Journal of Chromatography A 1653 (2021) 462442

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Two-dimensional high-performance thin-layer chromatography for the characterization of milk peptide properties and a prediction of the retention behavior – a proof-of-principle study

Mascha Treblin[a], Tobias von Oesen[b], Lisa-Carina Class[a], Gesine Kuhnen[a], Ingrid Clawin-Rädecker[b], Dierk Martin[b], Jan Fritsche[b], Sascha Rohn[a,c,*]

[a] University of Hamburg, Hamburg School of Food Science, Institute of Food Chemistry, Grindelallee 117, Hamburg D-20146, Germany
[b] Department of Safety and Quality of Milk and Fish Products, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Hermann-Weigmann-Straße 1, Kiel D-24103, Germany
[c] Department of Food Chemistry and Analysis, Technische Universität Berlin, Institute of Food Technology and Food Chemistry, TIB 4/3-1, Gustav-Meyer-Allee 25, Berlin 13355, Germany

ARTICLE INFO

Article history:
Received 31 May 2021
Revised 21 July 2021
Accepted 22 July 2021
Available online 29 July 2021

Keywords:
2D-HPTLC
HPTLC-MALDI-TOF-MS/MS
Peptide characterization
Milk proteins
Tryptic hydrolysis

ABSTRACT

High-performance thin-layer chromatography (HPTLC) is a suitable method for the analysis of peptides and proteins due to a wide selection of stationary and mobile phases and various detection options. Especially, two-dimensional HPTLC (2D-HPTLC) enables a higher resolution compared to one-dimensional HPTLC in the separation of complex peptide mixtures. Similar to 2D electrophoresis, characteristic peptide patterns can be obtained, allowing a differentiation of ingredients based on varying protein origins.

The aim of this study was to evaluate 2D-HPTLC with regard to its suitability for the characterization of proteins/peptides and to verify whether it is possible to predict the retention behavior of peptides based on their properties. As models, the five most abundant milk proteins α-lactalbumin, β-lactoglobulin, α-, β-, and κ-Casein were used. In order to determine the repeatability of the peptide separation by 2D-HPTLC, each tryptic protein hydrolyzate was separated eight times. The standard deviations of the retardation factors for the separated peptides varied between 1.0 and 11.1 mm for the *x-coordinate* and 0.5–7.3 mm for the *y-coordinate*. It was also shown that after the chromatographic separation, peptides of the individual protein hydrolyzates were located in specific areas on the HPTLC plate, so that a clustering could be obtained for the whey proteins' as well as the caseins' hydrolyzates. For establishing correlations between the properties of the peptides and their retardation factors, 51 of 85 selected peptides were identified by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS). On this basis, statistically significant correlations ($\alpha = 0.05$) between the retardation factors of the peptides and their isoelectric points, as well as the percentage of anionic and non-polar amino acids in the peptides were established. Finally, it was investigated, whether the retardation factors for peptides can be predicted on the basis of a linear regression of the percentage of non-polar amino acids in a peptide. For this purpose, a mixture of artifical (synthetic) peptides ($n = 14$) was separated by 2D-HPTLC and the measured retardation factors were compared with the corresponding retardation factors calculated. Absolute deviations of 0.3–17.9 mm were obtained. In addition, the universal applicability of the method to other protein sources other than milk proteins (animal protein) was tested using a mixture of pea peptides (plant protein, $n = 3$) resulting in absolute deviations of 0.7–8.6 mm.

1. Introduction

Proteomics is a powerful research tool for protein characterization and biomarker discovery [1]. For analyzing protein mixtures, shotgun proteomics, also called bottom-up approach, is often applied. For this purpose, proteins are enzymatically hydrolyzed and the resulting complex peptide mixtures are usually analyzed by

* Corresponding author at: Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Department of Food Chemistry and Analysis, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355 Berlin, Germany.
E-mail address: rohn@tu-berlin.de (S. Rohn).

https://doi.org/10.1016/j.chroma.2021.462442
0021-9673/

high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) [2]. An alternative for characterizing complex peptide mixtures with low equipment requirements and minimal costs can be high-performance thin-layer chromatography (HPTLC). The advantages of HPTLC include the choice of a wide variety of stationary phases and eluents, the simultaneous analysis of multiple samples, and various detection options [3–15]. For a separation of complex peptide mixtures, two-dimensional HPTLC (2D-HPTLC) seems suitable in particular, as it offers increase resolution compared to one-dimensional HPTLC (1D-HPTLC). Similar to 2D gel electrophoresis, 2D-HPTLC enables the evalution of compound patterns, as it also separates analytes in two different directions, but based on the use of two eluents with different properties. For example, by choosing a basic and an acidic solvent system, the ionizable groups of the peptides change their degree of dissociation according to their specific *pK* values. This leads to varied retardation factors (R_f) and thus to a better separation of the analytes [11–19]. In addition, separation selectivity can be increase by switching between a normal phase (NP) and reversed phase (RP) system. This can be achieved by differences in the polarity of the mobile phase and the strength of the ion pairing reagent. These many degrees of variation possibilities for changing the separation selectivity are not given in 2D gel electrophoresis.

However, when 2D-HPTLC is used without subsequent coupling to mass spectrometry (MS) or effect-directed analysis, only the information about the R_f values of individual peptides can be obtained. Thus, it is not possible to make predictions about certain properties of the peptides, such as the isoelectric point (IEP) or the amino acid composition, based on the R_f values alone [19,20]. For this reason, correlations between the properties of the peptides and their R_f values need to be established so that a characterization of peptide properties is also possible without immense instrumental effort. On the one hand, this would allow to characterize the peptides separated by 2D-HPTLC according to their R_f values. On the other hand, this could be a way to make predictions about the R_f values of peptides with known amino acid sequences. The correlations between the peptide properties and their retention behavior could be described mathematically via regressions and, based on this, the retardation factors could be calculated via the properties of the peptides. This would simplify, for example, the identification of biomarkers, the selection of internal standards, or the analysis of posttranslational modifications (PTM). PTM are often discriminated during HPLC separation, due to a loss in the pre-column or at the beginning of the separation column [19,21].

In the past, 1D- and 2D-HPTLC have already been applied for studying protein hydrolyzates. The characterization of certain properties of the peptides (e.g., hydrophilicity), PTM (e.g., phosphopeptides), or even intra- and intermolecular interactions (e.g., protein-protein-interactions, polyphenol-protein-interactions), have been among the studies [3,6,15,16,18,19,21]. Nontheless, the repeatability of 2D-HPTLC for the separation of complex peptide mixtures has not yet been sufficiently investigated, which is probably due to the limited studies on 2D-HPTLC of peptides. The determination of the repeatability would be crucial for verifying the applicability of the method for routine analysis.

Additionally, numerous ways of coupling to mass spectrometry were described for the identification of TLC-separated peptide bands or spots and also a mapping of the identified peptides was performed [3,7,11,16,18,19,22–25]. However, mass spectrometric identification of the separated peptides is not ideal: Until now, MS analyses have been performed mainly from non-derivatized HPTLC plates. This was done by, using either undirected lane scans or by analyzing only individual regions in comparison to derivatized reference plates with the same samples and separation conditions. Consequently, two plates needed to be prepared for the determination of the R_f values, which is laborious and cost-consuming [3,11,16,19,24]. Although both, the lane scans and the determination of specific regions of non-derivatized plates, provided good results for peptide identification so far, these methods also had their drawbacks. An example for a lane scan would be the direct coupling of 2D-HPTLC with matrix-assisted laser desorption/ionization (MALDI) with time-of-flight (TOF) after coating the plate with the (MALDI) matrix. This leads to diffusion of the analytes on the plate and thus, to a loss of intensity and shifting of R_f values [7,11,24]. Another possibility for direct MS-coupling with a lane scan would be the desorption electrospray ionization mass spectrometry (HPTLC/DESI-MS). However, this method identifies hydrophilic peptides with small R_f values more poorly in comparison to hydrophobic peptides in the presence of an NP system. This is caused by stronger binding of the peptides to the polar stationary phase and thus, a more difficult desorption [7,16]. When a reference plate is used, it cannot be guaranteed that the R_f values on both plates are identical, which sometimes results in analytes only partially measured or not being measured at all. For this reason, a mass spectrometric investigation of already derivatized peptides from the same HPTLC plate after full resolvatisation of the peptides is of great interest. This should neither cause any loss of intensity or shift in R_f values, nor should it discriminate polar peptides. Bakry et al. described an approach by derivatizing the peptides with fluorescamine prior to HPTLC separation, which allowed them to determine the exact R_f values of the analytes based on exposure with UV light [20]. Another possibility in MS-coupling is to use an MS-interface that extracts the peptides from a specific spot on the HPTLC plate and transfers them directly to the MS. However, the selected spot is limited to diameters of approx. 4 mm. Consequently, for complex mixtures with many spots such as protein hydrolyzates, an isolated extraction of single spots is often difficult [26].

To avoid MS-identification at all, a method for characterizing peptides separated by 2D-HPTLC, being solely based on its R_f values, which means *x*- and *y-coordinates*, would be useful. However, there are not many methods yet to determine the properties of peptides based on their R_f values or, conversely, to predict the R_f value areas of known peptides based on their amino acid composition under given chromatographic conditions. Up to now, only the group of Baczek et al. predicted the retention behavior of homologous peptides as a function of the content of acetonitrile in the mobile phase but not related to the peptide properties [27]. A year later, Baczek and Sparzak predicted the retardation factors for 30 peptides at a given content of ionic liquid in the mobile phase [28]. That time, however, they also included the properties of the peptides, such as the *n*-octanol/water partition coefficient and the refraction index, in their calculations. For a 2D-HPTLC system, no studies predicting the retention behavior of peptides are known yet.

Consequently, it was the aim of this study to characterize the peptides of tryptic protein hydrolyzates using 2D-HPTLC and to determine the capabilities and limitations of this method. For the different experiments, the five most abundant milk proteins (α-lactalbumin, β-lactoglobulin, α-, β- and κ-casein) were selected as model proteins. It was intended to evaluate the repeatability of the separation and to determine whether the peptides of the different milk protein hydrolyzates are located in different areas on the HPTLC plate after the two-dimensional separation. Furthermore, it should be determined, whether correlations can be established between the properties of the peptides and their R_f values. It was hypothized that R_f values can be predicted for peptides of different protein origins based on their amino acid sequence.

