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Component-Resolved Diagnostic of Cow's Milk Allergy by Immunoaffinity Capillary Electrophoresis–Matrix Assisted Laser Desorption/Ionization Mass Spectrometry

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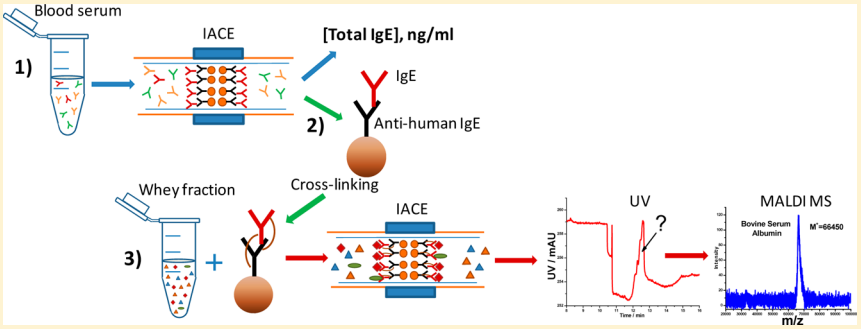
Supporting Information



ABSTRACT: Component-resolved diagnostic (CRD) of cow's milk allergy has been performed using immunoaffinity capillary electrophoresis (IACE) coupled with matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). First, total IgE quantification in the blood serum of a milk allergic patient by the IACE-UV technique was developed using magnetic beads (MBs) coated with antihuman IgE antibodies (Abs) to perform the general allergy diagnosis. Then, the immunocomplex of antihuman IgE Abs with the patient IgE Abs, obtained during the total IgE analysis, was chemically cross-linked on the MBs surface. Prepared immunosupport was used for the binding of individual milk allergens to identify the proteins triggering the allergy by IACE with UV and MALDI MS detection. Then, allergy CRD was also performed directly with milk fractions. Bovine serum albumin, lactoferrin, and α-casein (S1 and S2 forms, as was revealed by MALDI MS) were found to bind with the extracted IgE Abs, indicating that the chosen patient is allergic to these proteins. The results were confirmed by performing classical enzyme-linked immunosorbent assay of total and specific IgE Abs. The present IACE-UV/MALDI MS method required only 2 μL of blood serum and allowed the performance of the total IgE quantification and CRD of the food allergy not only with the purified allergen molecules but also directly with the food extract. Such an approach opens the possibility for direct identification of allergens molecular mass and structure, discovery of unusual allergens, which could be useful for precise personalized allergy diagnostic, allergens epitope mapping, and cross-reactivity studies.

Cow's milk allergy is a widespread food-related immunological disorder especially common during infancy and childhood.[1] Being one of the first alimentary products introduced into an early childhood diet, cow's milk frequently triggers the development of IgE-mediated hypersensitivity to its components by the human organism.[2] In this case, the allergic patients display various allergic reactions (rhinitis, asthma, urticarial, diarrhea, etc.) to whey milk proteins like α-lactalbumin, β-lactoglobulin, to caseins, or to both.[3,4] In clinics, to distinguish these symptoms from the non-IgE-mediated milk hypersensitivity[5] and to diagnose milk allergy, skin prick tests and the detection of cow's milk specific IgE antibodies (Abs) by enzyme-linked immunosorbent assays are recommended.[6] The confirmation of the diagnosis is typically performed by oral food challenge, while atopy patch tests can be also performed.[6,7] These methods of allergy diagnosis provide a general conclusion about the absence or presence of hypersensitivity to cow's milk. For more detailed information about the allergy origin and potential outgrowth prognosis, component resolved diagnostic (CRD) of cow's milk allergy should be performed.[2] Microarray-based CRD employs purified natural or recombinant allergens for precise identification of primary sensitizers provoked milk allergy development for each particular patient.[8] The possibility to study cross-reactivity between allergens of the different nature[9] and to constantly monitor the level of specific IgE Abs for various individual allergens for the prognosis of milk tolerance development[10] make the CRD an attractive method for clinical analysis.

Commercially available microarray systems for various food allergies, like ImmunoCAP ISAC-CRD 103 (Phadia, Sweden),[11]

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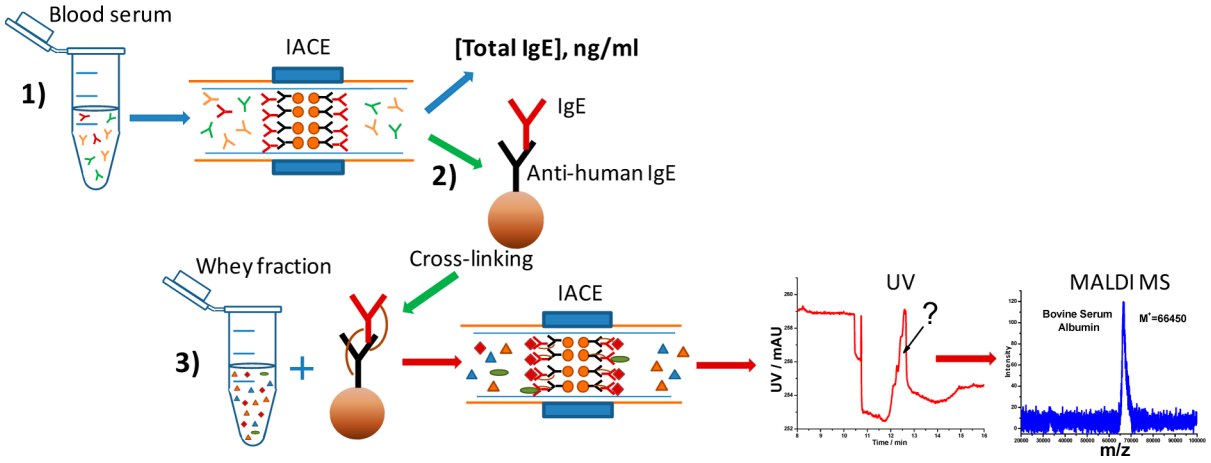
Scheme 1. General Experimental Workflow[a]



[a](1) Total IgE quantification in blood serum of the milk allergic patient by IACE-UV analysis. (2) IgE Abs extraction using the IACE protocol and further IgE Abs fixation on the MBs surface by cross-linking reaction. (3) CRD of cow's milk allergy by IACE analysis with UV and MALDI MS detection using MBs with a cross-linked immunocomplex.

as well as the ones under laboratory development[12−14] are based on the principal of classical two step immunoassay with fluorescence detection. Surface plasmon resonance[15] and electrochemical immunosensors,[16,17] magnetophoretic immunoassay,[18] interferometric reflectance imaging sensors,[19] and affinity probe electrophoresis[20] are also used to detect the level of total and specific IgE Abs in human blood serum. In the listed analytical methods, the allergy diagnosis is realized via the detection/quantification of specific IgE Abs using purified recombinant allergens from a standard list, defined for every type of food allergy.[21] Unexpected or rare allergens are normally disregarded by these techniques. A good example is bovine IgG Abs, which were detected as milk allergens by two-dimensional immunoblotting followed by mass spectrometric detection due to the use of diluted skimmed milk samples.[22]

