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Complete List of Authors:	Pomastowski, Paweł; Nicolaus Copernicus University, Chair of Environmental Chemistry & Bioanalytics Walczak, Justyna; Nicolaus Copernicus University, Chair of Environmental Chemistry & Bioanalytics Gawin, Marta; Jagiellonian University, Laboratory of Physicochemical Analysis and Structural Research Bocian, Szymon; Nicolaus Copernicus University, Chair of Environmental Chemistry & Bioanalytics Piekoszewski, Wojciech; Jagiellonian University, Laboratory of Physicochemical Analysis and Structural Research Buszewski, Boguslaw; Nicolaus Copernicus University, Faculty of Chemistry, Department of Environmental Chemistry and Bioanalytics

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ARTICLE TYPE

HPLC separation of casein components on diol-bonded silica column with MALDI TOF/TOF MS identification

Paweł Pomastowski, [a] Justyna Walczak[a], Marta Gawin[b], Szymon Bocian[a], Wojciech Piekoszewski[b,c] and Bogusław Buszewski*[a]

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The presented work is a comparative study of three home-made bonded silica columns employed to separation of components of bovine milk casein (α-, β- and κ-casein). A gradient elution HPLC procedure was proposed for separation of the protein components and the performed tests proved superiority of diol-bonded silica over octadecyl- and amine-bonded silica columns. Matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometer (MALDI-TOF/TOF MS) was utilized as a detector for off-line identification of the components of casein fractions. The evaluated method enabled good and fast separation of the examined casein components along with their detailed MS characterization.

Introduction

Bovine milk casein (CN) has recently attracted growing interest of many researchers.[1-5] It belongs to the group of phosphoproteins, and can be divided into four families according to the homology of their primer amino acid sequence (primary structure), namely α_{s1}-, α_{s2}-, β- and κ-CN.[6] Casein is a source of exogenous and endogenous amino acids and a precursor of biologically active peptides characterized by immunogenic, antioxidant, antibacterial, antiviral, antihypertensive, antitumor and opioid properties.[1,7] Moreover, bioactive casein forms exhibit potential nutraceutical, medical and pharmaceutical applications.[7]

Casein is obtained in precipitation process of skim milk.[1] The desire to understand the behavior of casein (and its peptides) in a living organism imposes the need to obtain the protein components separated. For this purpose, a method is required that is characterized by high selectivity and resolution of the process. Beside electrophoretic techniques of protein separation, high performance liquid chromatography plays a dominant role in the analysis of proteins and peptides.[2,3-5] Among the chromatographic systems reversed-phase chromatography (RP-HPLC) and hydrophilic interaction liquid chromatography (HILIC) have been often employed to separation of components of casein.[1,7]

The selection of an appropriate analytical column and composition of a mobile phase have a major impact on the separation process of proteins.[8] Silica based stationary phases modified with alkyl groups are typically used for separation of casein components in RP-HPLC system, including stationary phases with butyl (C4), pentyl (C5), octyl (C8) and octadecyl (C18) groups. However, this type of columns has several limitations in the case of separation of proteins, mainly insufficient reproducibility, selectivity and resolution of the chromatographic process.[2,9] This problem may be overcome to some extent by application of columns with polymeric sorbents, hydrophilic carboxyl groups, amino or diol ligands.[2,3,7]

Another problem of liquid chromatography (LC) analysis of casein results from the utilized detection system. Conventional UV-VIS detectors do not allow for full characterization of the separated proteins and peptides.[2] This problem has been elegantly solved by combining separation techniques with mass detectors.[10,11] Matrix-assisted laser desorption/ionization mass spectrometry technique (MALDI MS) is one of the most useful tools in proteomics[12] enabling identification of proteins, peptides and analysis of their post-translational modifications (PTMs).[13]

The purpose of this study was separation of bovine casein components with the use of lab-made diol-bonded silica stationary phases. Casein from bovine milk was separated in a reversed-phase (RP) mode and fractionated. Characterization of the collected fractions was realized in off-line mode via combination of HPLC with MALDI-TOF/TOF MS.

Experimental section

Materials and methods

Chemicals

Casein standards of α_s- (a mixture of α_{s1}, α_{s2}-), β- and κ-casein (from bovine milk), acetonitrile, methanol, trifluoroacetic acid, ammonium acetate, formic acid, urea, hydrochloric acid, ammonium hydroxide solution, ammonium bicarbonate, sulphuric acid and proteomics grade trypsin were purchased from Sigma Aldrich (Steinheim, Germany). Ultra-pure water from a Milli-Q water system (Millipore, Bedford, MS, USA) was used throughout the work.

