoferrin. Identification of this protein was further confirmed by PMF (Fig. E3, b) with 4 detected peptides, listed in Online Repository Table III. The CRD performance with casein fraction of UHT milk (Fig. E3, 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 (Fig. E3, d and Online Repository Table IV).

Egg allergy

The developed CRD method was further tested 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-IgE 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 $1 \text{ mg} \cdot \text{mL}^{-1}$. To avoid non-specific interactions with lipids and other components, two additional washing 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 Fig. E4. 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 antibodies specific to lysozyme and ovotransferrin (Fig. E4, b and insert) and patient N°3 to ovalbumin (Fig. E4, 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 (Fig. E4, 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.

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(8) and trypsin inhibitor properties(9). 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 (Fig. E5) confirmed the CRD of egg allergy obtained in Fig. E4. 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 antibodies 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. All peptides identified in these PMF analyses are listed in Online Repository Table V. Application of PMF for patient N°4 yielded only peptides originating from keratin contaminants and trypsin autolysis.

With these tests on egg allergy, we have shown that the method originally developed for milk allergy is easily transposed to other allergy 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.

Clinical case: detailed medical history

A 57 years old patient, working as cheese-maker, 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 centre 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, *Dermatophagoïdes pteronyssinus* and *farina*, latex). Epidermal tests with the European Standard Serie (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 tests (ImmunoCAP, Phadia/ThermoScientific, Sweden) were negative for the following milk proteins: α -lactalbumin (nBos d4), β -lactoglobulin (nBos d5), casein (nBos d8) and bovine serum albumin (nBos d6).

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). This test yielded no positive IgE specificity. The patient total IgE level was measured at 86 kU/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.

Supplementary Tables

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

Protein	Observed <i>m/z</i>	Corresponding peptide sequence	Position	Known modifications
Lactoferrin, LF	647.5	CVPNSK	534-539	
	659.6	AIAEKK	67-72	
	1266.5	RWQWRMKK	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	VDSALYLGSRYLTTLKNLR	333-351	
BSA	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	FWGKYLYEIARR	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	ATEEQLKTVMENFVAFVDKCCAADDK	562-587	
αS1-casein	831.2	EDVPSEK	99-105	
	2192.8	VPQLEIVPNSAEERLHSMK	121-139	(1xMSO)
	2321.1	QMEAESISSSEEIVPNSVEQK	74-94	
	2321.1	EDVPSEKRYLGYLEQLRLK	99-117	
	2321.1	LKKYKVPQLEIVPNSAEER	116-134	(PHOS: 130)
	2656.7	QMEAESISSSEEIVPNSVEQK	74-94	(1xMSO, 4xPHOS)
αS2-casein	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	TVDMESTEVFTKKTKLTTEEK	153-173	(1xPHOS)
	2725.6	FPQYLQYLYQGPIVLNPWDQVK	107-128	(TPO: 124)
	2927.8	NANEEYSIGSSSEESAEEVATEEVK	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*.

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

Protein	Observed <i>m/z</i>	Corresponding peptide sequence	Position	Known modifications
Lactoferrin, LF	875.3	NGKNCPDK	640-647	
	888.3	SCHTAVDR	475-482	
	1016.5	KSCHTAVDR	474-482	
	1063.6	NLNREDFR	582-589	
	1079.0	RAFALECIR	58-66	
	1344.7	VVWCAVGPEEQK	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	EDLIWKLLSKAQEKFGKNK	283-301	
	2535.3	NDTVWENTNGESTADWAKNLNR	564-585	
BSA	927.5	YLYEIAR	161-167	
	977.5	NECFLSHK	123-130	
	1138.6	CASIQKFGER	223-232	
	1465.7	VGTRCCTKPESER	456-468	
	1490.8	FGERALKAWSVAR	229-241	
	1519.9	LKPDPNTLCDEFK	139-151	
	1601.9	FWGKYLYEAIIR	157-168	
	1875.0	IETMREKVLASSARQR	205-220	
	1987.0	VCLASSARQLRCASIQK	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*.

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

Protein	Observed m/z	Corresponding peptide sequence	Position	Known modifications
Lactoferrin, LF	904.4	CVPNSKEK	534-541	
	922.4	AFALECIK	59-66	
	1320.5	SFQLFGSPPGQR	304-315	
	1982.9	CVPNSKEKYYGYTGAFK	534-550	

* Allergy CRD was performed using optimized immunomagnetic separation procedure with acetic acid elution, while the digestion using trypsin loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*.

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

Protein	Observed <i>m/z</i>	Corresponding 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	RELEELNVPGEIVESLSSEESITR	16-40	(3xPHOS)
α S1-casein	1267.6	YLGYLEQLLR	106-115	
	1337.6	HIQKEDVPSEK	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	
	1050.5	KISQRYQK	181-188	
	1098.5	AMKPWIQPK	204-212	
	1195.5	NAVITPTLNR	130-140	
	1251.6	EQLSTSEENSK	141-151	
	1251.6	TKVIPYVRYL	213-222	
	1761.9	LTEEEKNRLNFLKK	168-181	
	1864	FALPQYLKTVYQHQQK	189-203	
	1983.1	TVYQHQQAMKPWIQPK	197-212	
	1991.2	KTKLTEEEKNRLNFLK	165-180	
	1991.2	TKLTEEEKNRLNFLKK	166-181	
	2331.4	AMKPWIQPKTKVIPYVRYL	204-222	
	2556.4	NAVITPTLNREQLSTSEENSKK	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*.

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

Patient	Protein	Observed <i>m/z</i>	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	CNFCNAVVESNGTLTSLSHFGKC	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	CNFCNAVVESNGTLTSLSHFGKC	189-210	3xCYS_CAM
N°2	Ovotransferrin	893.4	VAAHAVVAR	266-274	
		914.4	IRDLLER	623-629	
		1015.3	KSCHTAVGR	471-479	CYS_CAM
		1037.3	DEKSKCDR	372-379	CYS_CAM
		1047.4	YFGYTALR	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	TGTCNFDEYFSEGCAPGSPPNSR	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	FDKLPFGGDSIEAQCGTSVNVHSSLR	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	CNFCNAVVESNGTLTLSHFGKC	189-210	3xCYS_CAM

* Allergy CRD was performed using optimized immunomagnetic separation procedure with acetic acid solution while the digestion using trypsin loaded MOSF was conducted following the digestion protocol detailed in *Materials and Methods*.

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Supplementary Figure Legends

Fig. E1. 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 ($200\text{ }\mu\text{g mL}^{-1}$) 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.

Fig. E2. 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. All recognized peptides are marked with an asterisk of corresponding colour.

Fig. E3. 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 colour.

Fig. E4. 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).

Fig. E5. 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 colour.