Table 1
Protein origin, amino acid sequences, and composition of the whey peptide and the pea peptide mixtures.

whey peptide mixture protein origin	amino acid sequence	composition [vol%]
α-LA	EQLTKCEVFR	9.35
α-LA	CEVFR	5.61
α-LA	ALCSEK	5.61
α-LA	IWCK	5.61
β-LG	VYVEELKPTPEGDLEILLQK	9.35
β-LG	SLAMAASDISLLDAQSAPLR	9.35
β-LG	LSFNPTQLEEQCHI	5.61
β-LG	TPEVDDEALEK	7.48
β-LG	LIVTQTMK	5.61
β-LG	IDALNENK	5.61
β-LG	ALPMHIR	5.61
β-LG	IVTQTMK	5.61
β-LG	QLEEQCHI	5.61
β-LG	PMHIR	14.0
Pea peptide mixture		
legumin A	IESEGGLIETWNPNNK	33.3
legumin A	QEEEEDEDEER	33.3
vicilin	ILENQK	33.3

2. Material and methods

2.1. Materials

2.1.1. Reagents

Acetone, acetic acid, ammonia, and methanol (HPLC-grade) were purchased from VWR International GmbH (Darmstadt, Germany). Fluorescamine and iodoacetamide were obtained from AppliChem GmbH (Darmstadt, Germany). 2-Butanol, sodium carbonate, and sodium bicarbonate were purchased from Grüssing GmbH (Filsum, Germany). Acetonitrile (MS-grade), ammonium bicarbonate, dithiotreitol (DTT), trifluoroacetic acid (TFA), and urea were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). 2,5-Dihydroxybenzoic acid and trypsin from porcine pancreas with a specific activity of 13,000–20,000 BAEE units/mg protein were purchased from Merck KGaA (Darmstadt, Germany). Pyridine was obtained from Fisher Scientific GmbH (Schwerte, Germany). The peptide calibration standard II for MALDI-TOF-MS analysis was purchased from Bruker Daltonik GmbH (Bremen, Germany). Purified water (18.2 MΩ) was obtained from a water purification system (ELGA LabWater, Veolia Water Technologies Deutschland GmbH, Celle, Germany). Unless otherwise specified, ACS grade was used for all reagents.

2.1.2. Proteins

The five most abundant milk proteins were chosen as model proteins for subsequent tryptic hydrolysis: α-lactalbumin (α-LA, $\geq$ 85% purity), β-lactoglobulin (β-LG, $\geq$ 85% purity), α-casein (α-CA, $\geq$ 70% purity), β-casein (β-CA, $\geq$ 98% purity) and κ-casein (κ-CA, $\geq$ 70% purity) were all purchased from Merck KGaA (Darmstadt, Germany).

2.1.3. Peptides

For the prediction of the R_f values of the peptides with known amino acid sequences, synthetic whey and pea (*Pisum sativum*) peptides without any modifications (98% purity) were purchased from Synpeptide Co. Ltd. (Shanghai, China). Peptide sequences were selected based on a *in silico* tryptic digest of α-LA, β-LG, legumin A, and vicilin with one or zero miscleavages (Table 1). Peptide sequences were determined using the UniProtKB protein database in combination with the protein cleaver PeptideMass from ExPASy.

2.1.4. HPTLC plates

HPTLC silica gel 60 plates (20 × 10 cm) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Methods

2.2.1. Sample preparation

Tryptic hydrolysis of proteins. According to Morschheuser et al. [4], α-LA and β-LG were dissolved in purified water, whereas α-, β- and κ-CA were dissolved in 10 mM carbonate buffer (protein concentration = 4 mg/mL). Caseins were dissolved in a carbonate buffer to maintain a neutral pH value to ensure their solubility. At pH values below 6, solubility is diminished [29]. The samples were first incubated on an orbital shaker (200 rpm, 30 min) and then centrifuged (2200 × g, 5 min). Five hundred microliters of the supernatant was lyophilized and used for hydrolysis according to Gianansanti et al. [2]. Briefly, samples were digested using trypsin for 12 h at 37 °C (protein:enzyme ratio 75:1) and cleaned-up by solid phase extraction using Sep-Pak® C18 cartridges (Waters GmbH, Eschborn, Germany). The cartrdiges were conditioned with 100% (v/v) acetonitrile, equilibrated, and washed with 0.6% (v/v) acetic acid in water. The desalted peptides were eluted with an elution buffer made out of 80% (v/v) acetonitrile and 0.6% (v/v) acetic acid in water. At last, the eluate was lyophilized, re-dissolved in 500 µL of elution buffer, and applied to 2D-HPTLC.

Synthesized peptide mixtures. The synthetic peptide standards were dissolved in purified water (peptide concentration = 2 mg/mL). A whey peptide mixture, consisting of 14 peptides and a pea peptide mixture, consisting of three peptides, were prepared. The amino acid sequences of the peptides and their mixing ratios are shown in Table 1. The mixing ratios were calculated based on a previously performed 1D-HPTLC experiment (data not shown). There, equal volumes of the synthetic peptide standards were applied onto a HPTLC plate, separated in the first dimension, and derivatized with fluorescamine. The intensities of the individual peptide bands were determined visually and compared with each other. The standard solutions of peptides that showed weaker bands make up larger percentage of the volume in the peptide mixture and *vice versa.*

2.2.2. Two-dimensional high-performance thin-layer chromatography (2D-HPTLC)

2D-HPTLC was performed following a protocol described by Pasilis et al., Tscherch et al., and Morschheuser et al. [3,4,11]. Primarily, silica gel HPTLC plates were pre-washed with methanol and activated at 100 °C for 10 min. Ten microliters each of the protein hydrolyzates and the pea peptide mixture or 15 µL of the whey peptide mixture were applied using an HPTLC autosampler (ATS4, CAMAG AG, Muttenz, Switzerland). Samples were sprayed as spots (1 mm bands) under a flow of nitrogen 8 mm from the left edge and 8 mm from the lower edge of the HPTLC plates (10 × 10 cm). For the following chromatographic separations twin-through chambers were used. The solvent system of the first dimension consisted of 2-butanol/pyridine/ammonia/purified water (39/34/10/26; v/v/v/v), that of the second of 2-butanol/pyridine/acetic acid/purified water (44/32/8/20; v/v/v/v). The development of plates was carried out up to a solvent migration distance of 80 mm under room temperature and atmospheric pressure. After each development the remaining solvents were evaporated overnight (12 h). For protein/peptide specific derivatization, the fully developed plates were immersed in a fluorescamine solution (0.05% in acetone) for 1 s at a speed level of 1 using the chromatogram immersion device III (CAMAG AG, Muttenz, Switzerland). After the solvent was evaporated, the analytes were visualized under UV light (254 nm,

366 nm) using a photodocumentation system (TLC visualizer, CAMAG AG, Muttenz, Switzerland). To determine the repeatability of the method, 2D-HPTLC measurement was performed eight times for each of the five milk proteins (c.f. Section 2.1.2), with one measurement including development in both directions.

2.2.3. Identification of separated peptides

Resolvatization of the separated peptides. Fifteen to twenty of the derivatized peptide spots per protein hydrolyzate were selected for an identification of the amino acid sequence. The main selection criteria were the intensity of the peptide spots and the separation from other spots in the chromatogram. The *x-coordinates* over the entire width and the *y*-coordinates over the entire length of the selected peptide spots were determined using the software winCats (version 1.4.8, CAMAG AG, Muttenz, Switzerland). The spots on the silica gel were scraped out with a scalpel and transferred to a reaction vessel with a paintbrush. One and half milliliter of 60% (v/v) acetonitrile and 0.1% (v/v) formic acid in water was added. The suspensions were incubated on an orbital shaker (400 rpm, 30 min) and then centrifuged (1700 × g, 10 min, 4 °C). The whole supernatants were filtered with a 0.45 µm regenerated cellulose filter (25 mm, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The filtrate was lyophilized, re-dissolved in 15 µL of 60% (v/v) acetonitrile and 0.1% (v/v) formic acid in water, and applied to MALDI-TOF-MS/MS.

MALDI-TOF-MS/MS. The peptide solutions were mixed with an equal volume of matrix (2,5-dihydroxybenzoic acid, 20 mg/mL in 30% (v/v) acetonitril in water). Approximately, an aliquot of 0.5 µL of the mixture was spotted onto a ground steel target and allowed to crystallize on air. For the MALDI-TOF-MS/MS analysis an ultrafleXtreme™ mass spectrometer equipped with a smartbeam-II™ laser (Bruker Daltonik GmbH, Bremen, Germany) was used. The mass spectra were aquired in positive reflector mode in a mass range of *m/z* 340-4,000. An external calibration of the mass spectrometer was performed with the peptide calibration standard II (dissolved in 125 µL 30% (v/v) acetonitrile in water). The LIFT™ technique with a mass range of *m/z* 10-2,500 was used for tandem mass spectrometry of selected signals. For mass spectrum analysis, flexAnalysis software (version 3.3, Bruker Daltonik GmbH, Bremen, Germany) was used.

Peptide identification by database search. For the interpretation of MS/MS spectra, the UniProtKB protein database was used in combination with the protein cleaver PeptideMass from ExPASy, resulting in a list of peptides of a theoretical tryptic degradation with up to two miscleavages. If a particular peptide was thereby suspected, the *b*- and *y*-fragments of the peptide were calculated using the PROTEOMICS TOOLKIT Fragment Ion Calculator (Institute for Systems Biology, Seattle, USA) and compared with the signals of the fragment spectrum in flexAnalysis. When the complete sequence or, in some cases, at least a large proportion of the calculated fragments were found, the peptide spot was considered as identified.

2.2.4. Statistical analysis

Repeatability of 2D-HPTLC. For each protein hydrolyzate ($n = 8$), 15–20 peptides were selected for determination of repeatability. For each spot, the *x*- and *y-coordinates* were determined with the winCats software. The data was tested for normal distribution by using the Kolmogorov-Smirnov test ($\alpha = 0.05$). When a normal distribution was given, outliers for the *x* -and *y-coordinates* were checked for each peptide spot according to Dixon's Q test ($\alpha = 0.05$). Finally, the mean and standard deviation (SD) were calculated from the cleaned values.

Correlations between peptide properties and retention behavior. For the identified peptides, the amino acid composition was used to determine the percentage of anionic, cationic, uncharged polar, and non-polar amino acids of the peptide. The molecular weight (MW) and IEP of the peptides were obtained using the PROTEOMICS TOOLKIT Fragment Ion Calculator (Institute for Systems Biology, Seattle, USA). Subsequently, the *x*- and *y-coordinates* were plotted against the different properties of the peptides and the Pearson correlation coefficients were calculated for the presumed linear correlations. The statistical significance of the correlation was calculated on the basis of a two-sided *t*-test ($\alpha = 0.05$).