All chemicals for MALDI-MS analyses were supplied at the highest commercially available purity from Fluka Feinchemikalien GmbH (part of Sigma Aldrich). Ground steel

targets (Bruker Daltonik, Bremen, Germany) were used for sample deposition. α-cyano-4-hydroxycinnamic acid (HCCA), 2,5-dihydroxybenzoic acid (DHB) (both with optional addition of 1% orthophosphoric acid) and sinapinic acid (SA) were employed as matrices for MALDI analysis of tryptic digest and intact proteins, respectively (dried droplet method). Peptide Calibration Standard II and Protein Calibration Standard I and II, all from Bruker Daltonik, were used for external calibration.

Instrumentation

Shimadzu Nexera UHPLC system (Tokyo, Japan) equipped with a binary solvent delivery system (LC-30AD), SPD-M20A UV-diode array detector, an autosampler (SIL-30AC), a column thermostat (CTO-20AC) and data acquisition station, was exploited for chromatographic analyses. LabSolutions (Shimadzu, Tokyo, Japan) software was used for data collection and analysis.

Chromatographic separations were performed with the use of home-made columns (125 × 4.6 mm) packed with different materials based on 5µm silica particles (Table 1). Three types of stationary phases were employed, namely: octadecyl-, amine- and diol-bonded silica.[14-16] Silica gel with the pore diameter of 300Å was chosen as a support for stationary bonded phase synthesis, since this pore size is recommended for large molecules. The specific surface area (S_{BET}) of bare silica gel was 110 m^2/g. The synthesis procedures are shown in Fig. 1a. The obtained materials were characterized using ^{13}C CP/MAS NMR spectroscopy. The registered spectra are shown in Fig. 1b. For each spectrum, the number of a signal corresponds to the carbon atom in the structure of the bonded phase.

Table 1 Physicochemical characterization of the stationary phases

Type of stationary phase	Pore diameter of bare silica [Å]	Carbon load [%]	Coverage density [µmol/m^2]	Column dimension [mm]
C18	300	7.95	3.36	125 × 4.6 i.d.
Amino		1.35	3.59	
Diol		1.02	1.32	

