



Immunological analysis of food proteins using high-performance thin-layer chromatography-immunostaining



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ABSTRACT

The chromatographic analysis of intact proteins is still challenging, especially when biological functions as antigenicity of proteins or peptides are in the focus. Traditional immunoassays provide information about the entirety of antigenic proteins/peptides, e.g., in ELISA assays. On the other hand, when focusing on the investigation of (cross) reactivity of antibodies, Western blot following gel-electrophoresis represents the method of choice. However, gel-electrophoresis is limited by the molecular weight and therefore, not suitable for peptides ≤ 3 kDa or proteins ≥ 250 kDa. Furthermore, for gaining detailed information about the protein sequence (e.g., via mass spectrometric analysis), a so called *in-gel digest* needs to be performed following electrophoretic separation and is therefore elaborate and accompanied by a significant loss of structural, and even more severe, conformational information. Here, protein analysis using HPTLC seems to be a promising alternative due to the high level of variability regarding the chromatographic system (multiple mobile and stationary phases, even mixed) and manifold detection as well as hyphenation possibilities.

This study exemplarily focused on the immunological investigation of proteins in milk following thin-layer chromatographic separation. The detection of these antigens is mandatory, as they might trigger allergenic reactions in sensitized people. Besides the proof of its applicability on different stationary phase materials, the newly developed immunoassay can be used as an approach for semi-quantitative estimation of antigenic proteins. In addition to the analysis of intact food allergens, also analyzing peptides thereof is worth considering which can be realized using HPTLC-immunostaining as well.

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1. Introduction

Milk is considered to be a precious nutritional source, especially for infants. Concurrently, components such as caseins and whey proteins are well-known food allergens which can cause severe consequences for allergic consumers. The only solution to protect allergic persons is avoiding such foods [1]. In order to secure health of allergic people, European Regulation requests the declaration of ingredients, as well as processing additives to caution sensitized consumers about hidden allergens with the Directive 2003/89/EC amending the Directive 2000/13/EC [2]. Hidden allergens are allergenic ingredients, e.g., in complex foods, that cannot be recognized by the average consumer and can be therefore not expected [3].

Besides hen's egg allergy, cow's milk allergy (CMA) represents the most common food allergy in infants and young children [4,5]. In contrast, CMA in adults is rare, but according to Lam et al. [6]

might be fatal under certain circumstances [6]. Usually, allergic reactions to cow's milk occur against the casein fraction 'Bos d 8' and the whey proteins α -lactalbumin (Bos d 4) and β -lactoglobulin (Bos d 5) [7]. In addition to intact proteins, also fragments of the protein structure might still sustain epitope sequence so their investigation must not be neglected.

Besides foodstuffs containing milk proteins in an obvious way (e.g., milk, yoghurt) hidden allergens endanger allergic persons in particular: For example, casein fractions are frequently applied as fining agents in wine production. In that case, they are aiding agents for clarifying the wine and eliminating undesirable effects on organoleptic qualities, but are removed as a precipitate to a certain extent at the end [8,9]. However, it cannot be ruled out that parts of the caseins remain in the final wine [9].

Traditionally, ELISA methods are used for the quantification of antigenic proteins and peptides, determining the amount of proteins with an affinity towards specific antibodies. There, the assay permits a quantification of the all-embracing antigenicity, solely. Qualitative investigation of samples containing antigenic proteins is done by immunostaining following electrophoretic

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separation and Western blot. The Western blot technique makes use of the negative charged proteins after electrophoretic separation and enables the transfer of the proteins to a membrane (e.g., nitrocellulose), where they are stained with specific antibodies [10]. One of the main drawbacks of gel electrophoretic approaches is the loss of information about the protein structure, caused by denaturing and redox active conditions. Additionally, gel electrophoresis is limited in molecular size, so the immunogenic analysis of peptides ≤ 3 kDa remains a challenging task [11,12].

Currently, LC–MS methods are the method of choice for analyzing peptides in general. Combinations of a chromatographic separation and mass spectrometric identification of the peptides and its origins are performed [13]. This is why MS analysis possesses a large benefit compared to ELISA or Western blot methodologies. Further, it is possible to analyze different antigens simultaneously. However, the antigenicity of the target protein(s) is not considered in LC–MS approaches, being the main drawback of this analytical procedures regarding allergen evaluation.

A combination between a highly variable chromatographic separation and an immunological staining associated with mass spectrometric identification would be desirable. At this point, high-performance thin-layer chromatography (HPTLC) exhibits excellent properties to accomplish requirements mentioned: Due to the diversity of different solvent systems and available stationary phases, the whole range of polarity can be covered. Biller et al. [14] demonstrated a strategy for developing solvent systems for the analysis of (intact) proteins using HPTLC and therefore also providing a solid base for the development of targeted detections [14]. In parallel, various publications describe the successful separation of (tryptic) peptides, occasionally accompanied by structure-function assays [15–20]. Additionally, the principle of developing the plates in two dimensions (2D–HPTLC) further improves resolution in peptide separation [15]. The assumption is near that HPTLC separation preserves the structural properties of the proteins enabling the execution of effect-directed assays following chromatographic separation e.g., using microorganisms, cell culture cells, aptamers, or antibodies. Effect directed assays are a popular way to determine specific properties of the analytes, as for example radical scavenging or antimicrobial effects, following the chromatographic separation of the compounds [21,22]. A few HPTLC antibody-based staining procedures have been already described in the literature: Meisen et al. [23] presented the detection of glycosphingolipids, whereas Morschheuser et al. [18] used antibodies for a selective detection of phosphorylated peptides [18,23].

Bioassays for the analysis of intact food proteins ('allergens') following HPTLC are not described yet, but might complement the traditional approaches. It remains still unclear, if certain food processing steps alter the antigenicity of food proteins. For instance, long storage times may lead to reactions between different food components (e.g., sugars, secondary plant metabolites) and the proteins, which might have an influence on the antigenicity of the protein, as well. Supplemented by the manifold hyphenation possibilities such as coupling HPTLC with MALDI- or ESI–MS [16,18,24,25], proteins/peptides of interest could be further analyzed in parallel and thus, complement the sum parameters observed by traditional ELISA methods.