3. Results and discussion

3.1. Determination of the repeatability of the method

One goal of this study was to determine the repeatability of this method in order to test its applicability to other questions such as biomarker selection or the study of intra- and intermolecular interactions. For this purpose, the five most abundant milk proteins were chosen as model proteins and each protein was tryptically hydrolyzed and separated eight times by 2D-HPTLC. Figs. 1a, d and 2a, d, g show example chromatograms for the whey proteins and caseins, respectively. The chromatographic conditions were chosen according to Tscherch et al.: A basic solvent system consisting of 2-butanol/pyridine/ ammonia/purified water (39/34/10/26; v/v/v/v) was used for the development of the HPTLC plates in the first dimension, and an acidic solvent system containing 2-butanol/pyridine/acetic acid/purified water (40/32/12/20; v/v/v/v) was used in the second dimension. The acidic solvent system, however, required optimization, as the separated peptides did not provide satisfactory spot sharpness. For this reason, the amount of acetic acid was reduced by 0.4 mL in the eluent and the amount of 2-butanol was increase by the same volume. As a result, the basic mobile phase consisted of 2-butanol/pyridine/ammonia/purified water (44/32/8/20; v/v/v/v) [11].

Own preliminary experiments compared different stationary phases and showed that proteins and peptides achieved best separation and band/spot sharpness, when silica gel 60 was used. Pasilis et al. also concluded that a better separation was obtained using silica gel plates in comparison to cellulose plates [3]. For this reason, also in this method, silica gel 60 was used as stationary phase.

For further analysis, 15–20 peptide spots per protein were selected. Figs. 1b, e and 2b, e, h show the spots identified. The *x*- and *y-coordinates* of each identified spot were determined on each developed HPTLC plate; the mean values and SD calculated from these are shown in Table 2. It was always possible to assign the spots to their identification numbers, as the different milk protein hydrolyzates had consistent spot patterns. In Figs. 1c, f and 2c, f, i, the mean values of the *x*- and *y-coordinates* of the peptide spots were plotted in coordinate systems and the SD were given as error bars. It is apparent that the SD are smaller in the lower left and upper right corners than in the middle of the HPTLC plates. This could be explained by the fact that a particularly large number of peptides with similar polarities and a similar number of dissociable groups are retarded in the middle region of the HPTLC plate. These peptides compete for interaction with the silanol groups of the stationary phase. Thus, small changes in the composition of the stationary phase lead to larger standard deviations in the middle of the plate than in the corners, where only a few peptides compete with each other. The SD varied between 1.0 and 11.1 mm for the *x-coordinates* and between 0.5 and 7.3 mm for the *y-coordinates*. Only Bakry et al. so far determined the repeatability at least for 1D-HPTLC separation of proteins and peptides [20]. For the (non-hydrolyzed) proteins insulin, cytochrome c, lysozyme, and myo-

Table 2
Mean values, SD and RSD of the *x*- and *y-coordinates* of the identified peptide spots for the five milk protein hydrolyzates; *n*: number of values for the determination of the *x*- and *y-coordinates* after an outlier test according to Dixon's Q test ($\alpha = 0.05$).

Protein	Spot number	Mean *x*-coordinate [mm]	SD *x*-coordinate [mm]	RSD *x*-coordinate [%]	n	Mean *y*-coordinate [mm]	SD *y*-coordinate [mm]	RSD *x*-coordinate [%]	n
α-LA	1	21.9	2.2	10.20	8	11.3	2.1	18.9	8
α-LA	2	21.4	1.8	8.3	8	17.9	3.6	20.2	8
α-LA	3	27.0	2.2	8.2	8	17.6	3.4	19.4	8
α-LA	4	30.1	2.5	8.2	8	24.0	5.9	24.5	8
α-LA	5	33.5	2.9	8.7	8	24.3	5.7	23.6	8
α-LA	6	39.6	3.3	8.3	8	30.8	5.8	18.9	8
α-LA	7	42.6	5.3	12.5	8	29.5	5.3	18.0	8
α-LA	8	44.5	4.3	9.6	8	33.5	7.3	21.9	8
α-LA	9	48.1	5.0	10.4	8	33.4	7.3	21.8	8
α-LA	10	39.0	2.0	5.1	8	40.0	5.8	14.5	8
α-LA	11	46.8	3.5	7.5	8	41.1	5.3	12.9	8
α-LA	12	50.1	6.3	12.5	8	45.5	5.8	12.7	8
α-LA	13	61.0	4.0	6.5	8	55.3	4.6	8.3	8
α-LA	14	64.5	4.9	7.6	8	52.0	5.6	10.7	8
α-LA	15	64.0	3.4	5.3	8	59.1	4.6	7.7	8
β-LG	1	10.4	2.3	21.8	8	6.8	0.5	6.9	8
β-LG	2	13.3	3.3	25.1	8	7.5	0.5	7.1	8
β-LG	3	11.4	3.9	33.9	8	12.3	1.4	11.3	8
β-LG	4	17.4	6.5	37.3	8	11.9	1.2	10.5	8
β-LG	5	19.9	5.8	29.0	8	17.5	1.9	11.0	8
β-LG	6	23.9	10.8	45.2	8	20.1	2.6	12.9	8
β-LG	7	23.0	8.5	37.0	8	25.1	3.2	12.8	8
β-LG	8	32.9	6.9	21.0	8	26.6	2.5	9.4	8
β-LG	9	29.8	9.8	33.0	8	37.5	3.4	9.0	8
β-LG	10	34.9	9.9	28.3	8	37.4	3.5	9.4	8
β-LG	11	31.3	9.6	30.8	8	42.3	4.7	11.1	8
β-LG	12	36.3	11.1	30.6	8	41.6	4.7	11.2	8
β-LG	13	24.1	8.9	36.7	8	47.0	5.2	11.0	8
β-LG	14	36.3	9.5	26.1	8	47.1	4.9	10.4	8
β-LG	15	45.7	7.5	16.5	8	50.6	5.5	10.8	8
β-LG	16	55.6	5.8	10.3	8	59.4	5.7	5.1	8
β-LG	17	70.4	1.1	1.5	8	66.9	4.6	6.8	8
α-CA	1	14.0	3.5	25.3	8	11.3	1.0	9.2	8
α-CA	2	11.1	2.5	22.8	8	16.8	1.8	10.9	8
α-CA	3	19.0	4.8	25.0	8	12.3	1.0	8.4	8
α-CA	4	19.5	6.4	32.7	8	18.4	1.8	9.6	8
α-CA	5	21.9	7.4	33.6	8	21.4	2.3	10.6	8
α-CA	6	24.5	8.1	32.9	8	25.3	2.0	7.8	8
α-CA	7	23.0	8.3	35.9	8	28.4	2.7	9.6	8
α-CA	8	11.4	2.4	21.5	8	36.8	2.4	6.5	8
α-CA	9	17.4	4.3	25.0	8	38.0	2.0	5.3	8
α-CA	10	22.5	9.2	41.0	8	35.3	2.3	6.6	8
α-CA	11	24.1	9.5	39.5	8	39.6	1.7	4.3	8
α-CA	12	32.1	10.8	33.7	8	42.0	2.1	5.1	8
α-CA	13	32.5	11.2	34.6	8	39.3	1.6	4.0	8
α-CA	14	40.4	10.8	26.6	8	44.4	2.3	5.1	8
α-CA	15	47.4	11.7	24.7	8	42.8	2.4	5.6	8
α-CA	16	48.1	11.8	24.5	8	46.3	2.3	4.9	8
α-CA	17	47.5	9.3	19.6	8	50.5	1.5	3.0	8
α-CA	18	52.6	7.9	29.3	8	53.9	1.2	2.3	8
α-CA	19	58.0	6.2	10.7	8	56.8	2.0	3.5	8
α-CA	20	60.6	6.0	9.8	8	59.1	2.0	3.4	8
β-CA	1	14.0	1.2	8.5	8	10.1	1.9	18.6	8
β-CA	2	18.0	1.4	7.9	8	16.0	3.9	24.5	8
β-CA	3	30.3	2.6	8.6	8	15.8	3.5	22.4	8
β-CA	4	35.4	3.5	9.9	8	15.8	4.4	27.7	8
β-CA	5	33.0	1.2	3.5	7	19.1	4.9	25.8	8
β-CA	6	25.1	2.5	9.9	8	22.1	5.3	24.0	8
β-CA	7	39.9	1.2	3.0	7	22.5	5.6	24.9	8
β-CA	8	45.3	1.3	2.8	7	26.1	5.3	20.3	8
β-CA	9	42.9	1.2	2.8	7	29.3	5.2	17.7	8
β-CA	10	42.7	1.0	2.2	7	32.6	5.2	15.9	8
β-CA	11	48.6	1.5	3.1	7	32.3	6.2	19.1	8
β-CA	12	38.6	1.1	2.9	7	38.1	5.8	15.1	8
β-CA	13	46.3	1.0	2.1	7	40.5	6.4	15.9	8
β-CA	14	60.4	1.8	2.9	8	40.8	5.0	12.4	8
β-CA	15	54.7	1.3	2.3	7	47.5	4.6	9.6	8
β-CA	16	51.1	1.2	2.4	7	48.5	4.4	9.2	8
β-CA	17	51.0	1.2	2.3	7	52.9	3.2	6.1	8
β-CA	18	65.5	1.3	4.5	8	54.0	3.2	5.9	8
κ-CA	1	11.9	1.7	14.5	8	8.8	1.9	21.8	8

(*continued on next page*)

Table 2 (*continued*)