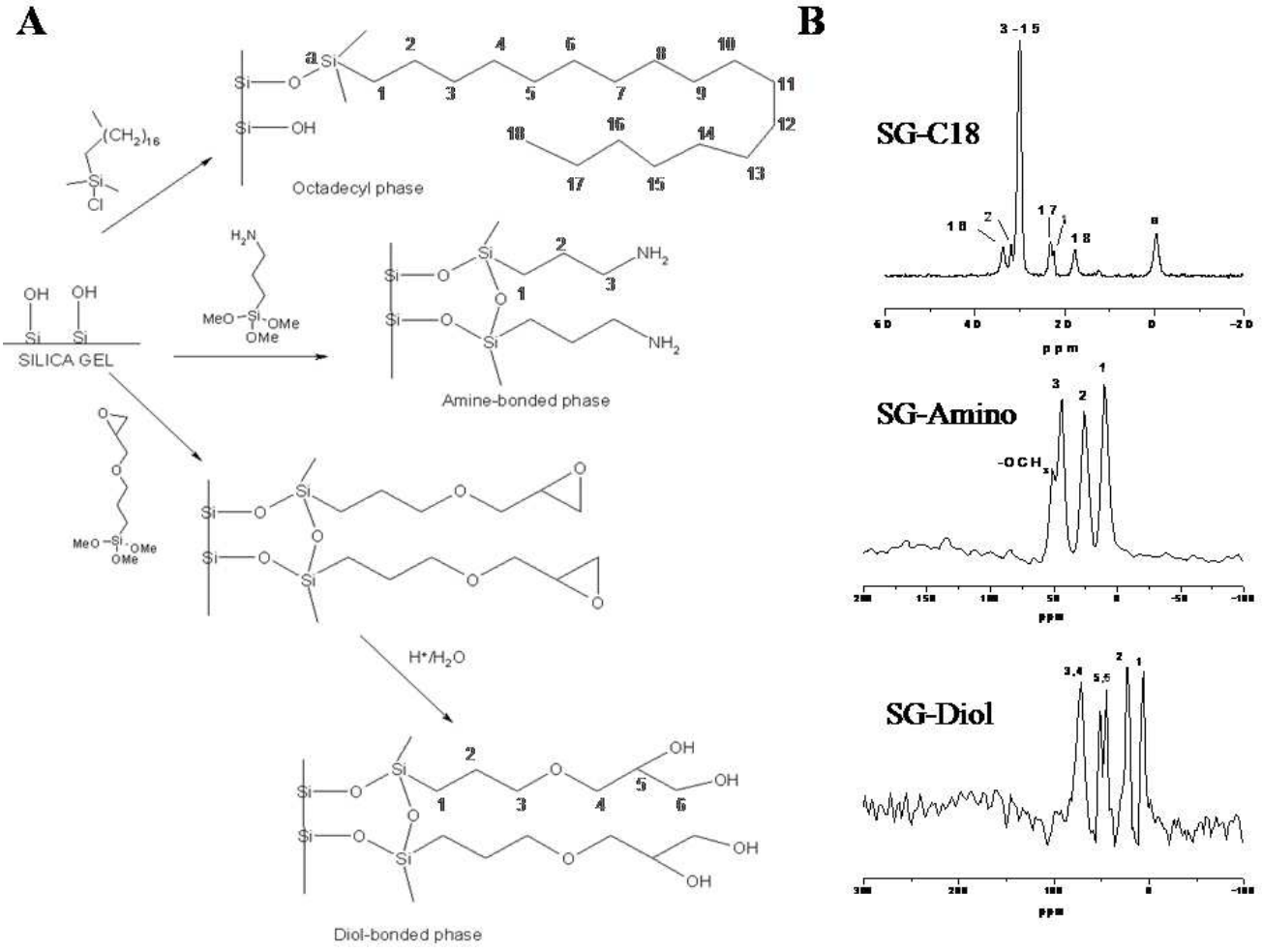

Fig. 1 a) Reaction scheme for the synthesis octadecyl-, amine- and diol-bonded stationary phase, b) ^{13}C NMR spectra of the synthesized stationary phases

Mass spectrometric analysis was conducted with the use of ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) equipped with a modified Nd:YAG laser (smartbeam II™) operating at the wavelength of 355 nm and the frequency of 2 kHz. PMF (Peptide Mass Fingerprint) spectra of tryptic digests of casein components were recorded in reflectron positive mode, within m/z range of 500-4000, and applying the acceleration voltage of 25 kV. Fragment spectra were acquired with the use of

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LIFT-PSD (Post-Source Decay) technique in m/z range of 20-1000. MS/MS spectra were calibrated internally on *immonium ions*.[17] MS spectra of intact proteins were obtained in linear positive mode in m/z range of 10.000-50.000, applying the acceleration voltage of 25 kV.

All mass spectra were acquired and processed with the dedicated software: flexControl and flexAnalysis, respectively (both from Bruker Daltonik). In the case of tryptic digests of casein components, the obtained MS and MS/MS peak lists were submitted to MASCOT search for protein identification with the use of BioTools and ProteinScape software (both from Bruker Daltonik) using non-standard search parameters: cysteines modified by carbamidomethylation and phosphorylation. Mass tolerance was set to 0.1 Da for MS and 0.3 Da for MS/MS analysis.

The two-dimensional gel electrophoretic analysis of casein components was performed in two modes. Isoelectrofocusing (IEF) was performed in PROTEAN IEF Cell (BioRad, Hercules, CA, USA) and the second dimension was developed in the Clever Scientific chamber (Cleaver Scientific Ltd, Great Britain).

Casein isolation

Casein was obtained in acidic precipitation process of skim cow's milk. First, milk was warmed up to 31°C. Then, 0.1 M solution of hydrochloric acid was added, to reach pH 4.3. After complete precipitation of casein, the whole was heated at the rate of 1°C/min, under continuous stirring, to the temperature of 53°C. Once the precipitate was heated the whey was separated, the sediment mass was rinsed with water at the temperature of 25°C and left for 12 hours. The precipitated casein was washed twice with water at 30°C and 10°C, respectively. The whole was dried and triturated.[18]

Dried casein formed white matte and glossy grains. To verify quality of the obtained material, 2 mg of casein were suspended in 0.5 mL of ammonia solution (10% v/v), shaken and left at ambient temperature. After 4 minutes, the seeds of casein were completely dissolved.[18]

Preparation of casein for chromatographic separation

Fresh, pure casein was dissolved in 6 M urea at the concentration of 1 mg/mL. The solution was then centrifuged at 10.000 g for 10 min. The supernatant was subsequently filtered through 0.45 µm poly(tetrafluoroethylene) syringe filter (Restek) and subjected to HPLC separation.