Immunoaffinity capillary electrophoresis (IACE), a hybrid technique combining immunoaffinity extraction with capillary electrophoretic separation, is suitable for allergy diagnosis as well. It is a powerful tool for complex samples analysis due to the simultaneous performance of the selective analyte enrichment and purification followed by highly resolved electrophoretic separation.[23] IACE has become a well-established method for biomarkers determination,[24] proteomics,[25] and biomolecules interactions studies.[26] This technique with the use of MBs-based immunosupport and UV detection was already demonstrated for β-lactoglobulin analysis in milk.[27] The detection sensitivity was significantly improved when IACE was coupled with matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) for analysis of whey proteins in soy milk for hypoallergenic food quality control.[28] Application of MS detection opens the possibility of simultaneous structural analysis of several analytes with high detection sensitivity. In general, such approaches involving MS detection are important for allergens detection during food quality control and for allergen epitope mapping[29] but typically are not applied directly for allergy diagnosis. For example, IACE was already reported for the total IgE quantification in blood serum, but only using laser-induced fluorescence detection.[30]

Herein, a new type of milk allergy diagnostic based on MS detection is demonstrated. It does not require the specific IgE Abs detection or quantification and is able to identify for the milk allergic patient the whole spectrum of his sensitizers from cow's milk without the laborious and serum consuming two-dimensional immunoblotting procedure.[22] Such personalized allergy CRD was performed by specially developed IACE analysis with UV and MALDI MS detection with direct application of the patient blood serum and milk extracts. At the beginning, the total IgE quantification in the patient blood serum was performed using optimized MBs-based IACE with UV detection for general allergy diagnosis. Then, the IACE-based protocol was introduced for the efficient online IgE Abs extraction on MBs surface via the formation of the antihuman IgE−IgE Abs complex. After the immunocomplex stabilization by chemical cross-linking, this newly obtained immunosupport was used in the IACE-UV/MALDI MS analysis of milk fractions (as well as individual milk proteins) to identify milk sensitizers for the chosen patient. The proposed technique allowed total IgE quantification and sensitive allergy CRD with only 2 μL of blood serum, sufficient for the performance of 25−30 IACE runs during the allergen identification step. Feasible due to the MS detection, direct application of milk extracts provided the possibility of allergen mass and structure characterization. It allows the identification not only of typical but also unknown or unusual allergens normally disregarded by other diagnostic techniques. The presented format of CRD is suitable for any kind of food allergies and could be useful for allergens epitope mapping and cross-reactivity studies as well.

EXPERIMENTAL SECTION

Chemicals and Materials. Estapor tosyl-activated superparamagnetic beads of uniform size (1.29 μm diameter) were kindly offered by Merck Chimie SAS (Fontenay-sous-Bois, France). Blood serum of the patient allergic to the cow's milk was purchased from Bioreclamation LLC (NY). Control blood serum of the patient with no allergies was kindly offered by the Regional Blood Transfusion Service of canton Vaud, Switzerland. Human IgE control Abs and dimethyl suberimidate (DMS) were obtained from Pierce Biotechnology (IL). Monoclonal antihuman IgE Abs (0100-0414) were purchased from AbD Serotec (Oxford, U.K.). More information about chemicals and materials is presented in Supporting Information SI-1.

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General Workflow of the Performed Experiments. The general workflow of the experiments performed for the CRD of cow's milk allergy by IACE-UV/MALDI MS analysis is presented in Scheme 1.

As shown in Scheme 1, the first step consisted of the total IgE quantification in a blood serum of the milk allergic patient by IACE-UV analysis using MBs coated with antihuman IgE Abs. The same protocol was introduced at the second step to extract the IgE Abs and to further cross-link the immunocomplex of antihuman IgE-human IgE Abs formed on the MBs surface. During the third step, prepared immunosupport was utilized for the performance of personalized CRD by IACE analysis with UV and MALDI MS detection. The detailed description of each experimental step is presented below.

Magnetic Beads Derivatization. MBs were coated with antihuman IgE Abs according to the manufacturer protocol. A total of 2 mg of rinsed tosyl-activated MBs were mixed with 280 μL of coating buffer (0.1 M sodium borate, pH 9.5), 166 μL of 3 M ammonium sulfate in coating buffer, and 54 μL of Abs (1.5 mg/mL). The mixture was incubated overnight at room temperature under continuous moderate stirring to avoid MBs sedimentation. Afterward MBs were incubated with a blocking buffer containing 0.1% Tween 20 in 10 mM PBS (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) during 1 h at room temperature, were rinsed with washing buffer (0.05% Tween 20 in 10 mM PBS) and stored in 10 mM PBS, containing 0.025% of Tween 20 and 0.02% of sodium azide at 4 °C. To roughly estimate the amount of Abs bound to the MBs surface, a BCA (bicinchoninic acid) protein test (BCA kit, Pierce, Rockford) was carried out following the standard manufacturer protocol. The amount of Abs bound per 1 mg of MBs was defined as 42 μg. Prior to each experiment, MBs coated with antihuman IgE Abs were rinsed and diluted to a working concentration of 0.5 mg/mL with 10 mM PBS containing 0.05% Tween 20.

IACE-UV Experiments for Total IgE Analysis. During IACE-UV experiments for total IgE analysis carried out with a 7100 A CE apparatus (Agilent, Waldbronn, Germany), the following buffers were used: leading electrolyte (50 mM ammonium acetate, pH = 8); sample and washing buffer (10 mM PBS with 0.05% of Tween 20, pH = 7.4); elution buffer and terminating electrolyte (10% acetic acid with 0.1% TFA, pH = 1.5); separation buffer (10% acetic acid, pH = 2). All the solutions were loaded with a 40 mbar injection pressure into the fused silica separation capillary (50 μm i.d., 41.5 cm effective length, 50 cm total length) coated with 5% hydroxypropylcellulose (HPC) solution, as described elsewhere.[31]

The general principal scheme of IACE-UV analysis was shown previously.[28] MBs coated with antihuman IgE Abs (0.5 mg/mL) were loaded for 5 min into the separation capillary where they were trapped by two permanent cylindrical magnets (Nd−Fe−B, 4 mm diameter, 12 mm length), which were placed directly around the capillary at a distance of 14.5 cm from the inlet using a homemade Plexiglas holder. After 4 min injection of the washing solution, the standard solution of control IgE Abs (concentration range 0.24−2400 ng/mL) or diluted blood sera were injected for 10 min. The washing step was realized first by injecting sequentially washing buffer and deionized water for 5 min each, and second, by introducing the leading electrolyte for 10 min. The direct injection of the elution buffer during 220 (±10) s for releasing trapped analyte molecules was followed by the application of 24 kV CE separation voltage and UV signal detection at 200 nm during 20 min. At the end of the analysis, the capillary was flushed at high pressure, 950 mbar, to remove the MBs plug and clean the capillary.

IgE Abs Extraction and Covalent Fixation on MBs Surface. The IgE Abs extraction from the blood serum of a milk allergic patient was realized online inside the CE separation capillary following the IACE-UV protocol described above. Instead of the elution buffer injection and the high voltage application steps, MBs with extracted IgE Abs were washed out from the capillary at high pressure, 950 mbar, to a collection vial. Such extraction procedure was repeated 15 times. Collected MBs were rinsed and resuspended in 20 μL of washing buffer yielding the final MBs concentration of ∼0.135 mg/mL.