The focus of the present study was the investigation of an alternative approach for analyzing protein or peptide antigenicity following chromatographic separation. For this purpose, the study aimed at the evaluation of a suitable method using HPTLC to inquire intact as well as fragments of cow's milk allergens. In order to develop an appropriate indirect staining procedure, β -lactoglobulin from bovine milk was investigated initially using polyclonal antibodies directed against this single protein. In this context, statistical parameters of the developed methodology were investigated. To show further feasibilities, casein-containing sam-

ples such as milk powder and wine fining agents were tested. The newly developed method was compared to traditional SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as well as Western blot. Besides the analysis of intact proteins, also tryptic peptides thereof were investigated to gain further information about residual epitopes.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Calcium acetate monohydrate, magnesium acetate tetrahydrate, monopotassium phosphate, bromophenol blue, dihydrogen dioxide and potassium acetate were purchased from Merck KGaA, Darmstadt, Germany. Acetic acid, acetonitrile, acetone, formic acid, sodium chloride, and *tert*-butanol were purchased from VWR International GmbH, Darmstadt, Germany. Ammonium peroxydisulphate (APS), β -mercaptoethanol, trifluoroacetic acid (TFA) was purchased from AppliChem GmbH, Darmstadt, Germany and ammonium bicarbonate, acrylamide (purchased as Rotiphorese® Gel; 37.5:1), dithiothreitol (DTT), tris-HCl, glycerin, sodium dodecyl sulfate, 2-mercaptoethanol, 2,2-dihydroxyindane-1,3-dione (ninhydrin), polyethylene-sorbitan monolaurate (Tween20), tris(hydroxymethyl)-aminomethane, and ethanol were from Carl Roth GmbH & Co. KG, Karlsruhe, Germany, respectively. Ammonia and pyridine were purchased from Th. Geyer GmbH & Co. KG, Hamburg, Germany. Iodoacetamid, fluorescamine, 3,3',5,5'-tetramethylbenzidine, dioctyl sulfo succinate sodium salt, citric acid monohydrate, sodium hydrogen phosphate dehydrate, polyisobutyl methacrylate (PIBM) and N, N, N', N'-tetramethylethylenediamine were purchased from Sigma-Aldrich GmbH, Steinheim, Germany. All solvents were of HPLC grade, otherwise, ACS grade was used. Water was double distilled (ddH₂O).

2.1.2. Proteins

As model proteins bovine β -lactoglobulin, α -casein, β -casein, and porcine pancreatic insulin (all Sigma Aldrich GmbH, Steinheim, Germany) as well as crude caseins (Merck KGaA, Darmstadt, Germany) were chosen. Lyophilized milk powder (Lactoland Trockenmilchwerke GmbH, Dülmen, Germany) and a casein-based wine-fining agent (Erbslöh Geisenheim AG, Geisenheim, Germany) served as 'food' samples.

2.1.3. HPTLC plates

HPTLC silica gel 60 (catalog no. 105641), silica gel 60 RP–18 W F254s (catalog no. 114296, in the following abbreviated as RP–18W), RP–18 (catalog no. 105914), RP–8 F254s (catalog no. 113725), and cellulose plates (catalog no. 105786) were kindly provided by Merck KGaA, Darmstadt. All stationary phase materials were glass-backed. Silica gel 60 RP–8 F254s plates were of 10 × 10 cm size, while the other stationary phase materials had a size of 10 × 20 cm. The plates were trimmed in accordance to the number of samples before analyses.

2.1.4. Antibodies

The polyclonal primary antibodies were raised in rabbit against bovine β -lactoglobulin (GeneTex Inc., Irvine, USA). The concentration of the purified antibodies was 0.5 mg/L. Alternatively, an antibody against casein-species was used: the polyclonal anti-casein antibodies (c = 1.1 mg/mL) were raised in rabbits against one selected fining agent, described as potassium caseinate, and purchased from Eurogentec, Seraing, Belgium [26]. Secondary antibodies were HRP-conjugated goat anti-rabbit antibodies with a concentration of 0.25 mg/L, obtained from Dako GmbH, Glostrup, Denmark.

2.2. Methods

2.2.1. Sample preparation

β -Lactoglobulin was dissolved in pure ddH₂O, whereas the aqueous solution of insulin contained 0.05% formic acid for better solubility (protein concentration $c = 2$ mg/mL). The casein-based fining agent as well as the casein standards were dissolved in 10 mM carbonate buffer (protein concentration $c = 2$ mg/mL). After incubating on an orbital shaker (80 rpm, 30 min), samples were centrifuged (2200g, 5 min) and the supernatant was used for analysis. Furthermore, milk powder was dissolved in 10 mM carbonate solution ($c = 3$ mg/mL) and dialyzed (MWCO 3.5 kDa, Carl Roth GmbH & Co. KG, Karlsruhe, Germany) against 25 mM ammonium bicarbonate solution for 72 h to remove remaining components e.g., sugars. All samples were used as described for thin-layer chromatography as well as electrophoresis.

In addition to the analysis of the intact proteins, β -lactoglobulin was also investigated in its hydrolyzed form: it was digested tryptically (trypsin, from porcine pancreas, Sigma-Aldrich GmbH, Steinheim, Germany) for 15 h at 37 °C (protein:enzyme ratio 100:1). Digested samples were used after SPE clean-up, using RP18ec SPE cartridges (Merck KGa, Darmstadt, Germany) with 60% ACN (conditioning and washing step) and 0.2% aqueous formic acid (equilibration and elution). Finally, the eluate was dried using a gentle stream of gaseous nitrogen, re-dissolved in a defined volume of 0.2% aqueous formic acid, and applied to chromatography.

2.2.2. Electrophoresis

For electrophoretic separation discontinuous-SDS-PAGE was performed using a modified protocol [27]. Gels with 12% polyacrylamide with 4% stacking gels were used. Protein samples were dissolved in PBS buffer (phosphate buffered saline, pH 7.4) and mixed in equal amounts of sample buffer containing 0.1 M Tris-HCl, 20% glycerol, 4% sodium dodecyl sulfate, 4% 2-mercaptoethanol, and 0.5% bromophenol blue to a final concentration of 0.5 mg sample protein/mL. Before being subjected to gel electrophoresis, the samples were incubated for 5 min at 95 °C. As protein standard, Precision Plus Protein™ Standard (purchased from BioRad, Hercules, USA) was used. Electrophoresis of two gels was executed with 200 V, 70 mA and 300 W with duration of 55 min. The proteins were visualized using Coomassie blue following a slightly modified staining protocol according to Candiano et al. [28]. The proteins occurred as blue bands on a white background.

2.3. High-performance thin-layer chromatography (HPTLC)

2.3.1. Intact proteins

Silica gel and cellulose as well as different reversed-phase (RP) materials were investigated with regard to their applicability for HPTLC-immunostaining. Silica gel HPTLC plates were pre-washed with methanol and activated at 100 °C for 10 min. RP and cellulose HPTLC plates were used without any pre-treatments. Sample application was done in bands (6 mm) using an HPTLC autosampler (ATS4, CAMAG AG, Muttenz, Switzerland). The chromatographic separation was carried out in a saturated twin-trough chamber with specific solvent mixtures, depending on the applied stationary phase materials [14,15]. Chromatography was performed up to a migration distance of 70 mm. The remaining solvents were evaporated overnight (12 h).