Protein	Spot number	Mean *x*-coordinate [mm]	SD *x*-coordinate [mm]	RSD *x*-coordinate [%]	n	Mean *y*-coordinate [mm]	SD *y*-coordinate [mm]	RSD *x*-coordinate [%]	n
κ-CA	2	12.9	2.5	19.2	8	13.9	2.4	17.0	8
κ-CA	3	19.1	3.4	18.0	8	12.0	2.3	19.4	8
κ-CA	4	16.1	2.8	17.4	8	14.9	3.6	24.0	8
κ-CA	5	21.9	4.2	19.0	8	16.5	2.4	14.8	8
κ-CA	6	16.8	3.3	19.6	8	22.5	4.2	18.6	8
κ-CA	7	29.1	4.9	17.0	8	28.6	4.6	16.1	8
κ-CA	8	22.5	4.7	21.0	8	33.6	5.8	17.1	8
κ-CA	9	31.8	5.6	17.6	8	32.3	5.5	17.0	8
κ-CA	10	41.1	5.6	13.6	8	41.5	4.6	11.1	8
κ-CA	11	50.8	5.4	10.7	8	42.0	5.6	13.3	8
κ-CA	12	47.6	5.7	11.9	8	46.1	4.7	10.3	8
κ-CA	13	49.1	4.6	9.4	8	52.6	2.1	4.1	8
κ-CA	14	57.4	2.8	4.9	8	53.4	2.2	4.1	8
κ-CA	15	62.8	2.4	3.9	8	58.5	1.9	3.3	8

globin they found an average, relative standard deviation (RSD) for the R_f values of 3.7% and for standard peptides they determined average RSD ranging from 1.8–2.9%. The 2D-HPTLC method presented herein for the separation of milk protein hydrolyzates resulted in an average RSD for the R_f values of 16.5% for the *x-coordinates* and 12.9% for the *y-coordinates*. Thus, a worse repeatability is obtained, but it should be noted that this is a method with a double elution, so that a poorer result was to be expected due to increasing possibilities for diffusion, when using a 2D approach. In addition, Bakry et al. used special monolithic thin layers as stationary phase, which are characterized by a smaller layer thickness and the absence of a binder, which may also increase the separation efficiency of small molecules, peptides and proteins [20]. Another possibility that can lead to a decrease in repeatability is contamination on the HPTLC plate. For this reason, in the presented method, the silica gel plates used were preconditioned by developing them completely with methanol to remove adhering impurities. The plates were then treated at 100 °C for 10 min to evaporate the methanol and any water residues. Therefore, the influence of methanol and water on the repeatability of the peptide separation should be negligible.

It can be resumed that with average, absolute SD of 4.9 mm and 3.7 mm for the *x*- and *y-coordinates*, respectively, the repeatability of this method can be considered as sufficient, as due to the pattern formation of the peptide spots, an assignment of the spots to their identification numbers is possible at any time. Thus, quali-

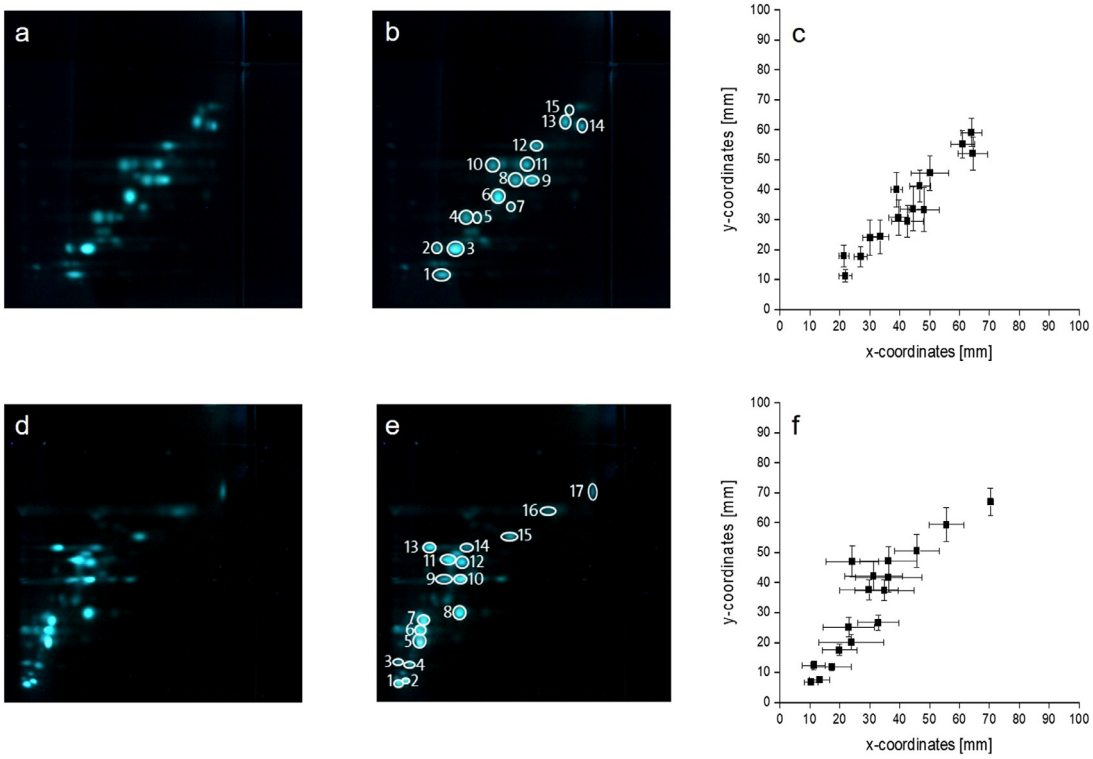

Fig. 1. a, d: 2D-HPTLC chromatograms of whey protein hydrolyzates resulting from tryptic digestion; b, e: Identified peptides of the hydrolyzates; c, f: SD of *x*- and *y-coordinates* of the selected peptide spots; a-c: α-lactalbumin, d-f: β-lactoglobulin. Numbers refer to Table 2 for means and SD of the *x*- and *y-coordinates* of the peptides and Table 3 for the amino acid sequences of the identified peptides. Development in the first dimension (*y-coordinates*) was performed with a basic solvent system consisting of 2-butanol/pyridine/ammonia/purified water (39/34/10/26; v/v/v/v). For the second dimension (*x-coordinates*), an acidic solvent system composed of 2-butanol/pyridine/acetic acid/purified water (44/32/8/20; v/v/v/v) was used.

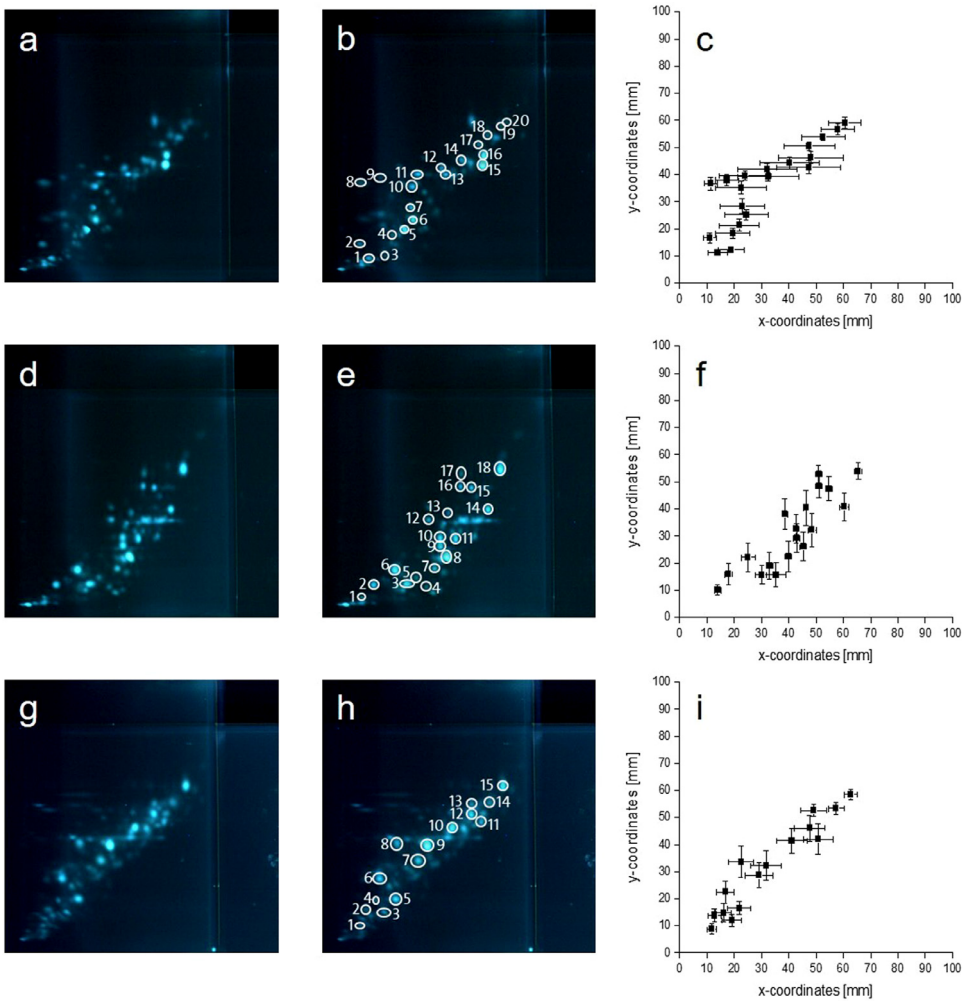

Fig. 2. a, d, g: 2D-HPTLC chromatograms of casein hydrolyzates resulting from tryptic digestion; b, e, h: Identified peptides of the hydrolyzates; c, f, i: SD of *x*- and *y*-*coordinates* of the selected peptide spots; a-c: α-casein, d-f: β-casein, g-i: κ-casein. Numbers refer to Table 2 for means and SD of the *x*- and *y*-*coordinates* of the peptides and Table 3 for the amino acid sequences of the identified peptides. Development in the first dimension (*y*-*coordinates*) was performed with a basic solvent system consisting of 2-butanol/pyridine/ammonia/purified water (39/34/10/26; v/v/v/v). For the second dimension (*x*-*coordinates*), an acidic solvent system composed of 2-butanol/pyridine/acetic acid/purified water (44/32/8/20; v/v/v/v) was used.

tative statements about the identity of spots can be made without constant mass spectrometric structural identification of the peptides. Furthermore, changes in the peptide pattern can be perceived, so that, for example, differences in the retention behavior of the peptides through intra- and intermolecular interactions can be detected. However, it must be considered that the more complex the protein mixture gets (e.g., in a dairy product), the more the error areas of the peptide spots overlap, so that an assignment to their identification numbers will become increasingly difficult. To facilitate the assignment of a large number of peptide spots, a targeted detection would be advantageous. Peptide spots of particular interest could thus be stained individually with e.g., specific immunostaining or dyes.