For the comparison, the off-line IgE Abs isolation procedure was performed as well. A volume of 20 μL of MBs coated with antihuman IgE Abs at 10 mg/mL concentration in washing buffer were mixed with 20 μL of blood serum diluted 10 times in the same washing buffer. After 30 min of incubation under agitation, the supernatant was discarded; MBs were rinsed 3 times with the washing buffer and resuspended in 80 μL of the same buffer to a final concentration of 2.5 mg/mL. Then, the solution of MBs with extracted IgE Abs was injected inside the CE separation capillary for 100 s at 40 mbar to perform analyte elution and separation steps of IACE-UV analysis. Obtained results were compared with online IgE Abs extraction, when 0.135 mg/mL MBs solution was injected inside the CE system for 300 s at 40 mbar.

In order to chemically stabilize the noncovalent immunocomplex of antihuman IgE Abs with patient IgE Abs on the MBs surface, a cross-linking reaction with dimethyl suberimidate (DMS) was performed following the manufacturer protocol. MBs, collected during IgE Abs extraction, were suspended into 15 μL of 200 mM triethanolamine, pH 9, and mixed with 75 μL of 20 mM DMS dissolved in 200 mM triethanolamine. After 30 min of incubation with slow agitation at room temperature, the cross-linking reaction was stopped by adding 15 μL of 50 mM Tris solution, pH 7.5, in which MBs were left for another 15 min of incubation. At the end, MBs were resuspended in 20 μL of 10 mM PBS containing 0.05% Tween 20.

CRD by IACE with UV and MALDI MS Detection. CRD of cow's milk allergy with milk whey proteins was realized using similar IACE protocol as the one described above for total IgE quantification but with minor modifications. MBs with extracted and cross-linked IgE Abs were loaded inside the capillary for 6 min as immunosupport, and individual milk whey proteins at fixed concentrations, or dilute milk whey fraction, were injected as a sample for 10 min. Meanwhile, the milk allergy CRD with individual caseins and casein fraction was performed using the same IACE protocol but also with addition of 0.1 M urea to the elution and separation buffers to increase proteins solubility.

After the CRD performance by IACE with UV detection, the same experiments were realized using MALDI MS detection. The hyphenation of IACE with MALDI-TOF-MS Microflex instrument (Bruker Daltonics, Bremen, Germany) was realized via a homemade automated fraction collection interface as described elsewhere.[28] More details are presented in SI-2 in the Supporting Information. For MALDI MS measurements of collected CE fractions, 2 mg/mL sinapinic acid solution in 70% acetonitrile, 29.9% of water, 0.1% TFA was used as a matrix. The instrument was operated in a positive linear ion mode, and an average spectrum from 500 laser shots at different spot locations was collected for each CE fraction spot.

Milk Fractions Preparation. For the CRD performance with direct application of the whole milk extract, skimmed milk

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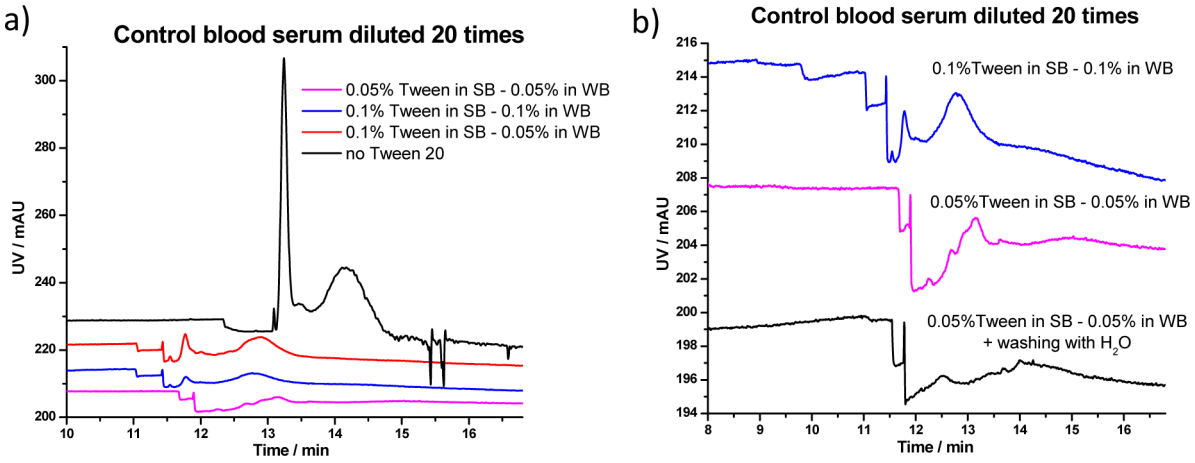

Figure 1. Nonspecific adsorption elimination on the example of control blood serum. (a) Application of various sample and washing buffers: only PBS (black line); PBS/0.1% Tween 20 in the sample buffer and 0.05% Tween 20 in the washing buffer (red line); PBS/0.1% Tween 20 in both buffers (blue line); PBS/0.05% Tween 20 in both buffers (pink line). b) Effect of additional washing step: 5 min washing with PBS/0.1% Tween 20 (blue line); 5 min washing with PBS/0.05% Tween 20 (pink line); 5 min washing with PBS/0.05% Tween 20 followed by 5 min washing with deionized water (black line). Conditions: typical IACE protocol with 5 min of MBs (0.5 mg/mL) loading, 10 min sample injection, system washing, sample elution, and application of 24 kV during 20 min for the CE separation; CE-UV electropherograms at 200 nm, HPC coated capillary, total/effective length 41.5/50 cm, 50 μm i.d., injection pressure of 40 mbar. PBS concentration: 10 mM. SB: sample buffer, WB: washing buffer.

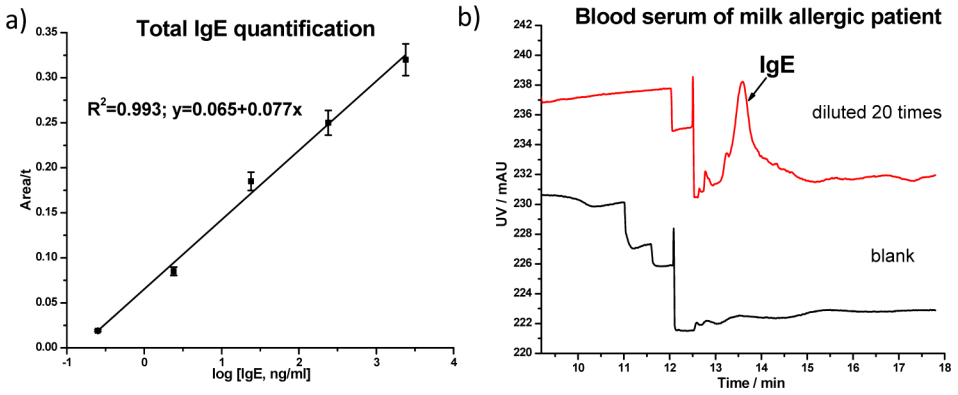

Figure 2. Total IgE quantification by IACE-UV analysis. (a) Calibration curve of the corrected IgE peak areas vs log[IgE]. (b) Example of the electropherogram obtained during total IgE quantification in the 20 times diluted patient blood serum (red line) and in the blank sample (only sample buffer, black line). Conditions: 10 mM PBS/0.05% Tween 20 as sample and washing buffers; system washing with 5 min of washing buffer and 5 min with deionized water. Other experimental conditions were the same as in Figure 1

powder was dissolved in deionized water at 20 mg/mL concentration. The casein fraction was precipitated from 2 mL of solution by adding 50 mM ammonium acetate buffer, pH 4.5.[32] After centrifugation at 12 000*g* for 2 min, the supernatant with whey proteins was separated from the casein precipitate and was ready to use. Casein precipitate was washed with the ammonium acetate buffer and was dissolved in 2 mL of 25 mM ammonium bicarbonate buffer, pH 8.5. The same protocol was applied for ultrahigh-temperature (UHT) treated milk which was defatted by centrifugation at 12 000*g* for 5 min before further manipulations. Prior to the IACE analysis, whey and casein fraction solutions were diluted 20 times with the sample buffer.