Analyses on silica gel plates were performed using a solvent system containing 2-butanol/pyridine/ammonia/ddH₂O (39/20/10/31; v/v/v/v) according to Biller et al. [14]. Cellulose plates were developed with a mixture consisting of 2-butanol/pyridine/ammonia/ddH₂O (32/30/11/25; v/v/v/v) [14].

Concerning chromatographic separation on RP layers, the mixture acetonitrile/TFA/ddH₂O (50/3.75/46.25; v/v/v) was applied [14].

2.3.2. Tryptic peptides

Chromatographic analysis of digested β -lactoglobulin was done similarly to the separation of intact proteins: samples were applied on silica gel material and developed in a twin-trough chamber. The composition of the mobile phase consisted of 2-butanol/pyridine/ammonia/ddH₂O (39/34/10/26; v/v/v/v) [15].

2.3.3. Protein/peptide specific staining

For protein/peptide specific derivatization, ninhydrin was used on cellulose stationary material, exclusively, while staining of proteins on RP materials and silica gel phases was done with fluorescamine. Subsequent to chromatographic separation, the developed and dried plate was dipped in ninhydrin (0.5% in pyridine/acetic acid 5:1; v/v) [29] or fluorescamine (0.05% in acetone) [30,31] staining solution for 2 s and evaporation was carried out at room temperature for 10 min, subsequently. Afterwards, cellulose plates were heated to 70 °C for 5 min. Proteins appeared as violet bands on white ground. The visualization of fluorescently stained proteins was without further heating. Proteins were detected at white light (ninhydrin) or ultraviolet light (UV 366 nm; fluorescamine), respectively, using a photodocumentation system (TLC visualizer, CAMAG AG, Muttenz, Switzerland). The proteins occurred as fluorescent bands on a dark ground.

2.3.4. High-performance thin-layer chromatography-immunostaining (HPTLC-IS)

As an extension to an all-embracing protein staining as described in the preceding section, a specific detection of single proteins using antibodies was developed. Subsequent to chromatographic separation, the plates were immunostained (IS). The staining protocol was adapted according to traditionally immunoblot procedures using indirect staining [26].

Following the chromatographic separation and evaporation of the mobile phase, the HPTLC plate was transferred into a small vessel. In order to inhibit unspecific binding of the antibodies to the surface, the plate was initially incubated with Tween20 as blocking reagent. For this purpose, a blocking solution containing 0.5% Tween20, 0.9% sodium chloride, and 0.6% tris(hydroxymethyl)-aminomethane was used. In the following, this buffer is called buffer A.

After incubating the plates with blocking solution two times for 15 min, blocking solution was removed and replaced by the primary antibody solution (1:20,000 (for intact proteins) 1:12,000 (for peptides) in buffer A). The plate was incubated for 2 h with primary antibodies, followed by three times washing with buffer A. Coating with the secondary antibody was performed for 1 h (diluted 1:2000 in buffer A) and was complemented by a washing step (three times with buffer A). Prior to detection, the pH value was lowered by incubating the plate for 1 min in a solution consisting of 0.06% Tris-HCl (pH 6.0). Finally, the detection was executed by incubating the plate in a dyeing solution containing 0.06% 3,3',5,5'-tetramethylbenzidine, 0.2% diethyl sulfosuccinate sodium salt, 0.7% citric acid monohydrate, 1.8% sodium hydrogen phosphate dihydrate, 25% ethanol, and 1.5% dihydrogen dioxide. This composition was modified after Steinhoff et al. [32]. All incubation procedures were executed on an orbital shaker at 70 rpm and room temperature. The finally conducted incubation using the detection medium was carried out until blue bands appear on white background. Subsequently, the analytes were detected at white light and documented using a photodocumentation system (TLC visualizer, CAMAG AG, Muttenz, Switzerland).

2.4. Statistical analysis

The HPTLC-IS methodology of intact proteins was validated with regard to following parameters: linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ). β -Lactoglobulin served as target molecule and was separated on silica gel as mentioned above. Following the documentation of the plate, data processing was performed using the image processing software Image J (developed at the National Institutes of Health (US); open source) [33]. Linearity was validated using a seven-point calibration curve ($n=4$) and was verified by means of Mandel's fitting test. Homogeneity of variance was assigned regarding all values obtained [34,35]. Limit of detection (LOD) and limit of quantification (LOQ) were determined based using the calibration method on DIN 32645:2008-11 ($n=5$) via the calibration line (concentrations between 30 ng–150 ng) [34,36]. Accuracy was determined by analyzing the closeness of individual densitometric measurement of the band (same concentration of β -lactoglobulin, $c=0.5 \mu\text{g}$, $n=6$).

3. Results and discussion

3.1. Method development HPTLC-Immunostaining of intact proteins

The aim of study was to develop a protein antigenicity assay directly on a HPTLC plate following chromatographic separation. The so called overlay assay technique was chosen due to its flexible possibilities for its adaptation on the current analytical question [37]. The schematic process of a so called overlay assay is described as follows: After separation of the samples using (HP)TLC the analytes are detected while being immobilized on the stationary phase material of the (HP)TLC plate. Different overlay assays are described in the literature using different media, such as agar (Rahalison et al., [38]), or specific proteins (Dowler et al., [39]).

When starting, chromatographic conditions were in the focus: To analyze the model proteins β -lactoglobulin and insulin, the most common silica gel materials, as well as different RP and cellulose plates were tested. These stationary phases should only present the range of possibilities. A final procedure should be applicable likewise in accordance with the respective analytical question. β -Lactoglobulin plays a key role in various publications on food allergenicity [5,6,40,41]. Insulin was chosen as model protein for method development with regard to hydropathy and isoelectric point [14]. However, with the solvent systems chosen, both proteins showed good separation on all materials, except cellulose. As future analytical questions might deal with chromatography on cellulose, the basic feasibility of an immunological staining protocol on cellulose was investigated, nonetheless. Current HPTLC methods for determining proteins and peptides can be significantly improved regarding their chromatographic capacity: In several studies, the spot of application still showed a more or less intensive signal even after chromatographic separation [14,16,17].