3.2. Clustering of milk protein hydrolyzates

After the mean values and SD for the *x*- and *y*-*coordinates* have been determined for the single milk protein hydrolyzates, it should be investigated in which areas of the HPTLC plate the peptide spots are located. Furthermore, it should be checked, whether the peptides of a protein hydrolyzate cluster in a certain area of the chromatogram. Looking again at the coordinate systems of Figs. 1c, f and 2c, f, i, it is obvious that the peptide spots are all located approximately on an imaginary diagonal at a 45 ° angle between the *x*- and *y*-axes after chromatographic separation. This means that the basic and the acidic solvents have comparable elution properties. The variation from a high to a low pH value should lead to change in the degree of dissociation of ionizable groups of the peptides and thus, to different retention behaviors [11,15]. However, Gwarda and Dzido found that different pH values only lead to small changes in separation selectivity and rather increase separation efficiency [15]. According to Gwarda et al., a large difference between the R_f values in two dimensions of the chromatogram requires a change between a NP and a RP system [6]. In the present method, only a NP system was used. When taking a closer look at the coordinate systems, it is noticeable that the hydrolyzates of the different proteins all behave somewhat differently and mostly lie either above or below the imaginary diagonal. In order to clarify the differences in the chromatographic behavior of the different protein hydrolyzates, the mean values of the *x*- and *y*-*coordinates* were transferred to a mutual coordinate system for the whey proteins and for the caseins, respectively. Fig. 3a shows the peptides of the whey protein hydrolyzates. It is quite obvious that two clusters formed: The peptides of the α-LA hydrolyzate have lower R_f values in the first dimension and higher R_f values in the second dimension compared to the peptides of β-LG. Only a few peptides

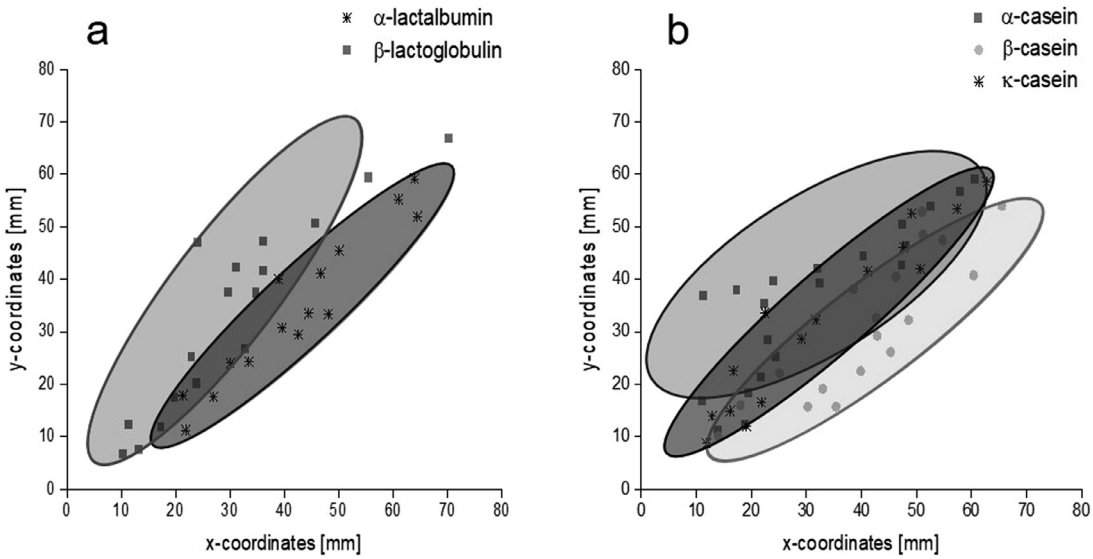

Fig. 3. Clustering of the selected peptides of whey protein hydrolyzates (a) and casein hydrolyzates (b). Development in the first dimension (*y-coordinates*) was performed with a basic solvent system consisting of 2-butanol/pyridine/ammonia/purified water (39/34/10/26; v/v/v/v). For the second dimension (*x-coordinates*), an acidic solvent system composed of 2-butanol/pyridine/acetic acid/purified water (44/32/8/20; v/v/v/v) was used.

overlap at the level of the imaginary diagonal between the *x*- and *y*-axes. This indicates that it is reasonable to to predict from which whey protein a peptide spot on the HPTLC plate originates. Nevertheless, it should be considered that despite the eightfold repetition of the separation per protein hydrolyzate, random formation of the two clusters cannot be completely excluded. As the whey proteins α-LA and β-LG show very similar properties in terms of polarity and conformation, no major differences in separation are expected [30]. When looking at the distribution of the peptides of the casein hydrolyzates in Fig. 3b, three areas can be identified in which the peptides of the three caseins are located. The peptides of κ-CA lie along the imaginary diagonal between the *x*- and *y*-axes. In comparison, the peptides of α-CA usually have higher R_f values in the first dimension and lower R_f values in the second dimension, whereas the peptides of β-CA are located below the imaginary diagonal, so that the R_f values are lower in the first dimension and higher in the second dimension. However, it can be seen that when comparing three protein hydrolyzates, the area of overlap of the clusters becomes larger. As a consequence, in complex protein mixtures it is not possible to assign a peptide to its protein origin based on its R_f values. When the protein origin of the individual peptides should be determined via the clustering method, a separation system specifically adapted to the respective proteins should be applied. In addition, other evaluation technqiues (than simple comparison of overlapping) should be considered as well in the future. There are several possibilities for evaluation such as image analysis of the rectangles that are confined by standard deviations of the *x*- and *y-coordinates* or more advanced methods taking the resolutions of the *x*- and *y*-axes into account.

However, for appropriate formation of peptide clusters, differences in protein properties should be considered. Consequently, a distinction can be made between acidic vs. basic or polar vs. nonpolar or further differentiations (e.g., cytosolic proteins *vs.* transmembrane proteins). Thereby, the more different the proteins are, the better the method will work. Ideally, the obtained peptide clusters would then be located in opposite regions of the HPTLC plate. The overall aim should be to obtain the best separation of total peptide clusters, rather than individual peptides over the entire extension of the HPTLC plate. Hence, not only the individual peptides, but the entire peptide patterns would be compared. The presence/absence of characteristic peptides for proteins would indicate the presence/absence of a protein in the mixture under investigation. Furthermore, post-translational modifications in a peptide pattern could also be identified in direct comparison to the peptide pattern of the corresponding native protein, either by detecting new peptide spots or by the disappearance of other peptide spots.

3.3. Mass spectrometric identification of peptide spots

The next step was to identify the amino acid sequences of the selected peptides by mass spectrometric analysis in order to subsequently establish correlations between the retention behavior and the properties of the peptides. For this purpose, the developed HPTLC plates were first derivatized with fluorescamine and the spots visible at UV light were assigned to the identified spots. The fluorescamine derivatisation made it possible to determine the exact *x*- and *y-coordinates* from these spots. The stationary phase including the analytes was then manually removed in the desired area and resolvated in several steps. The resulting solution was mixed with a matrix solution and applied directly to a ground steel target for MALDI-TOF-MS/MS analysis. A plate scan by means of a MALDI adapter was deliberately avoided, as it was found in preliminary tests that the chromatography plates containing aluminium as a carrier material due to needed conductivity for mass spectrometry do not show good separation performance. In addition, diffusion of the analytes occurs during the coating process with MALDI matrix and the detection limit is much higher. Biller likewise described the phenomenon of analyte diffusion in the immersion process for matrix application [31]. In contrast, the indirect method presented herein makes it possible that the desired peptides are studied, as the derivatized peptides can be targeted directly and thus, no reference plate has to be prepared. A similar approach was described by Bakry et al., who pre-derivatized their peptides with fluorescamine [20]. This allowed the matrix to be sprayed or spotted directly onto the peptide spots after UV detection for mass spectrometric analysis by MALDI-TOF-MS. Subsequently, the peaks of both the underivatized peptides and the corresponding peptides derivatized with fluorescamine were detected in the MALDI spectrum. In the method presented herein, both variants of the peptide were also found, with mass differences of $m/z = 260–262$. For example, in the MALDI spectrum of spot No. 1

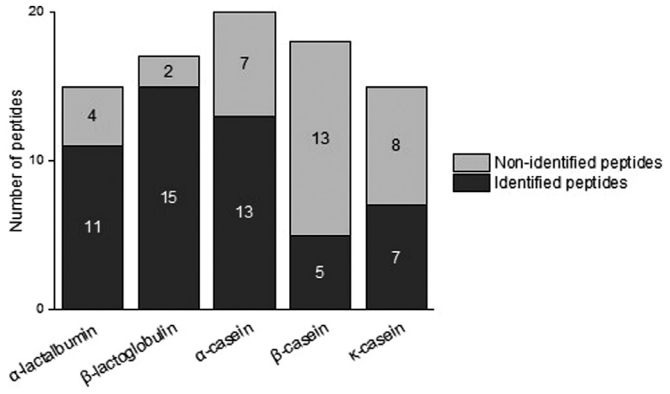

Fig. 4. Identification rates of amino acid sequences of milk peptides determined by MALDI-TOF-MS/MS.

of β-LG, *m/z* 1635.75 and *m/z* 1896.73 were measured with a mass difference of 261 Da between the two signals. *m/z* 1635.75 can be assigned to the $(M+H)^+$ ion of the peptide TPEVDDEALEKFDK and *m/z* 1896.73 to the same fluorescently labeled peptide.

When using tandem mass spectrometry, the obtained *b*- and *y*-fragments could be compared with the theoretically calculated fragment ions by the protein database UniProt in combination with the peptide cutter Expasy and the PROTEOMICS TOOLKIT Fragment Ion Calculator. Thus, the amino acid sequence of 51 peptides of the 85 investigated peptide spots were reliably identified by mass spectrometry, which corresponds to an identification rate of 60% of the measured peptide spots. The identification rates of the different protein hydrolyzates are shown in Fig. 4, whereby these differ markedly between the different protein origins. While β-LG showed an identification rate of 88% with 15 out of 17 peptides, β-CA had an identification rate of only 28% with 5 out of 18 peptides. Table 3 lists the identified amino acid sequences for the investigated peptide spots of the five protein hydrolyzates. It is evident that it was particularly difficult to determine the amino acid sequence of peptides with higher R_f values. As an NP system was used, it is easier to identify polar peptides with this method. This is in contrast to Pasilis et al., who used the HPTLC/DESI-MS method for mass spectrometric peptide identification and came to the conclusion that hydrophilic peptides with lower R_f values are more difficult to determine due to stronger binding to the polar stationary phase [3,16]. This problem is apparently avoided in the method presented herein, when removing the entire silica gel including the analyte in the desired area and then extracting with a polar solvent mixture of of 60% (v/v) acetonitrile and 0.1% (v/v) formic acid in water. The polar extraction agent could possibly be the reason for the poorer identification rate of the non-polar peptides, so that in the future, it might be possible to extract peptide spots with higher R_f values with more apolar solvents. Furthermore, the analysis is also concentration-dependent, as often larger peptide spots on the HPTLC plates were easier to identify than smaller ones. An increase in the volume of the applied samples on the HPTLC plates should be avoided, as the resolution and spot sharpness would be reduced due to an overloading with the analytes.