RESULTS AND DISCUSSION

Optimization of Total IgE Quantification by IACE-UV Analysis. As a first step, the total IgE quantification procedure for a general allergy diagnostic was developed. The injection of the blood serum, one of the most complex biological fluids, typically causes the strong nonspecific adsorption of various proteins in the analytical systems. HPC coating of the capillary inner surface was used to prevent protein adsorption on the capillary walls. Meanwhile, to minimize the nonspecific interactions in the system, a mixture of 10 mM PBS with Tween 20 (pH = 7.4) was used as sample and washing buffers. PBS and neutral detergent Tween 20 are known for their ability to quench protein adhesion[33] not only on MBs but also on the capillary walls. Moreover, PBS and Tween 20 are successfully applied in the analytical systems, such as the one under consideration, where the typical blocking reagents, like bovine or human serum albumins, milk casein, and ovalbumin, may interfere with the performed analysis.[30]

The efficiency of the nonspecific adsorption elimination was tested on the total IgE quantification by IACE-UV in the control blood serum of the patient with no allergies, i.e., with IgE Abs level below 240 ng/mL (100 IU/mL).[30] Prior to the analysis, the serum was diluted 20 times with various sample buffers. As displayed on the electropherogram in Figure 1a (black line), without Tween 20 the nonspecific adsorption in the system was very strong and significantly increased the analyte migration time. The addition of detergent decreased this effect, especially if the content of Tween 20 was the same in both sample and

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washing buffers, 0.1 or 0.05% (Figure 1a, blue and pink lines). The latter value of the detergent concentration showed the most effective suppression of nonspecific interactions.

Addition of the washing step with deionized water reduced protein adhesion on MBs and on the capillary walls even more (Figure 1a, green line), possibly due to its pH equal to 5.5, which is below the p*I* of the most abundant serum proteins, like human serum albumin and IgG Abs.[34,35] In this case, the lateral repulsion between adsorbed proteins and conformational unfolding help to decrease the nonspecific adsorption in the system.[33] The peak remaining at 12.50 min was systematic, while the peak at 14.00 min was corresponding to the IgE Abs presented in this serum. More details about nonspecific adsorption elimination are presented in SI-3 in the Supporting Information. Other IACE parameters, like the quantity of MBs to load, type of the leading and terminating electrolytes, the injection time of the elution buffer, and value of the CE separation voltage, were adopted from the previous studies.[28,30]

The optimized IACE-UV protocol was employed for the analysis of standard control IgE Abs solutions at 0.24−2400 ng/mL concentration range to generate a calibration curve for total IgE quantification. Good linearity (R^2 = 0.993) was achieved between the corrected analyte peak areas and logarithm of the analyte concentration (Figure 2a) as well as a good reproducibility for corrected peak areas (RSD = 7%, n = 3) and IgE Abs migration time (RSD = 4%, n = 5).

The LOD, calculated as 3 signal-to-noise ratios, was determined to be 0.24 ng/mL (0.1 IU/mL). The total IgE concentration in the blood serum of the milk allergic patient was detected by the developed IACE-UV analysis (Figure 2b) as 1940 ± 140 ng/mL (810 ± 60 IU/mL, n = 3), confirming the general allergy diagnosis. This value of IgE Abs concentration is in a good correlation (CV < 10%) with the data obtained for the analysis of the same sample by classical commercial ELISA: 2150 ± 160 ng/mL (900 ± 70 IU/mL, n = 3).

In comparison with the developed technique, total IgE quantification using cation exchange with nanocrystal clusters[36] and nanoparticle hybrid probes[37] is more sensitive 48 and 6 times, respectively. However, these methods require up to 100 μL of the sample, while the present IACE-UV analysis consumes only 2 μL of patient blood serum. Moreover, obtained LOD is 50 times better than the one of used commercial ELISA kit from Abnova (Heidelberg, Germany),[38] 3 and 10 times better than for previously reported affinity CE and IACE-based methods.[20,30] Taking into account the threshold value of IgE Abs concentration to diagnose an allergy (240 ng/mL or 100 IU/mL in blood serum),[30] it can be concluded, that the achieved sensitivity of IACE-UV analysis is suitable for total IgE quantification in clinical practice, where not only the high sensitivity but also low sample consumption is of primary importance.

Extraction of IgE Abs from Patient Blood Serum and Covalent Fixation on MBs Surface. The extraction of IgE Abs from the patient blood serum was performed online inside the capillary using the modified IACE-UV protocol. Elution buffer injection and high voltage application steps were replaced by flushing MBs with extracted IgE Abs out of the capillary into a collection vial.

The serum dilution and its injection time were optimized to provide the optimal IgE Abs collection efficiency with minimal influence of nonspecific interactions. As shown in Figure 3 (black and red lines), serum dilution 10 times provided extraction of the large amount of IgE Abs but also a high level of nonspecific interactions despite all preventive measures taken.

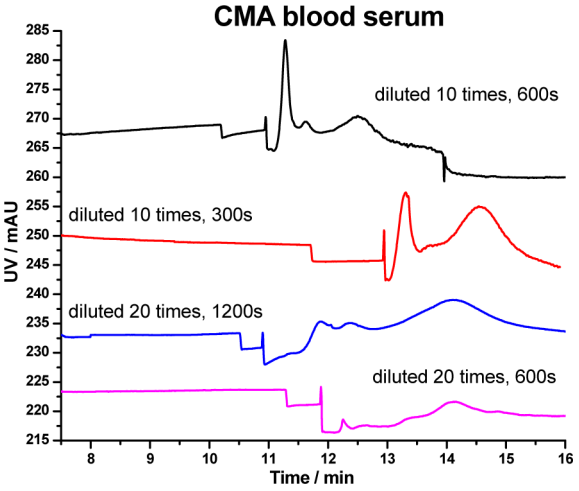

Figure 3. Optimization of the IgE Abs extraction parameters. Typical IACE protocol with two washing steps (5 min with PBS/0.05% Tween 20 and 5 min with deionized water). Sample injection: serum diluted 10 times and injected during 600s (black line), serum diluted 10 times and injected during 300 s (red line), serum diluted 20 times and injected 1200 s (blue line), serum diluted 20 times and injected 600 s (pink line). Other conditions: CE-UV electropherograms at 200 nm, HPC coated capillary total/effective length 41.5/50 cm, 50 μm i.d., injection pressure of 40 mbar.

If the serum was diluted 20 times and injected into the capillary during 1200 s, some nonspecific sample adsorption was still observed (Figure 3, blue line). The most effective suppression of nonspecific interactions in the system was achieved for 20 times diluted serum injected during 600 s (Figure 3, pink line), same parameters as for total IgE quantification procedure. Therefore, these values were used for the performance of IgE Abs extraction.