Subsequent to the evaluation of suitable chromatographic parameters, various well-established immunological staining procedures were tested, originally applied for immunostaining following Western blotting [18,32,42]. In this study, the method described by Steinhoff et al. [32] exhibited the most promising characteristics for transferring procedures for the detection of antigenic peptides following thin-layer chromatography [32]. Most immunological methods use proteins such as BSA or milk powder as a blocking reagent to reduce unspecific binding on the surface of the separating material [42,43]. As milk-based samples were analyzed in the present study, traditional protein blocking was unsuitable. With the use of the detergent Tween20 as blocking agent, the pro-

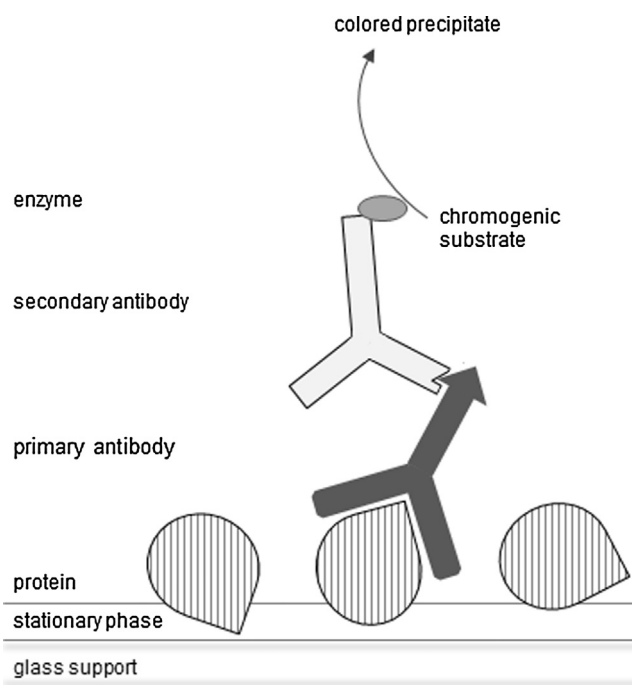


Fig. 1. HPTLC overlay assay scheme of an indirect antibody mediated detection of proteins. The detection was performed after HPTLC separation; stationary phase materials required a previous blocking step to inhibit unspecific bindings of antibody residues. Afterwards, the incubation was performed with protein-specific primary antibody followed by an overlay with secondary enzyme-labeled antibody, which recognizes the host of the respective primary antibody. Ligated antibodies are visualized by generating a colored precipitate originating from a chromogenic substrate.

cedure was applicable independently from the reactivity of the applied antibody [32,44].

In principle, the HPTLC overlay assay developed is closely related to the well-known indirect enzyme-linked immunosorbent assay using a chromogenic substrate (tetramethylbenzidine, TMB) which turns into a colored precipitate.

The first antibody possesses affinity towards the target protein and is applied following the initial blocking step. The secondary antibody is labeled with an enzyme (here, horseradish peroxidase, HRP) that exerts a reaction on the chromogenic substrate and therefore visualizes the first bound antibody. The basic principle of HPTLC-immunostaining (HPTLC-IS) is shown schematically in Fig. 1. The primary protein-binding antibody is associated with the enzymatically tagged secondary antibody, which is specific for the host animal of the primary antibody.

As the staining procedure was conducted in aqueous buffer, the HPTLC plate materials needed to be wettable and stable towards incubation in aqueous solution. To improve the stability of stationary phases, the polymer film former polyisobutyl methacrylate (PIBM) was used as a stabilizer as suggested in the literature [45]. It was observed that the stability of the surface material improved using PIBM. Additionally, the coloring of the background was reduced by this approach. However, the sensitivity of the immunostaining declined resulting from the use of PIBM, presumably caused by the hampered amenability of the epitopes. Comparing the results of approaches with and without fixation, the stationary phase materials stayed intact even without fixation, but coloring of the background was a little more intense compared to HPTLC-IS with fixation. On this account, coating with PIBM was resigned for all stationary phase materials as the slightly increased background coloring was negligible.

The original protocol described by Steinhoff et al. [32] prescribes an overnight incubation with the primary antibody [32]. Transferring this to the immunological staining following HPTLC in the

Table 1

Statistical parameters of HPTLC-IS analyzing β -lactoglobulin on silica gel using anti-bovine β -lactoglobulin antibodies.

Synonym	Value	RSD
Limit of detection	61.89 ng	23.58%
Limit of quantification	93.37 ng	22.35%
Accuracy	98.31%	4.8%
Coefficient of determination	0.992	1.6%

present study, it became obvious that the stationary phase material suffered from long incubation times combined with the mechanical stress resulting from the incubation on the orbital shaker. As the friction caused by the movement of the orbital shaker could not be omitted, the incubation time had to be reduced. As a result, concentration of the primary antibody was increased, while incubation time was conclusively set to a minimum of 2 h. The finally applied staining procedure is mentioned in the material and methods section and was realized without appreciable harm to stationary phase materials.

To eliminate potential structural deformations ('denaturation') of the proteins potentially caused by the solvents, proteins were renatured directly on the plate as described in Meisen et al. [42]. Following chromatography, the plates were incubated for 10 h in PBS solution (pH 7.2) to eliminate/dilute potential residues of the solvents and to refold the proteins when denatured reversibly. This procedure slightly increased the sensitivity of the immunological evidence, but led to broadened bands due to additional diffusion.

Fig. 2B shows the chromatograms gained using the finally developed immunological staining procedure. For comparison, a similar plate was stained with the protein-specific dye fluorescamine (Fig. 2A, HPTLC-Fluorescence Densitometry, HPTLC-FLD), coloring all proteins present. HPTLC-IS is even more specific depending on the antibody specificity. A band only occurred for the targeted protein β -lactoglobulin, exclusively, while band sharpness was comparable to traditionally applied fluorescamine staining.

The application of silica gel stationary phases exhibited superior results compared to the RP phases, presumably caused by a better wettability of silica gel and the corresponding more homogeneous distribution of the antibodies: Immunostained protein bands on RP-layers showed slightly inhomogeneous distributions within the band (please see Supplementary Material). However, HPTLC-IS succeeded even on non-polar surfaces, but required higher antibody concentrations and showed slight cross-reactivity to insulin, in this study. Although β -lactoglobulin showed no migration distance, the proof of concept for a possible immunostaining was also conducted on cellulose plates: the methodology succeeded even in this application (chromatograms not shown). Statistical parameters were evaluated at hand of the silica gel materials and shown in Table 1.

Briefly, concentrations with linear correlation were observed between 0.075 μ g and 2 μ g β -lactoglobulin. The linear relation was also confirmed by a residual analysis of the calibration standards and by means of Mandel's fitting test. Compared to other HPTLC-applications dealing with semi-quantitative estimations of various substances, the results achieved are very similar. For example, Widmer et al. [46] presented an HPTLC analysis of artemisinin, an antimalarial substance obtained from dried plant leaves. The published methodology showed linearity between 0.03 μ g and 0.120 μ g using densitometric evaluation [46]. A linear correlation of 1.25–6.25 μ g was determined by Reim et al. [47] when analyzing pea saponins. The results were achieved by densitometry and correlated very well with the concentrations applied [47]. Dealing with the analysis of similar proteins as in the current study, Morschheuser et al. [18] demonstrated the application of aptamers

as detection tool. Within that study, linearity between 0.1–1 μ g was shown [48].