3.4. Correlations between peptide properties and their retention behavior on 2D-HPTLC

On the basis of the peptides for which the amino acid sequence was determined by mass spectrometry, a further aim was to verify correlations between properties of the peptides and their *x*- and *y-coordinates* on 2D-HPTLC. Among the peptide properties studied were MW, IEP, and percentages of anionic, cationic, uncharged polar, and non-polar amino acids in the peptide (Table 3). The correlations of the respective milk peptide properties with the *x*- and *y-coordinates* were investigated by calculating the Pearson correlation coefficients. The statistical significance of the calculated correlations was tested with a two-sided *t*-test ($\alpha = 0.05$). In Table 4, the peptide properties are divided into statistically significant and statistically non-significant and the Pearson correlation coefficient *r* is given for each peptide property. An *r* value of +1 for a positive correlation and a value of -1 for a negative correlation would represent an ideal correlation. The best correlation coefficients are obtained for the percentage of anionic amino acids (r_x = -0.51, r_y = -0.65) and non-polar amino acids (r_x = 0.44, r_y = 0.57). In addition, it was found that there was a positive correlation between the IEP of the peptides and the *x*- and *y-coordinates*. This agrees with the negative correlation for the percentage of anionic amino acids, as they have a low IEP and thus, lead to stronger retention at the stationary phase. However, the polar, cationic amino acids, in contrast to the also polar anionic amino acids, had no significant influence on the retention behavior of the peptides. It was expected that with both – the cationic and anionic amino acids – polar retention mechanisms such as electrostatic interactions, hydrogen bonding, and distribution would occur through interactions with the silanol groups of the silica gel [6]. However, in the method presented herein, the influence of anionic amino acids on retention strongly predominated over cationic amino acids. In agreement with these findings, Gwarda et al. found that when using C18 silica-based sorbents and a mobile phase for a NP system, the basic amino acids were particularly strongly retained in comparison to other polar amino acids. The increase retention is probably due to the strong polar ion-ion interactions between the basic groups of the peptides and the acidic-free silanols of the stationary phase [6,15]. The uncharged polar amino acids did not have any effect on the retention of the peptides. Molecular weight also had no statistically significant effect, although a trend can be observed, at least for the *x-coordinates*, that with increasing MW, retention also increase.

In summary, for the method presented herein statistically significant correlations between the retention behavior of the peptides and the percentages of anionic and non-polar amino acids in the peptide as well as the IEP of the peptides were confirmed. This allows a characterization of the polarity and an estimation of the percentage of anionic amino acids of the separated peptides based on the retardation factors alone.

3.5. Prediction of x- and y-coordinates for peptides in 2D-HPTLC

Now that the statistically significant correlations between the peptide properties and the retention behavior of the peptides have been demonstrated, it should be checked, whether a prediction can be made about the *x*- and *y-coordinates* of the peptides based on their amino acid composition. At hand of the best Pearson correlation coefficients, the percentages of anionic and of non-polar amino acids in the peptide seemed to be the most suitable options for a prediction. However, as there were only two anionic amino acids (aspartic acid and glutamic acid), but eight non-polar amino acids (alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, and valine), the prediction should be based on the percentage of non-polar amino acids, because of a reduced susceptibility: When the content of one basic amino acid in the peptide changes, the calculated *x*- and *y-coordinates* change much more than when the content of one non-polar amino acid in the peptide changes. In Fig. 5, the *x*- and *y-coordinates* are each plotted against the percentage of non-polar amino acids in the peptide. For the linear correlations, coefficients of determination of $R^{2x} = 0.19$ and $R^{2y} = 0.32$ were found. The regression lines for the relationships between the non-polar amino acids (non-polar AA) and the *x*- and *y-coordinates* were as follows:

Table 3
Amino acid sequences and properties of peptides identified by MALDI-TOF-MS/MS.

Protein	Spot number	Amino acid sequence	MW [g/mol]	IEP	Anionic AA [%]	Cationic AA [%]	Uncharged, polar AA [%]	Non-polar AA [%]
α-LA	1	KILDK	615.40	8.59	20.0	40.0	0.00	40.0
α-LA	2	FLDDDLTDDIMCVK	1698.73	3.66	35.7	7.1	14.29	42.9
α-LA	2	EQLTK	617.34	6.10	20.0	20.0	40.00	20.0
α-LA	3	EQLTK	617.34	6.10	20.0	20.0	40.00	20.0
α-LA	3	ALCSEK	706.31	6.04	16.7	16.7	33.33	33.3
α-LA	5	QWLCEK	862.38	5.99	16.7	16.7	33.33	33.3
α-LA	6	ILDK	487.30	5.84	25.0	25.0	0.00	50.0
α-LA	8	CEVFR	709.30	6.00	20.0	20.0	20.00	40.0
α-LA	9	CEVFR	709.30	6.00	20.0	20.0	20.00	40.0
α-LA	10	CEVFR	709.30	6.00	20.0	20.0	20.00	40.0
α-LA	12	VGINYWLAHK	1199.65	8.57	0.0	20.0	30.00	50.0
α-LA	13	GYGGVSLPE	877.42	4.60	11.1	0.0	55.56	33.3
α-LA	14	GYGGVSLPE	877.42	4.60	11.1	0.0	55.56	33.3
β-LG	1	TPEVDDEALEKFDK	1634.77	4.02	42.9	14.3	7.14	35.7
β-LG	2	TPEVDDEALEK	1244.58	3.83	45.5	9.1	9.09	36.4
β-LG	3	CMENSAEPEQSLACQCLVR	2280.90	4.25	15.8	5.3	42.11	36.8
β-LG	4	IDALNENK	915.47	4.37	25.0	12.5	25.00	37.5
β-LG	5	IDALNENK	915.47	4.37	25.0	12.5	25.00	37.5
β-LG	6	VYVEELKPTPEGDLEILLQK	2312.25	4.25	25.0	10.0	20.00	45.0
β-LG	6	IIAEK	572.35	6.00	20.0	20.0	0.00	60.0
β-LG	7	GLDIQK	672.38	5.84	16.7	16.7	33.33	33.3
β-LG	8	IIAEK	572.35	6.00	20.0	20.0	0.00	60.0
β-LG	8	LSFNPTQLEEQCHI	1714.78	4.51	14.3	7.1	28.57	50.0
β-LG	9	LSFNPTQLEEQCHI	1714.78	4.51	14.3	7.1	28.57	50.0
β-LG	9	AASDISLLDAQSAPLR	1626.86	4.21	12.5	6.3	25.00	56.3
β-LG	9	LIVTQTMK	932.54	8.75	0.0	12.5	37.50	50.0
β-LG	10	TKIPAVFK	902.56	10.00	0.0	25.0	12.50	62.5
β-LG	10	IPAVFK	673.42	8.75	0.0	16.7	0.00	83.3
β-LG	10	AASDISLLDAQSAPLR	1626.86	4.21	12.5	6.3	25.00	56.3
β-LG	11	LIVTQTMK	932.54	8.75	0.0	12.5	37.50	50.0
β-LG	12	AASDISLLDAQSAPLR	1626.86	4.21	12.5	6.3	25.00	56.3
β-LG	12	IPAVFK	673.42	8.75	0.0	16.7	0.00	83.3
β-LG	13	ALPMHIR	836.47	9.80	0.0	28.6	0.00	71.4
β-LG	14	ALPMHIR	836.47	9.80	0.0	28.6	0.00	71.4
β-LG	15	ALPMHIR	836.47	9.80	0.0	28.6	0.00	71.4
α-CA	1	EDVPSER	830.38	4.14	42.9	14.3	14.29	28.6
α-CA	2	EGIHAQQK	909.47	6.85	12.5	25.0	37.50	25.0
α-CA	3	EDVPSER	830.38	4.14	42.9	14.3	14.29	28.6
α-CA	4	ITVDDK	689.36	4.21	33.3	16.7	16.67	33.3
α-CA	5	YVPLGTQYTDAPSFSDIPNPIGSENSEK	3025.42	3.92	14.3	3.6	46.43	35.7
α-CA	6	VNELSK	688.38	5.97	16.7	16.7	33.33	33.3
α-CA	10	HQGLPQEVLNENLLR	1758.94	5.40	13.3	13.3	33.33	40.0
α-CA	11	EPMIGVNQELAYFYPELFR	2315.13	4.25	15.8	5.3	26.32	52.6
α-CA	12	EPMIGVNQELAYFYPELFR	2315.13	4.25	15.8	5.3	26.32	52.6
α-CA	13	EPMIGVNQELAYFYPELFR	2315.13	4.25	15.8	5.3	26.32	52.6
α-CA	14	YPELFR	823.42	6.00	16.7	16.7	16.67	50.0
α-CA	14	QELAYFYPELFR	1574.78	4.53	16.7	8.3	25.00	50.0
α-CA	15	FFVAPFPEVGK	1236.65	6.00	9.1	9.1	9.09	72.7
α-CA	16	YLGYLEQLLR	1266.70	6.00	10.0	10.0	40.00	40.0
β-CA	8	AVPYPQR	829.44	8.79	0.0	14.3	28.57	57.1
β-CA	8	QEPVLGPVR	993.56	6.00	11.1	11.1	22.22	55.6
β-CA	9	AVPYPQR	829.44	8.79	0.0	14.3	28.57	57.1
β-CA	9	VLPVPQK	779.49	8.72	0.0	14.3	14.29	71.4
β-CA	10	AVPYPQR	829.44	8.79	0.0	14.3	28.57	57.1
β-CA	14	LLYQEPVLGPVR	1382.79	6.00	8.3	8.3	25.00	58.3
β-CA	18	GPFPIIV	741.44	5.53	0.0	0.0	14.29	85.7
κ-CA	3	**Q**EQNQEQPIR (modification: pyroglutamate)	1251.59	4.53	20.0	10.0	50.00	20.0
κ-CA	4	**Q**EQNQEQPIR (modification: pyroglutamate)	1251.59	4.53	20.0	10.0	50.00	20.0
κ-CA	5	**Q**EQNQEQPIR (modification: pyroglutamate)	1251.59	4.53	20.0	10.0	50.00	20.0
κ-CA	6	SCQAQPTTMAR	1249.53	7.96	0.0	9.1	54.55	36.4
κ-CA	9	FFSDK	642.30	5.84	0.0	20.0	20.00	60.0
κ-CA	10	VLSR	473.30	9.72	0.0	25.0	25.00	50.0
κ-CA	11	QEQNQEQPIR	1268.61	4.53	20.0	10.0	50.00	20.0

$$x\text{-coordinate} = 0.356 \times \%\ \text{non-polar AA} + 15.981 \quad (1)$$

$$y\text{-coordinate} = 0.448 \times \%\ \text{non-polar AA} + 9.7085 \quad (2)$$

To test the actual predictive power of the linear regressions obtained based on the milk protein hydrolyzates, a mixture of synthetic peptides Table 1) was separated by 2D-HPTLC using the method presented herein and the resulting *x*- and *y-coordinates* were measured ($n = 2$). The separated peptide spots were also an-

Table 4
Classification of peptide properties as statistically significant or statistically non-significant ($\alpha = 0.05$) for correlations with the *x*- and *y-coordinates* in the 2D-HPTLC chromatograms. Pearson correlation coefficients are given as r_x and r_y for the *x*- and *y-coordinates*, respectively.