The repetition of the extraction experiment was performed 15 times to collect a sufficient amount of MBs (20 μL, concentration ∼0.135 mg/mL) for further manipulations. It is worth mentioning, that the number of extraction experiments, as well as the number of serum injection/system washing cycles within one extraction, are not absolute and should be always adapted in accordance with the initial IgE Abs concentration in the blood serum. On the basis of the results obtained in the present work, it was assumed that the double sample injection/washing cycle should be applied, when the detected total IgE Abs concentration is below 400 ng/mL (167 IU/mL, 5 times smaller than in the blood serum used in the present work). More detailed explanations are provided in SI-4 in the Supporting Information.

In order to prove the efficiency of the proposed online IgE Abs extraction, the off-line IgE Abs isolation was performed for comparison, following the standard protocol proposed by MBs manufacturers.[39] The amount of isolated IgE Abs for both, online and off-line, extraction procedures was defined by injecting the corresponding MBs with extracted IgE Abs inside the CE system and performing analyte elution/separation steps of IACE-UV analysis (Figure 4a).

On the electropherogram obtained with MBs collected after online IgE Abs extraction (Figure 4a, red line), a large peak corresponding to IgE Abs was observed at 12.60 min. At the same time, on the electropherogram corresponding to the off-line IgE Abs extraction (Figure 4a, black line), only a small IgE Abs peak (at the LOD level) was obtained despite the use of higher MBs concentration and less diluted blood serum.

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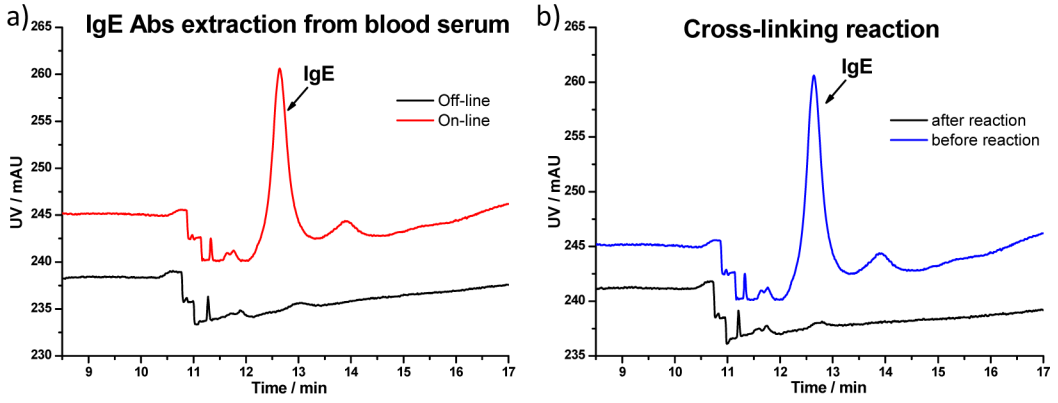

Figure 4. (a) IgE Abs extraction from the blood serum of milk allergic patient. Electropherograms obtained during IACE-UV analysis of IgE Abs extracted on MBs surface using online IACE protocol (red line) and off-line commercial protocol (black line). MBs loading: 100 s (2.5 mg/mL) and 300 s (0.135 mg/mL) for off-line and online protocols, respectively. (b) Chemical cross-linking of extracted IgE Abs. Electropherograms obtained during IACE-UV analysis of IgE Abs extracted on MBs surface using online IACE protocol: before (blue line) and after (black line) cross-linking reaction. MBs (0.135 mg/mL) loading: 300 s. Conditions: sample elution and CE separation steps were directly performed after MBs loading. Other experimental conditions were the same as in Figure 1

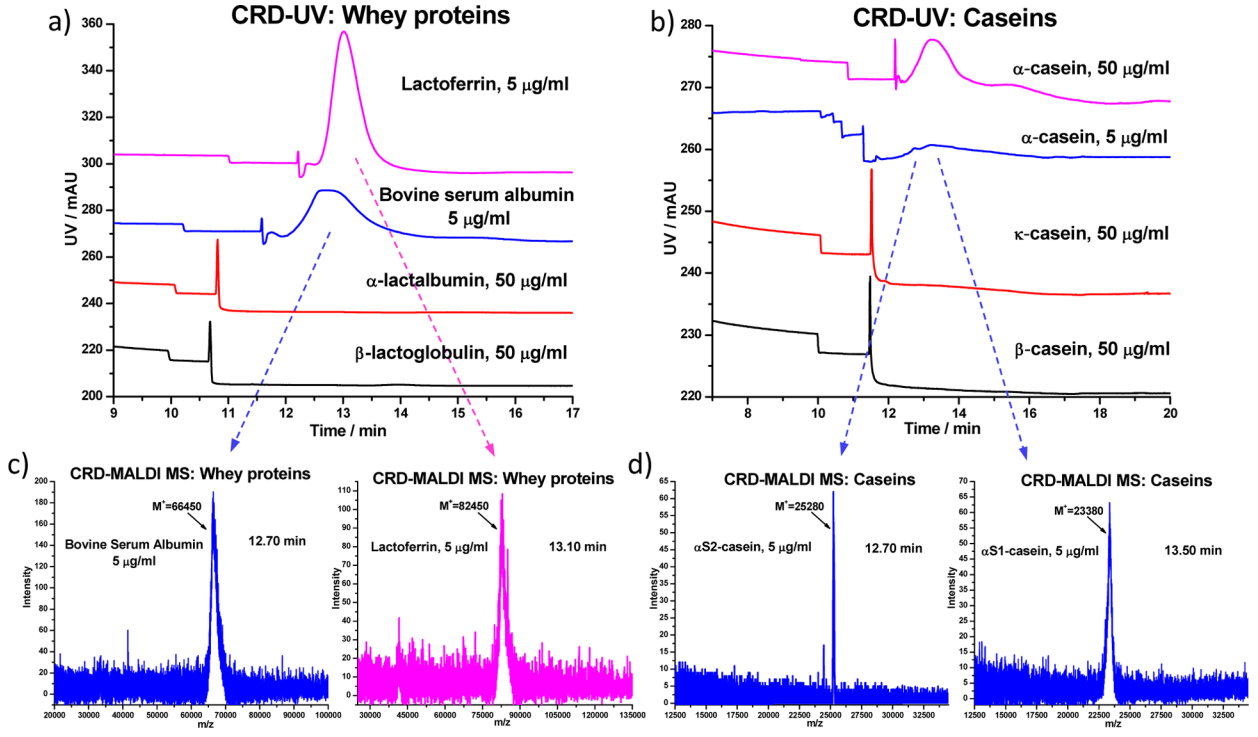

Figure 5. CRD of cow's milk allergy using solutions of individual purified allergens. (a) Electropherograms obtained during CRD by IACE-UV analysis of the milk whey proteins: β-lactoglobulin (50 μg/mL, black line), α-lactalbumin (50 μg/mL, red line), bovine serum albumin (5 μg/mL, blue line), and lactoferrin (5 μg/mL, pink line). (b) Electropherograms obtained during CRD by IACE-UV analysis of the milk caseins: β-casein (50 μg/mL, black line), κ-casein (50 μg/mL, red line), and α-casein (5 μg/mL, blue line and 50 μg/mL, pink line). (c) MALDI MS spectra collected during CRD by IACE-MALDI MS analysis of the milk whey proteins: bovine serum albumin (5 μg/mL, eluted at 12.70 min) and lactoferrin (5 μg/mL, eluted at 13.10 min). (d) MALDI MS spectra collected during CRD by IACE-MALDI MS analysis of the milk caseins: αS2-casein (eluted at 12.70 min) and αS1-casein (eluted at 13.50 min), total α-casein concentration of 5 μg/mL. Conditions: 402 s of MBs loading (0.135 mg/mL, with extracted and cross-linked IgE Abs). For caseins analysis, 0.1 mM urea was added to the elution and separation buffers. Other experimental conditions were the same as in Figure 2

This newly developed online extraction procedure based on IACE-UV protocol is more efficient than the off-line protocol and allowed the extraction of more IgE Abs by a smaller amount of MBs. This fact could be potentially explained by the high local concentration of MBs trapped inside the capillary and their better availability to the binding with IgE Abs presented in the blood serum.