In the present study, determination of LOD and LOQ was done on the basis of DIN standards using the calibration method (Table 1). The results achieved were on the same scale as the LOD and LOQ of other semi-quantitative HPTLC analyses. Reim et al. [47] showed values of 0.6 μ g $\pm$ 17% (LOD) and 2.1 μ g $\pm$ 5% (LOQ), while Morschheuser et al. [18] calculated a LOD of 0.063 μ g $\pm$ 19% and a LOQ of 0.112 μ g $\pm$ 19% [47,48]. "After the separation of sample components on TLC, these products are immobilized on the plate. They are easily accessed because they are open" and therefore influenced by exterior factors [49]. This is why semi-quantitative analysis must be conducted with samples and standards in the same run. This also explains the comparatively high relative standard deviation (RSD) of the LOD and LOQ, as they were calculated on the basis of between-batch results. This is countered by the relatively low RSD of the accuracy. Here, the samples were applied on the same plate as the calibration standard (within-batch results). In this case, the samples are treated the same way as the calibration samples. Consequently, the accuracy and the coefficient of determination (R^2), as well as their RSD are better quality parameters for proving the reliability of HPTLC-IS.

3.2. Proof of principle: application of HPTLC-IS on milk samples

To prove the feasibility of an immunological detection of antigens in (real) food samples, following their separation with HPTLC, casein standards as well as a commercially available milk powder and a wine-finishing agent based on milk were investigated with the HPTLC-IS protocol developed herein. In contrast to the antibody specific for β -lactoglobulin, in this case, antibodies with affinity to multiple proteins were used in order to show the wide scope of application. As caseins display a major class of CMA, antibodies with an affinity towards several casein species e.g., α - and β -caseins were applied.

For comparing HPTLC-IS with traditional methods, casein standards were also analyzed using SDS-PAGE, as well as HPTLC-FLD. Fig. 3 illustrates the electrophoretic as well as the chromatographic separation of isolated casein species (Fig. 3A–C, lanes 1 and 2). When comparing SDS-PAGE (Fig. 3A) and HPTLC-FLD (Fig. 3B), a well separation of the caseins can be observed. Based on the R_f -values of the pure α - and β -casein standards (Fig. 3A–C, lanes 1 and 2), the identity of the two distinct bands can be verified also for a crude preparation (Fig. 3A–C, lane 3). α -Casein is displayed with an "a" while β -casein is provided with "b". Besides the two main proteins, the crude casein standard as well as the β -casein fraction contained further proteins in traces, which was also apparent from the SDS-PAGE results (Fig. 3A, lanes 2 and 3). Taking their molecular weights into account, it can be assumed that these proteins were further milk proteins. The corresponding HPTLC-FLD separation (Fig. 3B, lanes 2 and 3) showed no additional band(s), except the application band. This might have had two different reasons: the protein amount was below the detection limit or the remaining protein(s) showed no separation with the applied solvent system. As reported by Biller et al. [14], thin-layer chromatographic separation is dependent on several characteristics of the protein and must be adapted individually for every analytical question [14]. The analysis of the trace proteins using HPTLC-IS was not focused in this study, but should be also realizable in principle, if necessary.

HPTLC plates were furthermore stained immunologically (Fig. 3C). When comparing fluorescamine detection (Fig. 3B) and immunological detection (Fig. 3C), it was observed that both casein species were detected successfully using HPTLC-IS, either as pure compounds as well as in the crude preparation.

As an extension to the analysis of single model proteins, real food samples based on caseins were analyzed with SDS-

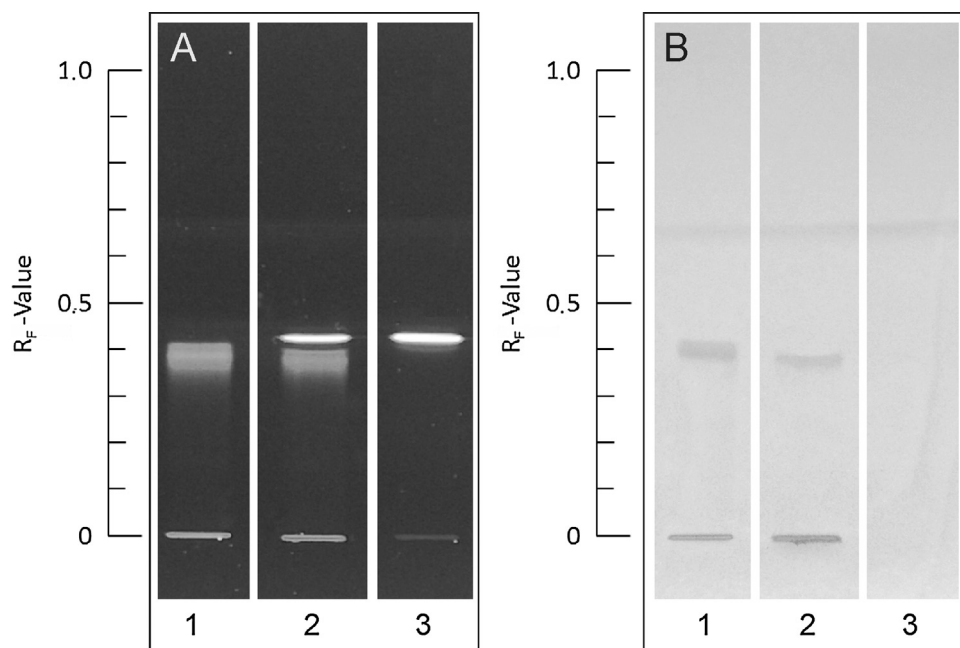


Fig. 2. HPTLC(-IS) chromatogram of model proteins β -lactoglobulin and insulin on silica gel, mobile phase consisted of 2-butanol/pyridine/ammonia/ddH₂O (39/20/10/31; v/v/v/v). (A) shows protein staining by the use of fluorescamine as a derivatization reagent (HPTLC-FLD; $\lambda = 366$ nm), (B) represents the corresponding chromatogram but followed by immunological staining (-IS) (detected at white light) to detect the antigen exclusively (here: β -lactoglobulin). Legend: (1) β -lactoglobulin, (2) mixture of insulin and β -lactoglobulin (3) insulin.