Preparation of casein for mass spectrometric analysis

Fractions of casein were collected manually every 30 s. Each fraction was evaporated on a Labconco CentriVap DNA concentrator (Kansas City, USA) and further processed in two manners. In the first case, the protein fractions were dissolved in acetonitrile containing 0.1% TFA, in the second one they were digested with trypsin at an enzyme to protein ratio of 1:12. Proteolytic digestion was carried out overnight in 50 mM ammonium bicarbonate solution containing 10% ACN at 37°C. Subsequently, 1% TFA aqueous solution was added to the digests to terminate the reaction.

Two-dimensional gel electrophoresis

A solution of extracted casein was transferred onto dialysis membrane with 1 kDa cut-off (Spectrum, USA), the dialysate was portioned into aliquots and evaporated at 35°C on Labconco CentriVap DNA concentrator (Kansas City, USA). 125 µg of casein was reduced and alkylated (ReadyPrep™ Reduction-Alkylation Kit, BioRad), dissolved in 125 µl rehydratation buffer (BioRad) and subjected to rehydratation on 7 cm IPG strip pH 4-7 (BioRad) over 12 hours. Isoelectric focusing was performed in PROTEAN IEF system with the voltage program of: 250 V for 20 minutes, 4000 V for 2 hours, 4000 V for 20 000 Vh with post-run voltage set at 500 V. IPG strips were equilibrated (ReadyPrep 2-D Starter Kit Equilibration Buffer I, II, BioRad) and immersed in 0.5% agarose containing 0.001% bromophenol blue. Electrophoresis was performed in polyacrylamide gels (10×10 cm; 12%, 37.5:1 acrylamide/bisacrylamide) in SDS-Glycine-Tris running buffer (10x premixed electrophoresis buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 following dilution to 1x with water, BioRad) at 90 V for 10 minutes and then at 220 V for 1h. Gels were subsequently stained with Bio-Safe™ Coomassie (BioRad), scanned (HP Deskjet F2280) and chosen protein spots were excised. In-gel digestion was performed as described in protocol of Mamone et al.[23] The obtained mixture of tryptic peptides was subjected to PMF.

Results and discussion

Separation process

Separation of casein components was realized in gradient elution. Three modifiers of mobile phase were tested, namely: trifluoroacetic acid (0.1% v/v), formic acid (0.1% v/v) and ammonium acetate (5 mM) in different gradient profiles. A modifier was added both to eluent A and B. The flow rate was 0.6 mL/min. The optimal gradient was as follows: 0 min 20% B, 2 min 20% B, 3 min 70% B, 3.8 min 50% B, 4.5 min 90% B, and from 7 to 35 min 20% B, where (A) is water and (B) is acetonitrile.

All the tested modifiers altered pH of the eluent, influenced selectivity and hydrophobicity of the molecules of interest by solvation and ionization, which affects quality of the separation. The type and concentration of a modifier applied in HPLC has a significant effect on the selectivity of protein separations. Formic acid is an often used additive and a good denaturant for proteins, however, upon its presence in the mobile phase the components of casein were not well separated. Application of ammonium acetate caused overlapping of the peaks from κ-casein and α-casein. Ammonium acetate increased pH of the mobile phase, which changed hydrophobicity of the protein, and as a result caused non-selective separation of casein forms.[5] In the presented study the best results were obtained upon addition of trifluoroacetic acid.