Antihuman IgE-patient IgE Abs immunocomplex obtained after online extraction was submitted to a cross-linking reaction for its covalent fixation on the MBs surface. Chemical cross-linking increased not only the stability of the obtained immunosupport but also the sensitivity of the analysis. It allowed avoiding the effect of the analyte signal masking by the Abs signal, observed, for example, for the use of protein-A coated MBs in IACE analysis.[27] In this case, the analyte of average molecular

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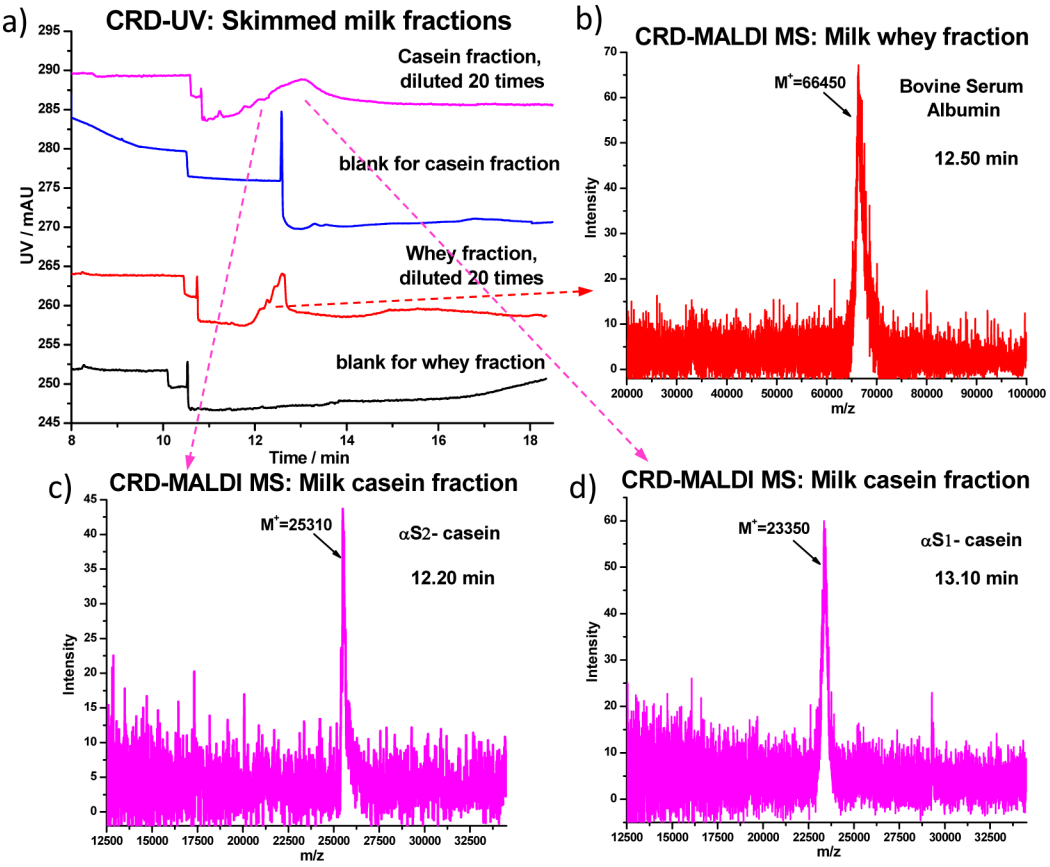

Figure 6. CRD of cow's milk allergy using skimmed milk fractions. (a) Electropherograms obtained during CRD by IACE-UV analysis of the milk fractions: blank for whey fraction (black line), whey fraction (diluted 20 times, red line), blank for casein fraction (blue line), casein fraction (diluted 20 times, pink line). Blank samples contained only sample buffer. (b) MALDI MS spectra collected during CRD by IACE-MALDI MS analysis of the milk whey fraction: bovine serum albumin (eluted at 12.50 min). (c, d) MALDI MS spectra collected during CRD by IACE-MALDI MS analysis of the milk casein fraction: αS2-casein (eluted at 12.20 min) and αS1-casein (eluted at 13.10 min), respectively. Experimental conditions were the same as in Figure 5

weight (~18.3 kDa) provided a weak UV signal neighboring with the large and broad signal of IgG Abs (~150 kDa molecular weight), due to their simultaneous elution from the MBs and further CE separation. As the IgE Abs molecular weight is even larger (~180 kDa), such an effect was expected to be more significant and was prevented by the performance of the cross-linking reaction with DMS, water-soluble imidoester-based cross-linker with spacer arm of 11.0 Å.

In-capillary cross-linking reaction could lead to the modification of the capillary walls and MBs by the cross-linker molecules in an unpredictable manner and thus could interfere with the analysis. Therefore, this reaction was performed off-line and its efficiency was checked by using MBs with extracted IgE Abs for IACE-UV analysis before and after cross-linking (Figure 4b). Before the reaction with DMS, a large peak corresponding to the extracted IgE Abs was observed on the electropherogram (Figure 4b, blue line). This peak disappeared after the cross-linking reaction (Figure 4b, black line), indicating that IgE Abs were covalently attached to the molecules of antihuman IgE Abs on the MBs surface and could not be eluted by the elution buffer any further.

Prepared immunosupport was stored in 10 mM PBS, containing 0.025% of Tween 20 and 0.02% of sodium azide at 4 °C without losing its immunological activity at least during 1 week. Moreover, its quantity was enough to perform 25−30 diagnostic experiments for the same patient, taking into account that one CRD by IACE analysis consumed around 0.5 μL of cross-linked MBs solution.

CRD by IACE Analysis with UV and MALDI MS Detection. Prepared MBs with covalently fixed patient IgE Abs were further used as a personalized immunosupport for the CRD performance by developed IACE analysis with UV and MALDI MS detection. Together with the total IgE quantification and IgE Abs extraction, the whole procedure required only 2 μL of patient blood serum.

First, CRD of cow's milk allergy was performed with individual purified bovine milk allergens, which were analyzed at concentrations of 5 or 50 μg/mL. For whey proteins, the IACE protocol was used without modifications, while for caseins 0.1 M of urea was added to the elution and separation buffers to increase proteins solubility at acidic pH. The addition of urea did not influence the CE separation but slightly decreased the sensitivity of MALDI MS detection. It resulted in a rather high noise level because of the analyte ion suppression effect caused by urea.