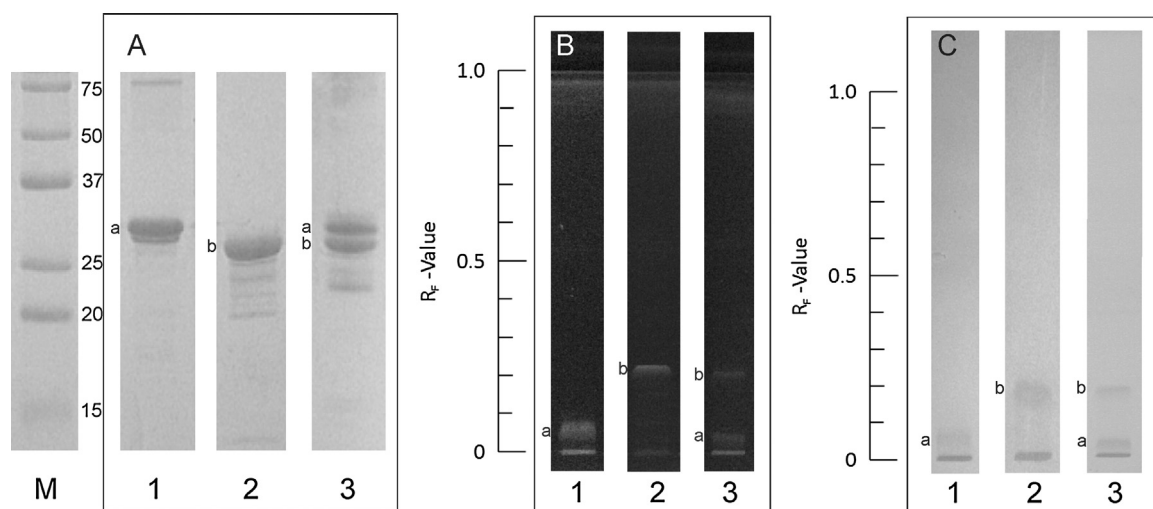


Fig. 3. Separation of different casein standards using traditional SDS-PAGE (A), HPTLC-FA (B) and newly developed HPTLC-IS (C) on silica gel (mobile phase: 2-butanol/pyridine/ammonia/ddH₂O (39/20/10/31; v/v/v/v)). Staining of proteins after gelelectrophoresis was performed protein specifically (colloidal coomassie detection, visualized at white light) while protein specific fluorescamine solution was used in 3 B ($\lambda = 366$ nm). Fig. 3C presents the specific detection of casein species using an anti-casein antibody (visualized at white light). Legend: (M) molecular weight marker (kDa), (1) α -casein "a", (2) β -casein "b", (3) mixed casein standard.

PAGE and HPTLC. In this context, milk powder and a wine-finishing agent were chosen. Both, milk powder and wine-finishing agent contained (as specified by the manufacturer) high amounts of α - as well as β -casein, which was also confirmed by SDS-PAGE (Fig. 4A). Additionally, the proteins were detected immunologically using Western blotting following electrophoresis (Fig. 4B), HPTLC with fluorescamine staining (Fig. 4C), and HPTLC-immunostaining (Fig. 4D). For comparing the results of the electrophoretic approach with HPTLC, it needs to be considered that proteins are heavily denatured in SDS-PAGE, while they remain more or less native in HPTLC as described above. For better visualization, bands belonging to α -casein are marked with "a", β -casein with "b" and β -lactoglobulin with "c", respectively.

The thin-layer chromatographically separation of lyophilized milk powder is shown in Fig. 4C (lane 2), the separation of the finishing agent in Fig. 4C (lane 3). As expected, each separation showed bands for α - and β -casein, respectively. These results are in agreement with the immunological detection following Western blotting (Fig. 4B) and following HPTLC (Fig. 4D): Both showed corresponding bands.

The study showed that HPTLC-IS succeeded in analyzing food samples regarding their antigenicity towards specific antibodies, but the newly developed methodology might also be used vice versa.

As mentioned in the material section, the applied antibody was raised against the casein based finishing agent (Fig. 4 lane 3). For this

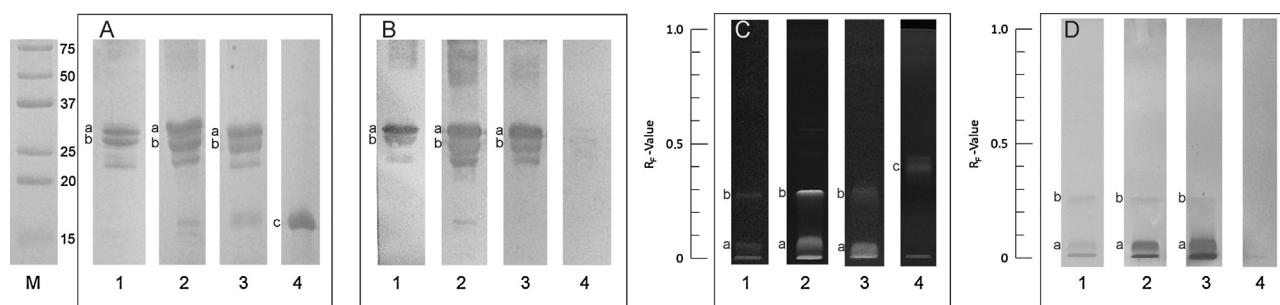


Fig. 4. Traditionally applied SDS-PAGE and Western blot of casein containing samples compared to unselectively stained HPTLC-FA and antibody based HPTLC-IS analysis on silica gel using a mobile phase of 2-butanol/pyridine/ammonia/ddH₂O (39/20/10/31; v/v/v/v). **Fig. 4A** shows the SDS-PAGE visualized with protein specific colloidal coomassie staining while **Fig. 4B** shows the Western blot of an identical gelelectrophoretic separation, using the anti-casein antibody. Both analyses were visualized at white light. **Fig. 4C** & **D** show the HPTLC-separation of the same samples: **Fig. 4C** presents the unspecific protein detection using fluorescamine ($\lambda = 366$ nm) and the corresponding selective detection via HPTLC-IS is represented in **Fig. 4D** (anti-casein antibody, visualized at white light). Legend: (M) molecular weight marker (kDa), (1) mixed casein standard, (2) milk powder, (3) casein based fining agent, (4) β -lactoglobulin "c".

reason the assumption is near that the antibody showed affinity to every protein being present in the fining agent.