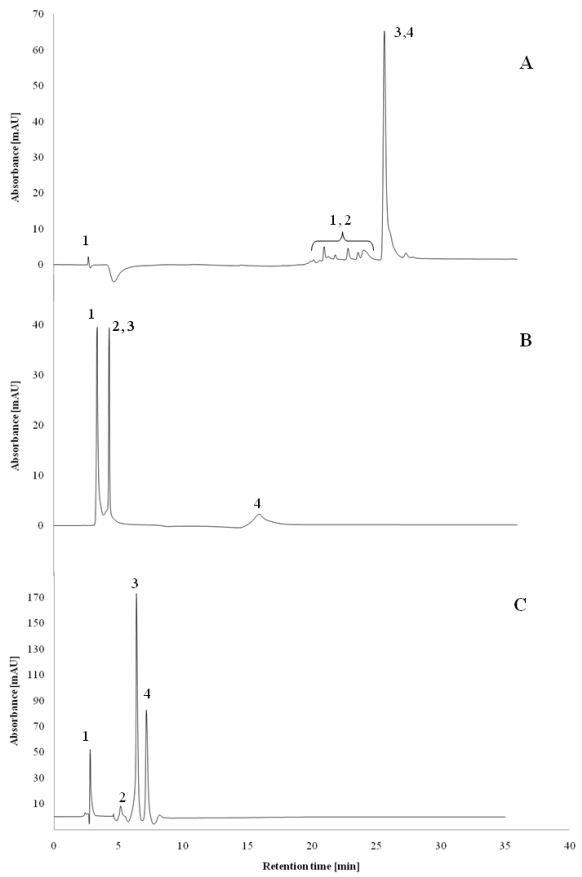

Fig. 2 Chromatograms of casein components separated with the use of: a) octadecyl-, b) amine-, c) diol-bonded column; 1- unidentified fraction, 2 - κ-casein, 3 - α-casein, 4 - β-casein. Separation was performed with the use of gradient elution at the flow rate of 0.6 mL/min, pH 2.2 and TFA as a modifier. The dead-time (t_0) was 2.46 min. Detection was performed by UV-diode detector (280 nm)

The chromatograms of casein components obtained on octadecyl, amine and diol column have been presented in Fig. 2. Sample components were eluted within 35 min giving four peaks, three of them originating from casein components (2 - κ-casein, 3 - α-casein, 4 - β-casein) and one was not identified. The reason for this may be insufficient purification of the casein extracted from cow's milk, or the first compound may be strongly solvated and does not interact with the mobile and the stationary phase.[2,3] Fig. 2a shows the chromatogram registered for octadecyl silica as a stationary phase. The separation was not satisfactory because peaks 1 and 2, as well as 3 and 4 overlapped. Furthermore, the analysis took as much as 30 minutes. Retention of casein in RP chromatography can be modified by pH of the mobile phase: pH below 7.5 provides stability of a stationary phase based on silica support. Using TFA as a modifier, amino groups of proteins are positively charged (protonation at pH < 7.5), however carboxyl groups are neutralized. In this way mobile phase containing trifluoroacetic acid (pH 2.2) suppresses ionization of acidic groups of peptides making the micelles of casein positively charged, since pI of casein is about 4.5.[19] According to submicelle model and Holt model, casein micelle consists of two parts.[6] The hydrophobic part is situated inside the micelle and is created by α- and β-casein. The hydrophilic outer part of the micelle is built by κ-casein.[20,21]

Fig. 2b presents the chromatogram of casein separation on amino-bonded stationary phase. The first peak was observed in the column void volume. Then, κ-, α-casein co-eluted (2,3) and β-casein (4) was eluted as the last one. In the experimental conditions, amino groups of the stationary phase were positively charged. This may cause repulsive electrostatic interactions (beside weak hydrophilic/hydrophobic interactions) between the charged surface of the stationary phase and casein particles. The formation of hydrogen bonds is another kind of retention forces in these conditions. In comparison with C18 stationary phase, the separation on amino phase provided shorter time of analysis, which may be caused by the presence of additional electrostatic repulsive interactions.

In the chromatogram presented in Fig. 2c all main components of casein were well separated: κ-casein (2), α-casein (3) and β-casein (4). The diol-bonded stationary phase demonstrated good selectivity of casein components separation in the shortest analysis time. This situation most probably results from the presence of a combination of attractive electrostatic and hydrophilic /hydrophobic interactions in the separation system, as well as hydrogen bonds between peptides and hydroxyl groups in the stationary phase. This phenomenon influences increased selectivity and resolution of the system.

Linearity of the developed method was tested for calibration curves. The correlation coefficient was 0.9939, 0.9989 and 0.9992, and reproducibility (RSD, %) 3.75, 0.45 and 5.21, for κ-casein, α-casein and β-casein respectively. This demonstrates a lack adsorption of casein on the column. Recovery values for κ-, α- and β-casein were 100, 97, 95%, respectively.

Additionally, a comparison of the chromatographic results with those obtained with the use of gel electrophoresis was performed. Supplement 1 shows a 2-D gel of cow's casein. Farrell et al.[19] showed that the isoelectric point (pI) of α_{s1}-casein, β-casein and κ-casein equals 4.92-5.00, 5.30-5.53 and 5.35-6.07, respectively. Hye-Hyun et al.[22] indicated that pI value of α_{s1}-, α_{s2}-, β- and κ-casein oscillates between 4.2-4.6, 4.8-5.1, 4.6-5.1 and 5.3-5.8 respectively. According to the proteomic data and spectrometric analysis of the excised casein spots identified α_{s2}-, β-, α_{s1}- and κ-casein, respectively. The image of gel was enclosed in Supplement 1. The described chromatographic separation of casein components is in agreement with the results of two-dimensional electrophoresis analysis, though electrophoretic analysis is more specific and more sensitive. Nevertheless, the chromatographic procedure is faster and more efficient in routine and quantitative analysis of caseins. Moreover, the fractions of casein obtained during chromatographic separation can be used in further physicochemical analysis e.g. a study of metal binding to casein components.