Statistically significant correlation			Statistically non-significant correlation		
IEP	$r_x = 0.26$	$r_y = 0.33$	MW	$r_x = -0.21$	$r_y = -0.02$
% anionic AA	$r_x = -0.51$	$r_y = -0.65$	% cationic AA	$r_x = -0.15$	$r_y = -0.12$
% non-polar AA	$r_x = 0.44$	$r_y = 0.57$	% uncharged polar AA	$r_x = 0.01$	$r_y = -0.05$

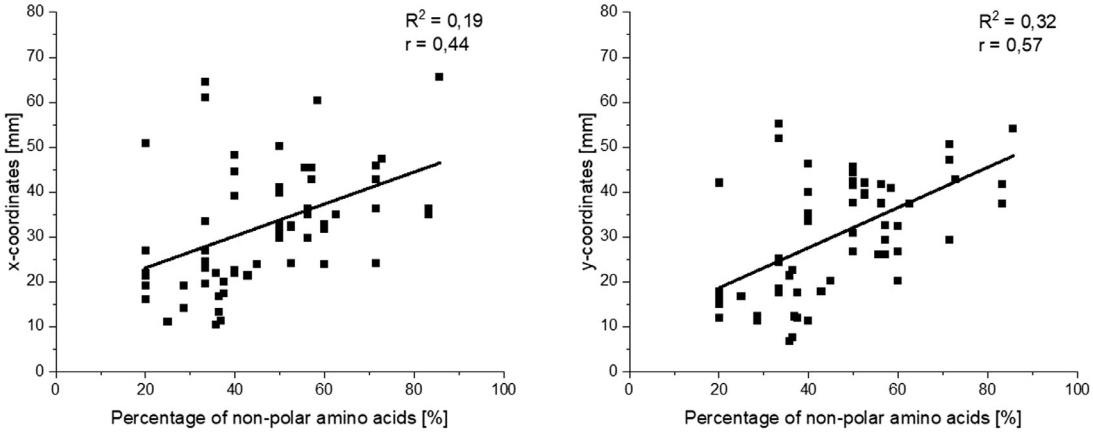

Fig. 5. Linear correlations of *x*- and *y-coordinates* of the identified peptides with regard to the percentage of non-polar amino acids in the peptides.

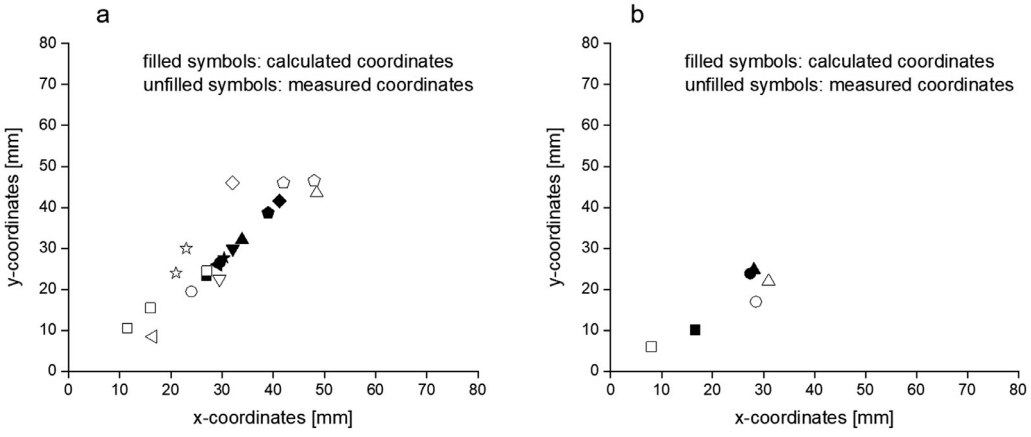

Fig. 6. Comparison of the coordinates calculated by linear regression based on the content of non-polar amino acids in the peptides (filled symbols) with the coordinates obtained by 2D-HPTLC ($n = 2$) of the whey peptide mixture (a) and the pea peptide mixure (b) (unfilled symbols). The various symbols represent the different identified peptide sequences (see Table 4).

alyzed by MALDI-TOF-MS/MS to verify the amino acid sequences. Based on the identified amino acid sequences, the theoretical *x*- and *y-coordinates* were then calculated via the linear regression lines (Eqs. (1) and ((2)) and compared with the measured values. In Fig. 6a, the filled symbols represent the calculated peptides and the unfilled symbols represent the corresponding measured peptides, with each different symbol standing for a different amino acid sequence. It can be seen that the measured peptide spots all lie around the calculated peptide spots. Particularly good predictions could be made for peptides with medium and high R_f values. Worse results were obtained for peptides with small R_f values. Absolute deviations of maximum 17.5 mm between the calculated (26.0 mm) and measured (8.5 mm) coordinates were found. This corresponds to a relative deviation of 67.3%. The absolute deviations of all peptides ranged from 0.3–15.2 mm (average: 7.8 mm) for the *x-coordinate* and from 1.4–17.5 mm (average: 7.2 mm) for the *y-coordinate*. The average, relative deviation for the R_f values is 25.3% for both the *x*- and *y-coordinates*. On the one hand, it is now possible to predict the *x*- and *y-coordinates* for peptides with a known amino acid sequence, so that, for example, the selection of internal standards is facilitated, as the subsequent position in the 2D-chromatogram can be selected. On the other hand, measured *x*- and *y-coordinates* can be used to calculate the content of non-polar amino acids in the peptide(s). Using linear regressions lines, it is possible to calculate the content of anionic amino acids in peptides with known R_f values.

Nontheless, it should be noted that the method was primarily tested using only a mixture of synthetic peptides, which all had sequences based on either α-LA or β-LG as protein. This means that the method was initially tested using only the same or very similar peptides that were used to develop the method. For this reason, it should be checked whether the method presented herein is also suitable for peptides of other protein origins. For this reason, a mixture of three peptides from pea (*Pisum sativum*) proteins (Table 1) was treated in the same way as the peptide mixture. Fig. 6b illustrates the comparison between the measured and cal-

culated *x*- and *y-coordinates* for the three pea peptides. The absolute deviations varied between 1.4 and 8.0 mm (average: 4.2 mm) for the *x-coordinates* and 2.6-6.7 mm (average: 4.3 mm) for the *y-coordinates*. The mean relative deviations with regard to the R_f values are 22.1% and 25.7% for the *x*- and *y-coordinates*, respectively. The absolute deviations for the prediction of the retention behavior of the pea peptides were even lower than for the whey peptides, although the linear regressions were determined on the basis of the milk protein hydrolyzates. However, when comparing the relative deviations, the values are in the same range. Unfortunately, only three synthetic peptides of a different protein origin were available for testing the method. Nontheless, it is reasonable to assume that the method presented herein for predicting the *x*- and *y-coordinates* in the 2D chromatogram can also be applied to proteins of other origins. In the future, the prediction power of other peptide standards or other protein origins should be further investigated.

Only Baczek and Sparzak predicted previously the retention behavior of 30 peptides based on their properties for 1D-HPTLC. They used the approach of quantitative structure-retention relationships (QSSR) for their calculations. However, they determined the dependent variable R_M, which is calculated from the R_f value using a logarithmic function [28]. Thus, for comparability of the results, the relative deviations should be compared. In the method presented herein, mean relative deviations between the measured and calculated *x*- and *y-coordinates* of 22.1-25.7% were determined. With the method according to Baczek and Sparzak, the experimentally determined values deviated from the calculated R_M values by an average of 107.5% [28]. Therefore, the calculation method presented herein shows a significantly lower relative deviation in comparison to Baczek and Sparzak and is even applicable for a two-dimensional system.

Nevertheless, it should be noted that the calculation of the *x*- and *y-coordinates* refers only to the percentage of non-polar amino acids and not to the percentage of anionic amino acids in the peptide, although both show a similarly high correlation to the retardation factors. This inaccuracy in the calculation formula explains in particular the higher deviations between the calculated and measured retention factors in the lower, left area of the plate, as peptides with a higher content of anionic amino acids are assumed to be located there due to stronger binding to the silanol groups. Furthermore, even though the sample set used to develop the method and the test set used to verify the method were very similar, mean relative deviations of 25.3% were obtained. This can probably be explained by the inaccuracy of the calculation formula. As well, differences in retention behavior may occur between peptides in complex mixtures and single standards. In complex peptide mixtures fewer interactions, especially ionic interactions, can occur with the stationary phase due to higher competition for the binding sites. An optimized equation would include all three peptide properties (non-polar amino acids, anionic amino acids, and IEP) for which a statistically significant correlation to the retention behavior was found. However, with the method presented herein, it was demonstrated that the prediction of retention behavior based on the amino acid composition of peptides is generally possible.

4. Conclusions

It was shown that the developed 2D-HPTLC method presented herein is in principle suitable for the separation of milk protein-based peptides and for the characterization of the polarity and an estimated percentage of anionic amino acids in them. The 2D-HPTLC method may also be applied to peptides of other protein origins such as pea peptides (*Pisum sativum*).

Milk protein hydrolyzates were demonstrated to form consistent peptide patterns with adequate repeatability of average RSD of 16.5% and 12.9% for the *x*- and *y-coordinates*, respectively. The peptides cluster in specific areas of the HPTLC plates. Therefore, the method could be applied in the future, for example, to characterize the protein and peptide profile of milk and dairy products. Moreover, following mass spectrometric identification of the fluorescently labelled peptide spots, statistically significant correlations were demonstrated between the IEP or the percentages of anionic and non-polar amino acids in the peptide and their corresponding *x*- and *y-coordinates*. Based on the content of non-polar amino acids in the peptide, it was even possible to make predictions about the retention behavior of peptides.