The electrophoretic separation of the casein-based wine fining agent (**Fig. 4A**, lane 3) showed a weak band with approx. 18 kDa which could be assigned to residual β -lactoglobulin. At a first glance, it can therefore be assumed that the antibody ligates moreover β -lactoglobulin, additionally to the caseins. Proofing the assumption that the applied anti-casein antibody showed also affinity towards β -lactoglobulin, the latter was investigated in parallel as a single standard: **Fig. 4A** & **B** (lane 4) shows the electrophoretic separation of β -lactoglobulin, while **Fig. 4C** & **D** (lane 4) illustrate the HPTLC separation, respectively. For better visualization the corresponding band is marked with a "c". Neither the chromatogram in **Fig. 4B** shows a band at a molecular weight similar to β -lactoglobulin (lane 4), nor does the HPTLC-IS chromatogram (**Fig. 4D**, lane 4) at the corresponding R_F-value. Both techniques led to the same conclusion: The antibody applied showed no affinity towards β -lactoglobulin, independently, if the β -lactoglobulin occurs in its native structure (HPTLC-IS, **Fig. 4D**) or in its denatured form (Western blot following SDS-PAGE, **Fig. 4B**).

3.3. HPTLC-Immunostaining of (tryptic) peptides

The present study already showed that intact proteins can be analyzed with regard to their antigenicity using immunological staining directly on a HPTLC plate. As some proteins keep their antigenicity even after hydrolysis (e.g. by using proteases), the antigenic analysis of corresponding peptides must not be neglected. Especially, proteins leading to CMA are of particular interest as often hydrolysate preparations are used for special hypoallergenic nutrition for infants. As mentioned above, the analysis of peptides using traditional methods such as Western blotting following gel electrophoresis is a challenging or rather impossible task, due to their low molecular weight. In contrast, separation of peptides using HPTLC succeeded in various publications [15,16,18,50]. On that account, the present study focused on the development of an immunological detection of tryptic peptides after HPTLC following the same scheme as HPTLC-IS of intact proteins (compare to **Fig. 1**). β -Lactoglobulin served as a model protein due to its relevance.

Initially, β -lactoglobulin was digested with a traditional protocol using the protease trypsin. To prove the presence of epitopes in the hydrolysate, samples were also analyzed with an established ELISA protocol [26] with slight modifications (ELISA data not shown). The results showed that the antigenicity of β -lactoglobulin was reduced compared to the intact protein, but a certain extent of antigenicity remained.

For the HPTLC approach, the evaluation of suitable chromatographic parameters was focused: various solvent systems were

applied to achieve an optimal HPTLC separation of the tryptic peptides. The finally established conditions are described in the materials and methods section. Subsequently, suitable parameters for the immunological overlay assay were evaluated based on similar protocols as for the intact proteins. The evaluation was done with regard to composition of the incubation medium in general, time and duration of the incubation steps, antibody and antigen concentrations, and blocking of the surface to inhibit unspecific binding. To evaluate possible cross-reactivities e.g., caused by high antibody concentrations, intact β -lactoglobulin as well as intact insulin were investigated in parallel. The finally compiled instruction yielded to separations as shown exemplarily in the chromatogram in **Fig. 5B**.

Peptides with remaining epitopes appear as sharp and distinct blue bands on white background. To differentiate antigenic and non-antigenic peptides, **Fig. 5A** shows the corresponding plate after peptide/protein specific staining using fluorescamine. It is obvious, that presumably 4 of approx. 15 separated peptides still have an epitope, despite the digestion of β -lactoglobulin. However, it cannot be excluded yet that HPTLC separation of the peptides was complete. As expected, the intact β -lactoglobulin (**Fig. 5B** lane 1) appeared also as distinct band on the application spot. Additionally, the chromatogram of the intact insulin showed a signal at the application spot, although the antibody applied did not possess affinity towards insulin. This interaction is presumably caused by the high concentrations of the antibody.

Nevertheless it can be assumed that HPTLC-IS possesses superior characteristics to screen peptide-containing samples with regard to antigenicity. When comparing band sharpness of HPTLC-FLD with HPTLC-IS, it is slightly decreased in the latter case, presumably caused by diffusional effects resulting from the incubation in aqueous solution. In order to estimate the correlation between quantity of an antigenic peptide and signal intensity of the corresponding band, dilution series were investigated. It became apparent that band intensity changed proportionately to the concentration of the tryptic digest applied (**Fig. 6**).

Besides the successful application of peptide HPTLC-IS on silica gel, its use on other stationary phases seems to be realizable without further complex efforts. As shown above (section *Method development HPTLC-Immunostaining of intact proteins*) immunological staining succeeded on a multitude of chromatographic systems irrespective of strength and/or pH value of the solvent system.

In case of deeper interest, antigenic peptides can be further analyzed by mass spectrometry. At this point, various hyphenation possibilities are worth considering: Coupling to ESI-MS is as well possible as HPTLC-MALDI-MS, but needs to be chosen individually. As the blocking reagent Tween20 tends to interfere with the signals in mass spectrometry, the protocol needs to be complemented

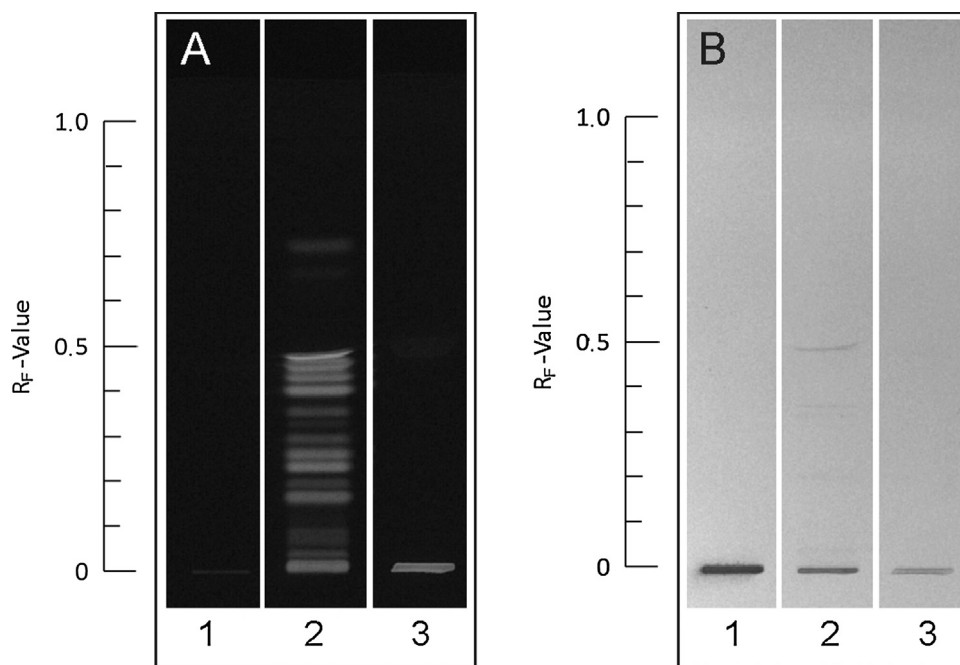


Fig. 5. HPTLC chromatograms of tryptic peptides as well as intact proteins (A) after derivatization with fluorescamine ($\lambda = 366$ nm), and (B) immunologically stained using the anti- β -lactoglobulin antibody (visualized at white light). The analyses were performed on silica gel using the peptide specific mobile phase 2-butanol/pyridine/ammonia/ddH₂O (39/34/10/26; v/v/v/v). Legend: (1) non-digested β -lactoglobulin, (2) tryptically digested β -lactoglobulin, (3) non-digested insulin.