MALDI MS analysis

Intact protein analysis was performed for three casein fractions collected from chromatographic separation. It should be noted that no signal was registered on a mass spectrum for the fraction with the first chromatographic peak. This situation probably results from artifacts formed during extraction or chromatographic process.[2,18] Fig. 3 shows MS spectra registered for the three kinds of casein components: α_{s1}-CN, β-CN and κ-CN. The analysis did not reveal the presence of α_{s2}-CN. The content of this isoform in bovine casein is only 10%.[6] The

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fraction α_{s2}-CN could be lost during the process of sample preparation for chromatographic analysis. The reason lies in greater hydrophobicity of this component and its tendency for aggregation such as formation of amyloidal fibril.[24] Moreover, isolated α_{s2}-casein in *in vitro* conditions and at room temperature is prone to form ribbon-like fibrils with the diameter of ca. 12 nm and the length of more than 1 mm, occasionally forming loop structures. Therefore, during the filtration process prior to chromatographic analysis α_{s2}-casein may be lost. Fibril formation can be minimalized when the intra- and intermolecular disulphide bonds in α_{s2}-casein are disrupted by a reducing agent, e.g. dithiothreitol, and then protected from re-formation by the alkylation process. Unfortunately, in the case of chromatographic analysis it is not advisable to use reducing/alkylation agents as they cause clogging of capillaries.

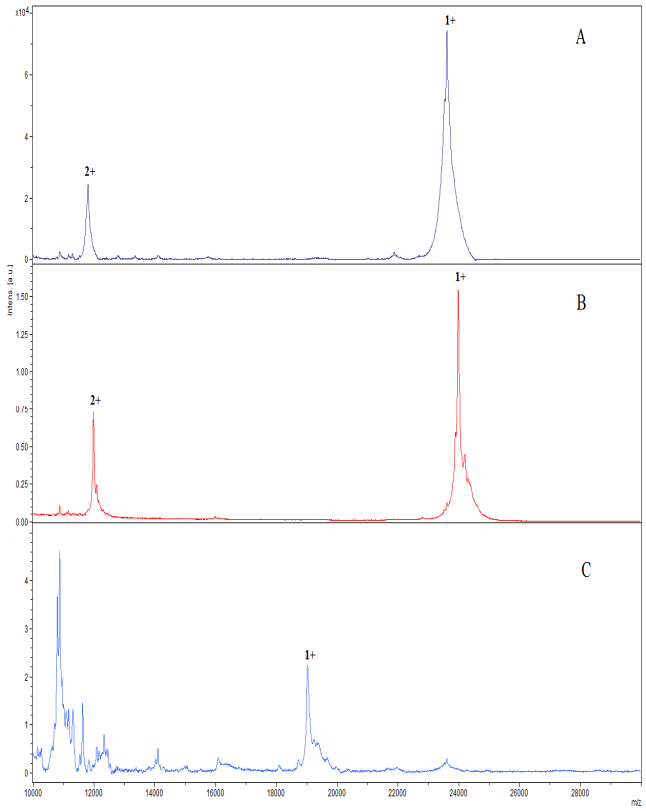

Fig. 3 Mass spectra of intact casein components: A) α_{s1}-CN (23610 Da), B) β-CN (23997 Da) and C) κ-CN (19000 Da)

MS analysis of the collected chromatographic fractions led to the conclusion that peaks 2, 3 and 4 observed on chromatograms belonged to κ-CN, α_{s1}-CN and β-CN, respectively. The measured molecular masses of α_{s1}-CN, β-CN and κ-CN were 23610 ± 2, 23997 ± 6 and 19000 ± 5 Da, respectively. The results are consistent with the literature data and proteomic database (Swiss-Prot).[10,11,22] The relatively small standard deviation (SD) values obtained for 10 series of measurements indicate stable ionization conditions in linear positive mode. Although MS spectra may give us a hint about the kind of isoform we are dealing with, they are insufficient for full characterization of the analytes of interest and must be supported with additional analyses.