Funding

The project is supported by funds of the *Federal Ministry of Food and Agriculture* (BMEL) based on a decision of the *Parliament of the Federal Republic of Germany* via the *Federal Office for Agriculture and Food* (BLE) under the innovation support programme (FKZ: 281A107916).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Mascha Treblin: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Tobias von Oesen:** Conceptualization, Writing – review & editing. **Lisa-Carina Class:** Formal analysis, Methodology. **Gesine Kuhnen:** Formal analysis, Methodology. **Ingrid Clawin-Rädecker:** Conceptualization. **Dierk Martin:** Project administration, Writing – review & editing, Funding acquisition. **Jan Fritsche:** Supervision, Writing – review & editing. **Sascha Rohn:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Acknowledgments

We are grateful to Jana Lüneburg and Janina Rehbock for excellent support in the laboratory. We are thankful to Maria Riedner, Gaby Graak, and Christine Christ for their mass spectrometric support. We further want to thank Katrin Schrader and Wolfgang Hoffmann for their valuable consulting about milk proteins and products.

References

[1] N. Amiri-Dashatan, M. Koushki, H. Abbaszadeh, M. Rostami-Nejad, M. Rezaei–Tavirani, Proteomics applications in health: biomarker and drug discovery and food industry, Iran. J. Pharm. Res. 17 (2018) 1523–1536.
[2] P. Giansanti, L. Tsiatsiani, T.Y. Low, A.J.R. Heck, Six alternative proteases for mass spectrometry-based proteomics beyond trypsin, Nat. Protoc. 11 (2016) 993–1006, doi:10.1038/nprot.2016.057.
[3] S.P. Pasilis, V. Kertesz, G.J. Van Berkel, M. Schulz, S. Schorcht, Using HPTLC/DESI-MS for peptide identification in 1D separations of tryptic protein digests, Anal. Bioanal. Chem. 391 (2008) 317–324, doi:10.1007/s00216-008-1874-6.
[4] L. Morschheuser, K. Mink, R. Horst, C. Kallinich, S. Rohn, Immunological analysis of food proteins using high-performance thin-layer chromatography-immunostaining, J. Chromatogr. A 1526 (2017) 157–166, doi:10.1016/j.chroma.2017.10.046.
[5] R. Gwarda, A. Tomczyszyn, A. Misicka, T.H. Dzido, Retention and separation efficiency of some synthetic oligopeptides in reversed-phase thin-layer chromatography, Acta Chromatogr. 27 (2015) 1–14, doi:10.1556/AChrom.27.2015.1.1.

[6] R. Gwarda, M. Aletańska-Kozak, D. Matosiuk, T.H. Dzido, Inversion of type of separation system in planar chromatography of peptides, using C18 silica-based adsorbents, J. Chromatogr. A 1440 (2016) 240–248, doi:10.1016/j.chroma.2016.02.064.
[7] G. Morlock, W. Schwack, Hyphenations in planar chromatography, J. Chromatogr. A 1217 (2010) 6600–6609, doi:10.1016/j.chroma.2010.04.058.
[8] J. Biller, L. Morschheuser, M. Riedner, S. Rohn, Development of optimized mobile phases for protein separation by high performance thin layer chromatography, J. Chromatogr. A 1415 (2015) 146–154, doi:10.1016/j.chroma.2015.08.048.
[9] L. Morschheuser, S. Mükusch, M. Riedner, H. Seitz, S. Rohn, High-performance thin-layer chromatography as a fast screening tool for phosphorylated peptides, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1008 (2016) 198–205, doi:10.1016/j.jchromb.2015.11.055.
[10] L. Morschheuser, H. Wessels, C. Pille, J. Fischer, T. Hünniger, M. Fischer, A. Paschke-Kratzin, S. Rohn, HPTLC-aptastaining - innovative protein detection system for high-performance thin-layer chromatography, Sci. Rep. 6 (2016) 6–13, doi:10.1038/srep26665.
[11] K. Tscherch, J. Biller, M. Lehmann, S. Rohn, One- and two-dimensional high-performance thin-layer chromatography as an alternative analytical tool for investigating polyphenol – protein interactions, Phytochem. Anal. 24 (2013) 436–445, doi:10.1002/pca.2459.
[12] M. Dolowy, A. Pyka, Application of TLC, HPLC and GC methods to the study of amino acid and peptide enantiomers: a review, Biomed. Chromatogr. 28 (2014) 84–101, doi:10.1002/bmc.3016.
[13] Y. Zhang, R. Chen, H. Ma, S. Chen, Isolation and identification of dipeptidyl peptidase IV-inhibitory peptides from trypsin/chymotrypsin-treated goat milk casein hydrolysates by 2D-TLC and LC-MS/MS, J. Agric. Food Chem. 63 (2015) 8819–8828, doi:10.1021/acs.jafc.5b03062.
[14] R. Bhusan, V.K. Mahesh, P.V. Mallikharjun, Thin layer chromatography of peptides and proteins: a review, Biomed. Chromatogr. 3 (1989) 95–104, doi:10.1002/bmc.1130030302.
[15] R. Gwarda, T.H. Dzido, The influence of pH on retention and migration of peptides in systems with octadecyl silica-based adsorbent by high-performance thin-layer chromatography and pressurized planar electrochromatography techniques, J. Chromatogr. A 1534 (2018) 179–187, doi:10.1016/j.chroma.2017.12.063.
[16] S.P. Pasilis, V. Kertesz, G.J. Van Berkel, M. Schulz, S. Schorcht, HPTLC/DESI-MS imaging of tryptic protein digests separated in two dimensions, J. Mass Spectrom. 43 (2008) 1627–1635, doi:10.1002/jms.
[17] I. Urbanova, F. Svec, Monolithic polymer layer with gradient of hydrophobicity for separation of peptides using two-dimensional thin layer chromatography and MALDI-TOF-MS detection, J. Sep. Sci. 34 (2011) 2345–2351, doi:10.1002/jssc.201100202.
[18] R.Ł. Gwarda, T.H. Dzido, Two-dimensional high-performance thin-layer chromatography of tryptic bovine albumin digest using normal- and reverse-phase systems with silanized silica stationary phase, J. Chromatogr. A 1312 (2013) 152–154, doi:10.1016/j.chroma.2013.08.082.
[19] V. Panchagnula, A. Mikulskis, L. Song, Y. Wang, M. Wang, T. Knubovets, E. Scrivener, E. Golenko, I.S. Krull, M. Schulz, H. Emil-Hauck, W.F. Patton, Phosphopeptide analysis by directly coupling two-dimensional planar electrochromatography/thin-layer chromatography with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, J. Chromatogr. A 1155 (2007) 112–123, doi:10.1016/j.chroma.2007.04.029.
[20] R. Bakry, G.K. Bonn, D. Mair, F. Svec, Monolithic porous polymer layer for the separation of peptides and proteins using thin-layer chromatography coupled with MALDI-TOF-MS, Anal. Chem. 79 (2007) 486–493, doi:10.1021/ac061527i.
[21] L. Morschheuser, S. Mükusch, M. Riedner, H. Seitz, S. Rohn, High-performance thin-layer chromatography as a fast screening tool for phosphorylated peptides, J. Chromatogr. B 1008 (2016) 198–205, doi:10.1016/j.jchromb.2015.11.055.
[22] J.F. Emory, M.J. Walworth, G.J. Van Berkel, M. Schulz, S. Minarik, Direct analysis of reversed-phase high-performance thin layer chromatography separated tryptic protein digests using a liquid microjunction surface sampling probe/electrospray ionization mass spectrometry system, Eur. J. Mass Spectrom. 16 (2010) 21–33, doi:10.1255/ejms.1041.
[23] A.I. Gusev, O.J. Vasseur, A. Proctor, A.I. Gusev, D.M. Hercules, A.G. Sharkey, Imaging of thin-layer chromatograms using matrix-assisted laser desorption/ionization mass spectrometry, Anal. Chem. 67 (1995) 4565–4570, doi:10.1021/ac00120a021.
[24] A.I. Gusev, A. Proctor, D.M. Hercules, Y.I. Rabinovich, D.M. Hercules, Thin-layer chromatography combined with matrix-assisted laser desorption/ionization mass spectrometry, Anal. Chem. 67 (1995) 1805–1814, doi:10.1021/ac00107a009.
[25] G.J. Van Berkel, V. Kertesz, Automated sampling and imaging of analytes separated on thin-layer chromatography plates using desorption electrospray ionization mass spectrometry, Anal. Chem. 78 (2006) 4938–4944, doi:10.1021/ac060690a.
[26] I. Vovk, G. Popović, B. Simonovska, A. Albreht, D. Agbaba, Ultra-thin-layer chromatography mass spectrometry and thin-layer chromatography mass spectrometry of single peptides of angiotensin-converting enzyme inhibitors, J. Chromatogr. A 1218 (2011) 3089–3094, doi:10.1016/j.chroma.2011.03.039.
[27] T. Baczek, M.P. Marszałł, R. Kaliszan, Ł. Walijewski, W. Makowiecka, B. Sparzak, Z. Grzonka, K. Wiśniewska, P. Juszczyk, Behavior of peptides and computer-assisted optimization of peptides separations in a normal-phase thin-layer chromatography system with and without the addition of ionic liquid in the eluent, Biomed. Chromatogr. 19 (2005) 1–8, doi:10.1002/bmc.405.
[28] T. Baczek, B. Sparzak, Ionic liquids as novel solvent additives to separate peptides, Z. Für Naturforsch. C 61 (2006) 827–832, doi:10.1515/znc-2006-11-1210.
[29] A.E. Post, B. Arnold, J. Weiss, J. Hinrichs, Effect of temperature and pH on the solubility of caseins: environmental influences on the dissociation of α S- and β-casein, J. Dairy Sci. 95 (2012) 1603–1616, doi:10.3168/jds.2011-4641.
[30] J. Wagner, C.G. Biliaderis, T. Moschakis, Whey proteins: musings on denaturation, aggregate formation and gelation, Crit. Rev. Food Sci. Nutr. (2020) 1–14 0, doi:10.1080/10408398.2019.1708263.
[31] J. Biller, Die Massenspektrometrische Charakterisierung Von Lebensmittelrelevanten Proteinen und Proteinderivaten Mittels HPTLC-MS-Verfahren, Verlag Dr. Hut, Munich, Germany, 2017.