Among whey proteins, bovine serum albumin and lactoferrin displayed binding with IgE Abs on the MBs surface during IACE-UV analysis (Figure 5a, blue and pink lines). As no α-lactalbumin and β-lactoglobulin peaks were detected on the electropherograms obtained with 5 μg/mL solutions of these proteins (data not shown), their concentrations were increased to 50 μg/mL (Figure 5a, black and red lines). However, no signal, i.e., no

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binding, was observed for such high sample concentrations either, ensuring that the nonspecific adsorption in the system was efficiently minimized and that the proteins did interact with immunosupport via a reaction with IgE Abs.

The CRD by IACE-MALDI MS provided the same results as for UV detection. No proteins signal was observed in any of the collected CE fractions in the case of 50 μg/mL α-lactalbumin and β-lactoglobulin solutions (data not shown), while bovine serum albumin and lactoferrin at 5 μg/mL concentration were easily detected on MALDI MS spectra from the corresponding CE fractions (Figure 5c). Therefore, it was concluded that only these two proteins reacted with extracted IgE Abs.

In the case of caseins, β- and κ-caseins did not bind with the immunosupport neither at 5 μg/mL (data not shown) nor at 50 μg/mL concentration (Figure 5b, black and red lines). A relatively small peak of α-casein was observed at 5 μg/mL concentration (Figure 5b, blue line). Its presence was confirmed by increasing the sample concentration to 50 μg/mL (Figure 5b, pink line), indicating that α-casein did react with IgE Abs. However, in the described conditions, it was difficult to distinguish based on the CE separation, which form of the protein, αS1- or αS2-casein, participated in the immune reaction with IgE Abs. The performance of CRD by IACE with MALDI MS detection solved this problem. As presented in Figure 5d, the broad peak on electropherograms (Figure 5b, blue and pink lines) corresponded to the overlapped peaks of two α-casein forms. αS2 and αS1-caseins were observed on MALDI MS spectra obtained from the CE fractions collected for 5 μg/mL sample concentration at 12.00 and 12.80 min, respectively.

Application of the MALDI MS as a detection method opened the possibility to facilitate and accelerate the diagnostic experiments by the direct use of milk fractions for the IACE analysis. Obtained results for the milk allergy CRD using whey and casein fractions from 20 mg/mL skimmed milk powder solution are presented in Figure 6. For the whey milk fraction, a peak at 12.50 min was obtained on the electropherogram (Figure 6a, red line). It corresponded to the bovine serum albumin, as was defined by the performance of the IACE with MALDI MS detection (Figure 6b).

However, lactoferrin was not detected in any of the collected CE fractions, as its concentration in whey fraction is very low. Therefore, a separate experiment with individual solution of this protein would be always necessary for the complete allergy CRD. The MS signal from bovine IgG Abs, a potential unusual milk allergen, was also not observed. As the concentration of bovine IgG Abs in the whey fraction is typically at the same level as the concentration of bovine serum albumin, obtained results indicate the absence of allergy to these immunoglobulins for the chosen patient.

During the casein fraction application for CRD, a broad peak at ~13 min was detected on the electropherogram (Figure 6a, pink line). CRD by IACE-MALDI MS analysis revealed that this peak corresponded to the two protein forms: αS2- and αS1-caseins were detected in the CE fractions collected at 12.20 and 13.10 min, respectively (Figure 6c,d).

CRD experiments by IACE with UV and MALDI MS detection were also performed using UHT milk fractions. Obtained results are presented in detail in SI-5 in the Supporting Information. The application of this type of cow's milk provided the same data, as for the skimmed milk fractions: bovine serum albumin, αS1- and αS2-caseins were detected as sensitizers for the chosen patient. Such result was expected owing to the similar origin, composition, and manufacturing processes of UHT and skimmed milk.

To prove again the effective suppression of the nonspecific interactions in the system (potential source of the false positive results), soy milk was used through CRD as a control sample and did not display any signal neither on the electropherograms nor on the MALDI MS spectra (data not shown). Therefore, from the performed CRD experiments with MALDI MS and UV detection, it can be concluded that the chosen patient is allergic to the bovine serum albumin, lactoferrin, αS1- and αS2-caseins. To check these results, classical qualitative ELISA of specific IgE Abs in the patient blood serum was performed using purified individual milk allergens. The positive responses about the presence of the specific IgE Abs were received for α-casein, bovine serum albumin, and lactoferrin (data not shown). These data confirmed the conclusion made by the CRD using developed IACE-UV/MALDI MS analysis. Moreover, from the obtained results it is possible to make a conclusion about potential cross-reactivity with beef allergy. As the chosen patient is allergic to bovine serum albumin, a protein largely present in beef, there is a probability for him to possess a beef allergy too, so-called cross-reactivity syndrome between cow's milk and beef.[40]

As was already mentioned, the presented analytical and diagnostic protocol requires only 2 μL of the patient blood serum for all manipulations. While commercial classical ELISA protocols[38,41] or microarrays systems,[11] as well as some techniques under laboratory development,[12−14,17,19] consume from 25 to 100 μL of blood serum. Once the solution of MBs with cross-linked IgE Abs is ready to use, it typically takes only around 55 min to perform the CRD with various allergens in contrast with 2−4 h of classical ELISA,[38,41] microarray protocol,[11] or laborious and serum consuming two-dimensional immunoblotting procedures.[22] At the same time, the amount of the immunosupport prepared at once for CRD by IACE analysis is sufficient for the performance of 25−30 diagnostic experiments. It can be stored at 4 °C during 1 week without alteration of its properties and biological reactivity and thus can be easily applied for other experimental work.

Another main advantage of the developed allergy diagnostic is the ability to detect for a particular patient all the compounds provoking an allergic response, owing to the direct application of the food extracts. It allows the identification of the standard allergenic substances as well as the discovery of the unusual and unexpected allergens, which could be difficult to realize by other methods. At the same time, the storage stability of patient IgE Abs-based immunosupport opens the possibility to perform additional studies, like allergen epitope mapping, cross-reactivity reactions, and interaction with allergen nitrated forms.

CONCLUSIONS

CRD of cow's milk allergy was developed using IACE with UV and MALDI MS detection. The foregoing total IgE quantification in the blood serum of milk allergic patient was realized by IACE-UV analysis with MBs-based immunosupport and allowed a general allergy diagnosis with the LOD of 0.24 ng/mL (0.01 IU/mL). The same IACE procedure was used for the IgE Abs extraction. Antihuman IgE- patient IgE Abs immunocomplex formed on MBs surface was chemically cross-linked, and newly obtained immunosupport was further employed for the CRD performance. IACE-UV protocol was efficient for CRD with individual purified allergens. Obtained results indicated that bovine serum albumin, lactoferrin, and α-casein were allergy

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sensitizers for the chosen patient. At the same time, the CRD based on IACE with MALDI MS detection provided reliable results not only for individual allergen solutions but also for the UHT and skimmed milk fractions. It revealed that both αS1- and αS2-caseins reacted with extracted IgE Abs.