More detailed study of the casein components was carried out after their tryptic digestion followed by analysis of the obtained fingerprints in positive reflectron mode.[11,23] Fig. 4 presents mass spectra of tryptic peptides obtained for the collected fractions of casein. In the case of α_{s1}-CN and β-CN the mass spectra contained relatively few unwanted signals[6,9,19], in contrast to the spectrum of κ-CN where many spurious signals could be observed. This situation probably results from the order of elution during the chromatographic separation. Upon separation of proteins in a hydrophilic interaction chromatographic system unfavorable cases of conformational change and/or partial denaturation of polypeptide chain can be observed.[3,5,7] κ-CN is characterized by the lowest molecular mass among all casein components[19], therefore some parts of polypeptides and other artifacts could co-migrate with κ-CN.[4,9,11]

Peak lists generated on the basis of the obtained PMF spectra were subjected to a database search (SwissProt, UniProt)[7,9,13,19,23] for protein identification. PMF results were confirmed by MS/MS search based on chosen peptides. Table 2 collects the measured and theoretical masses of the detected peptides. Sequence coverage (SC) for α_{s1}-CN and β-CN was 46-75% and 37-49%, respectively, and 33-35% for κ-casein. Higher SC values were obtained for DHB with the addition of 1% H_3PO_4.

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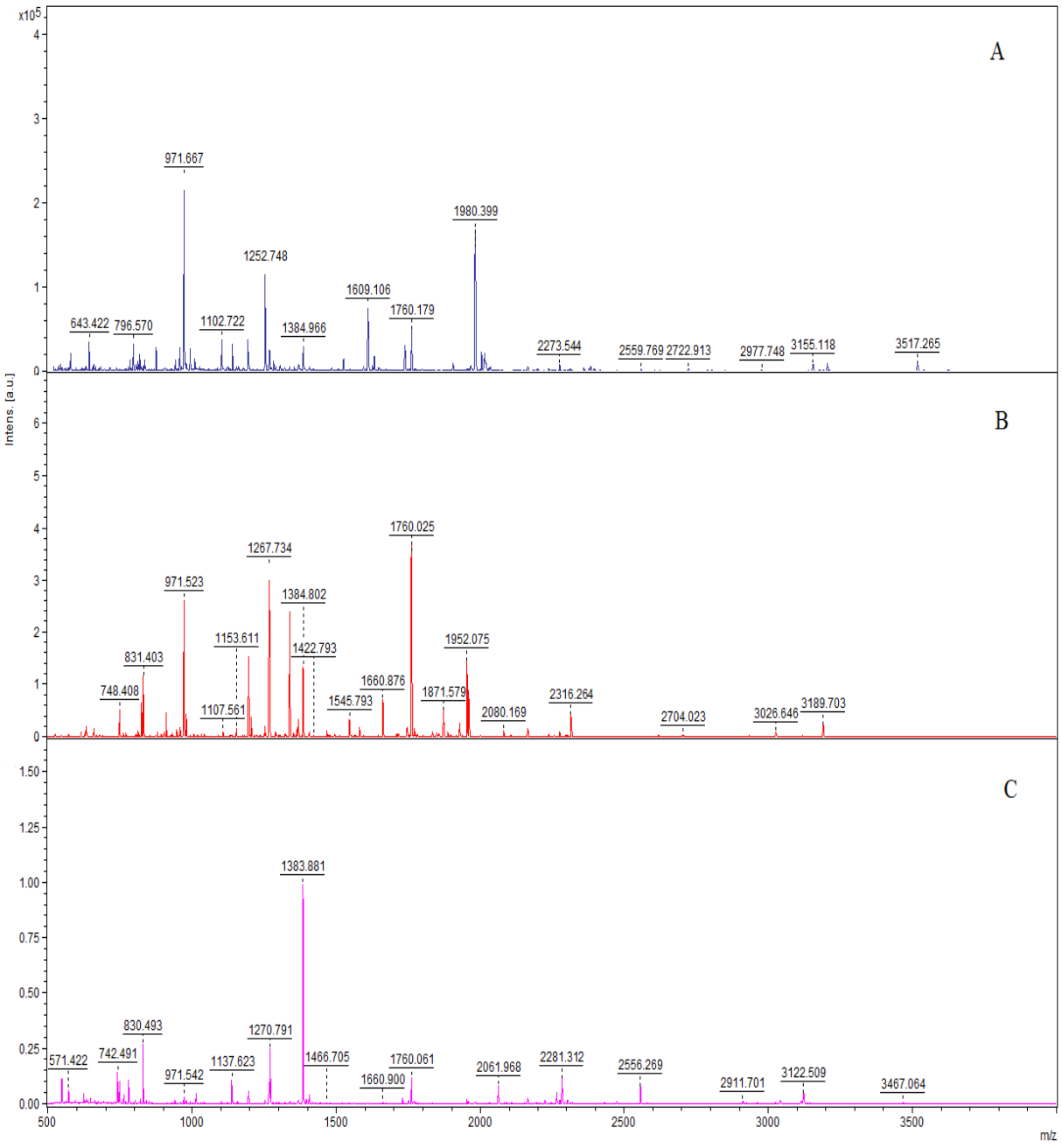