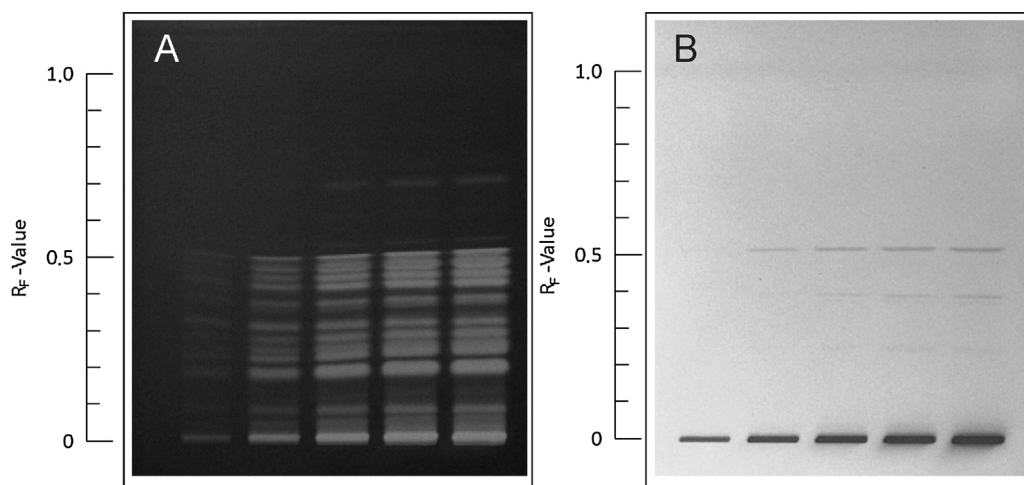


Fig. 6. Peptide HPTLC-IS on silica gel developed with 2-butanol/pyridine/ammonia/ddH₂O (39/34/10/26; v/v/v/v) – comparison different concentrations of digested β -lactoglobulin (increasing concentration from left to right, 3–60 μ g/band). Fig. 6A shows the unselectively stained chromatogram (fluorescamine staining solution, $\lambda = 366$ nm) and the corresponding immunologically detection is represented in Fig. 6B (anti- β -lactoglobulin antibody, visualized at white light).

in terms of an adequate blocking agent. However, in comparison to the use of Tween20 as blocking reagent, the traditionally use of BSA led to an enhanced coloring of the background in HPTLC-IS. This might be caused by the high concentration of the antibody and the associated cross-reactivity towards proteins in general. In case of an experimental set-up where antibodies with affinity towards BSA are used, the blocking agent must be adapted accordingly. First studies of mass spectrometric analyses after/in parallel to HPTLC-IS show promising results and should be further investigated.

Pecquet et al. [51] dealt with an identification of antigenic peptides obtained by tryptic hydrolysis of β -lactoglobulin. Therefore, peptides obtained by tryptic digest were fractionated in multitude runs of preparative chromatography and identified using mass spectrometry, subsequently. In that study ELISA was also conducted determining the peptides of β -lactoglobulin with affinity to IgE

antibodies obtained by human sera. All these elaborate single steps could be simplified by the use of peptide HPTLC-IS in combination with mass spectrometry.

4. Conclusion

Summing up, an immunological staining of intact as well as digested proteins following their separation via HPTLC directly on-plate (I) is less laborious than transferring proteins after gel-electrophoretic separation onto a membrane with consecutive staining. Furthermore (II) it displays more characteristics of the proteins, e.g., regarding their antigenic properties, as it does not destroy the tertiary and secondary structure of the protein. This is why the analysis of antigens using HPTLC-IS is able to reveal structural epitopes as well as continuous epitopes. Besides this, HPTLC-IS

(III) handles with decreased toxic substances than SDS-PAGE (i.e. acrylamide) and exhibits therefore an improved handling. Due to the versatility of HPTLC analysis (IV) as for example regarding mobile phase composition, conditions of HPTLC-IS can be individually adjusted on the basis of a present analytical problem. (V) In conjunction with the hyphenation possibilities of HPTLC, i.e. using mass spectrometry, HPTLC-IS offers excellent properties for the progressing analysis of (food) antigens.

As a conclusion for future work, HPTLC-IS combined with different mass spectrometric measurements enables the investigation of proteins and especially peptides of different origins. This methodology might solely be limited by suitable chromatographic conditions: manifold combinations have been already provided in the literature, mainly dealing with peptides [15,18,52–54]. In the present study, polyclonal antibodies were investigated for reasons of a broader applicability and accessibility. In contrast, identification of antigenic proteins and peptides would profit from the use of monoclonal antibodies.

The entire methodology benefits additionally from the two-dimensional approach, developing a sample in two different directions using two different solvent systems [15,17,50]. Initial tests of peptide 2D-HPTLC-IS showed promising results and should be investigated intensively in the future, as the chromatographic resolution might be increased in comparison to one-dimensional separation. The presented methodology of HPTLC-IS leads also to further amendments of already known analytical approaches. For example, using HPTLC-IS as an extension of a structure-function assay presented in Tschersch et al. [17] will give further insights in structure-function changes caused by intermolecular reactions during food processing or storage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.chroma.2017.10.046>.

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Figure: HPTLC(-IS) chromatogram of model proteins β -lactoglobulin and insulin on RP-materials: RP-8 (A), RP-18 (B) and RP-18W (C), mobile phase consisted of acetonitrile/TFA/ddH₂O (50/3.75/46.25; v/v/v). The left chromatograms (l) show protein staining by the use of fluorescamine as a derivatization reagent (HPTLC-FLD; $\lambda = 366$ nm), the right chromatograms (r) represent the corresponding chromatogram but followed by immunological staining (-IS) (detected at white light) to detect the antigen exclusively (here: β -lactoglobulin). Legend: (1) β -lactoglobulin, (2) insulin (3) mixture of insulin and β -lactoglobulin.