The whole analytical and diagnostic procedure required only 2 μL of patient blood serum, which was enough for carrying out 25−30 IACE runs during the CRD step. Application of the MALDI MS detection allowed precise CRD performance not only with purified allergens but also directly employing food extracts. Such diagnostic procedure could be useful for the personalized food allergy diagnosis including the identification of unusual and unexpected allergens. For each particular patient it can provide the list of primary sensitizers, identify nature and structure of these molecules, simplify the allergens epitope mapping, and cross-reactivity studies.

■ ASSOCIATED CONTENT

Supporting Information

Details about used chemical and materials, description of the IACE coupling with the MALDI MS, and optimization of the IgE Abs extraction process using the IACE-UV protocol. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Schouten, B.; van Esch, B. C. A. M.; van Thuijl, A. O. J.; et al. *J. Allergy Clin. Immunol.* **2010**, *125*, 1308−1314.
(2) Hochwallner, H.; Schulmeister, U.; Swoboda, I.; Spitzauer, S.; Valenta, R. *Methods* **2014**, *66*, 22−33.
(3) Shek, L. P. C.; Bardina, L.; Castro, R.; Sampson, H. A.; Beyer, K. *Allergy* **2005**, *60*, 912−919.
(4) Monaci, L.; Tregoat, V.; Hengel, A. J.; Anklam, E. *Eur. Food Res. Technol.* **2006**, *223*, 149−179.
(5) Monaci, L.; Losito, I.; De Angelis, E.; Pilolli, R.; Visconti, A. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 2009−2018.
(6) Fiocchi, A.; Schünemann, H. J.; Brozek, J.; et al. *J. Allergy Clin. Immunol.* **2010**, *126*, 1119−1128.e12.
(7) Dupont, C. *Ann. Nutr. Metab.* **2011**, *59* (Suppl 1), 8−18.
(8) Hochwallner, H.; Schulmeister, U.; Swoboda, I.; et al. *Clin. Exp. Allergy* **2010**, *40*, 1809−1818.
(9) Valenta, R.; Ferreira, F.; Focke-Tejkl, M.; Linhart, B.; Niederberger, V.; Swoboda, I.; Vrtala, S. *Annu. Rev. Immunol.* **2010**, *28*, 211−241.
(10) Ahrens, B.; Lopes de Oliveira, L. C.; Grabenhenrich, L.; Schulz, G.; Niggemann, B.; Wahn, U.; Beyer, K. *Clin. Exp. Allergy* **2012**, *42*, 1630−1637.
(11) http://www.phadia.com/en/Products/Allergy-testing-products/ImmunoCAP-ISAC/.
(12) Fall, B. I.; Eberlein-König, B.; Behrendt, H.; Niessner, R.; Ring, J.; Weller, M. G. *Anal. Chem.* **2003**, *75*, 556−562.
(13) Monroe, M. R.; Reddington, A. P.; Collins, A. D.; LaBoda, C.; Cretich, M.; Chiari, M.; Little, F. F.; Unlü, M. S. *Anal. Chem.* **2011**, *83*, 9485−9491.
(14) Cerecedo, I.; Zamora, J.; Shreffler, W. G.; Lin, J.; Bardina, L.; Dieguez, M. C.; Wang, J.; Muriel, A.; de la Hoz, B.; Sampson, H. A. *J. Allergy Clin. Immunol.* **2008**, *122*, 589−594.
(15) Rebe Raz, S.; Liu, H.; Norde, W.; Bremer, M. G. E. G. *Anal. Chem.* **2010**, *82*, 8485−8491.
(16) Lee, C.-Y.; Wu, K.-Y.; Su, H.-L.; Hung, H.-Y.; Hsieh, Y.-Z. *Biosens. Bioelectron.* **2013**, *39*, 133−138.
(17) Xu, D.; Xu, D.; Yu, X.; Liu, Z.; He, W.; Ma, Z. *Anal. Chem.* **2005**, *77*, 5107−5113.
(18) Hahn, Y. K.; Jin, Z.; Kang, J. H.; Oh, E.; Han, M.; Kim, H.; Jang, J.; Lee, J.; Cheon, J.; Kim, S. H.; Park, H.; Park, J. *Anal. Chem.* **2007**, *79*, 2214−2220.
(19) Monroe, M. R.; Daaboul, G. G.; Tuysuzoglu, A.; Lopez, C. A.; Little, F. F.; Unlü, M. S. *Anal. Chem.* **2013**, *85*, 3698−3706.
(20) Cheow, L. F.; Han, J. *Anal. Chem.* **2011**, *83*, 7086−7093.
(21) http://www.phadia.com/en-GB/5/Products/ImmunoCAP-Allergens/.
(22) Natale, M.; Bisson, C.; Monti, G.; Peltran, A.; Garoffo, L. P.; Valentini, S.; Fabris, C.; Bertino, E.; Coscia, A.; Conti, A. *Mol. Nutr. Food Res.* **2004**, *48*, 363−369.
(23) Guzman, N. A.; Blanc, T.; Phillips, T. M. *Electrophoresis* **2008**, *29*, 3259−3278.
(24) Guzman, N. A.; Phillips, T. M. *Electrophoresis* **2011**, *32*, 1565−1578.
(25) Guzman, N. A.; Phillips, T. M. *Anal. Chem.* **2005**, *77*, 61A−67A.
(26) Lü, C.; Li, H.; Wang, H.; Liu, Z. *Anal. Chem.* **2013**, *85*, 2361−2369.
(27) Chen, H.-X.; Busnel, J.-M.; Gassner, A.-L.; Peltre, G.; Zhang, X.-X.; Girault, H. H. *Electrophoresis* **2008**, *29*, 3414−3421.
(28) Gasilova, N.; Gassner, A.-L.; Girault, H. H. *Electrophoresis* **2012**, *33*, 2390−2398.
(29) Sancho, A. I.; Mills, E. N. C. *Regul. Toxicol. Pharmacol.* **2010**, *58*, S42−S46.
(30) Chen, H.-X.; Busnel, J.-M.; Peltre, G.; Zhang, X.-X.; Girault, H. H. *Anal. Chem.* **2008**, *80*, 9583−9588.
(31) Shen, Y.; Smith, R. D. *J. Microcolumn Sep.* **2000**, *12*, 135−141.
(32) Cunsolo, V.; Cairone, E.; Fontanini, D.; Criscione, A.; Muccilli, V.; Saletti, R.; Foti, S. *J. Mass Spectrom.* **2009**, *44*, 1742−53.
(33) Stutz, H. *Electrophoresis* **2009**, *30*, 2032−2061.
(34) Hamilton, R. G.; Abmli, D. *The Human IgG Subclasses*; Calbiochem: Baltimore, MD, 2001.
(35) Anderson, N. L. *Mol. Cell. Proteomics* **2002**, *1*, 845−867.
(36) Yao, J.; Han, X.; Zeng, S.; Zhong, W. *Anal. Chem.* **2012**, *84*, 1645−1652.
(37) Li, H.; Qiang, W.; Vuki, M.; Xu, D.; Chen, H.-Y. *Anal. Chem.* **2011**, *83*, 8945−8952.
(38) http://www.abnova.com/products/products_detail.asp?catalog_id=KA0216.
(39) https://www.lifetechnologies.com/order/catalog/product/65502?ICID=search-product.
(40) García, B. E.; Lizaso, M. T. *J. Investig. Allergol. Clin. Immunol.* **2011**, *21*, 162−170.
(41) http://www.phadia.com/en/Products/Allergy-testing-products/ImmunoCAP-Assays/sIgE/.