Fig. 4 Spectra of tryptic digests registered for: (A) κ-CN; (B) α_{s1}-CN; and (C) β-CN

Table 2 Identification of cow's casein peptides

Fraction No.	Isoform of casein	Mass [Da] measured	Mass [Da] theoret. [a]	Sequence coverage [%]	Sequence No.	Sequence [b]
2	κ-CN	643.42	643.31	33-35	38-42	FFSDK
		779.44	779.34		32-37	CEKDER
		1193.70	1193.54		108-118	(*S)CQAQPTTMAR
		1273.76	1273.51		108-118	SCQAQPTTMAR
		1609.11	1608.85		119-132	HPHPHLSFMAIPPK
		1625.08	1624.84		119-132	HPHPHLSFMAIPPK
		1737.23	1763.94		119-133	HPHPHLSFMAIPPKK
		1753.18	1752.94		119-133	HPHPHLSFMAIPPKK
		1980.40	1980.09		90-107	SPAQILQWQVLSNTVPAK
		3155.12	3154.60		90-118	SPAQILQWQVLSNTVPAKSCQAQPTTMAR
3	α_{s1}-CN	525.34	524.31	46-75	95 - 98	HIQK
		615.34	614.32		135 - 139	LHSMK
		748.41	747.36		209 - 214	TTMPLW
		831.40	830.38		99 - 105	EDVPSER
		910.50	909.47		140 - 147	EGIHAQQK
		946.54	945.51		50 - 57	EKVNELSK
		1267.73	1266.70		106 - 115	YLGYLEQLLR
		1337.73	1336.67		95 - 105	HIQKEDVPSER
		1384.80	1383.72		38 - 49	FFVAPFPEVFGK
		1580.61	1579.82		121 - 134	VPQLEIVPNSAEER
		1660.88	1659.79		121 - 134	VPQLEIVPN(*S)AEER
		1760.02	1758.94		23 - 37	HQGLPQEVLNENLLR
		1927.77	1926.68		58 - 73	DIG(*S)E(*S)TEDQAMEDIK
		1952.08	1950.95		119 - 134	YKVPQLEIVPN(*S)AEER
		1959.12	1958.15		2 - 18	KLLILTCLVAVALARPK
		2080.17	2079.06		99 - 115	EDVP(*S)ERYLGYLEQLLR
		2193.13	2192.13		121 - 139	VPQLEIVPNSAEERLHSMK
		2273.29	2272.09		121 - 139	VPQLEIVPN(*S)AEERLHSMK
		2316.26	2315.13		148 - 166	EPMIGVNQELAYFYPELFR
		2332.25	2331.12		148 - 166	EPMIGVNQELAYFYPELFR
		2678.15	2677.02		52 - 73	VNEL(*S)KDIG(*S)E(*S)TEDQAMEDIK
		2721.06	2719.91		74 - 94	QMEAE(*S)I(*S)(*S)(*S)EEIVPN(*S)VEQK
4	β-CN	646.36	645.32	37-49	115 - 120	EAMAPK
		662.36	661.31		115 - 120	EAMAPK
		742.51	741.44		218 - 224	GPFPIIV
		748.42	747.36		123 - 128	EMPFPK
		764.47	763.36		123 - 128	EMPFPK
		780.55	779.49		185 - 191	VLPVPQK
		830.49	829.44		192 - 198	AVPYPQR
		1013.59	1012.52		121 - 128	HKEMPFPK
		1029.59	1028.51		121 - 128	HKEMPFPK
		2061.97	2060.82		48 - 63	FQ(*S)EEQQQTEDELQDK
		2432.23	2431.04		45 - 63	IEKFQ(*S)EEQQQTEDELQDK

a) expected monoisotopic mass calculated on the basis of the amino acid sequence

b) phosphorylation sites of serine residues are indicated with an asterisk (*S)

MS/MS spectra of the selected casein peptides were also subjected to the analysis of phosphorylation sites with the use of ProteinScape. The obtained results have been collected in Table 2 and fragment mass spectra of chosen tryptic peptides of κ-, α_{s1}- and β-casein have been shown in Figure 5.

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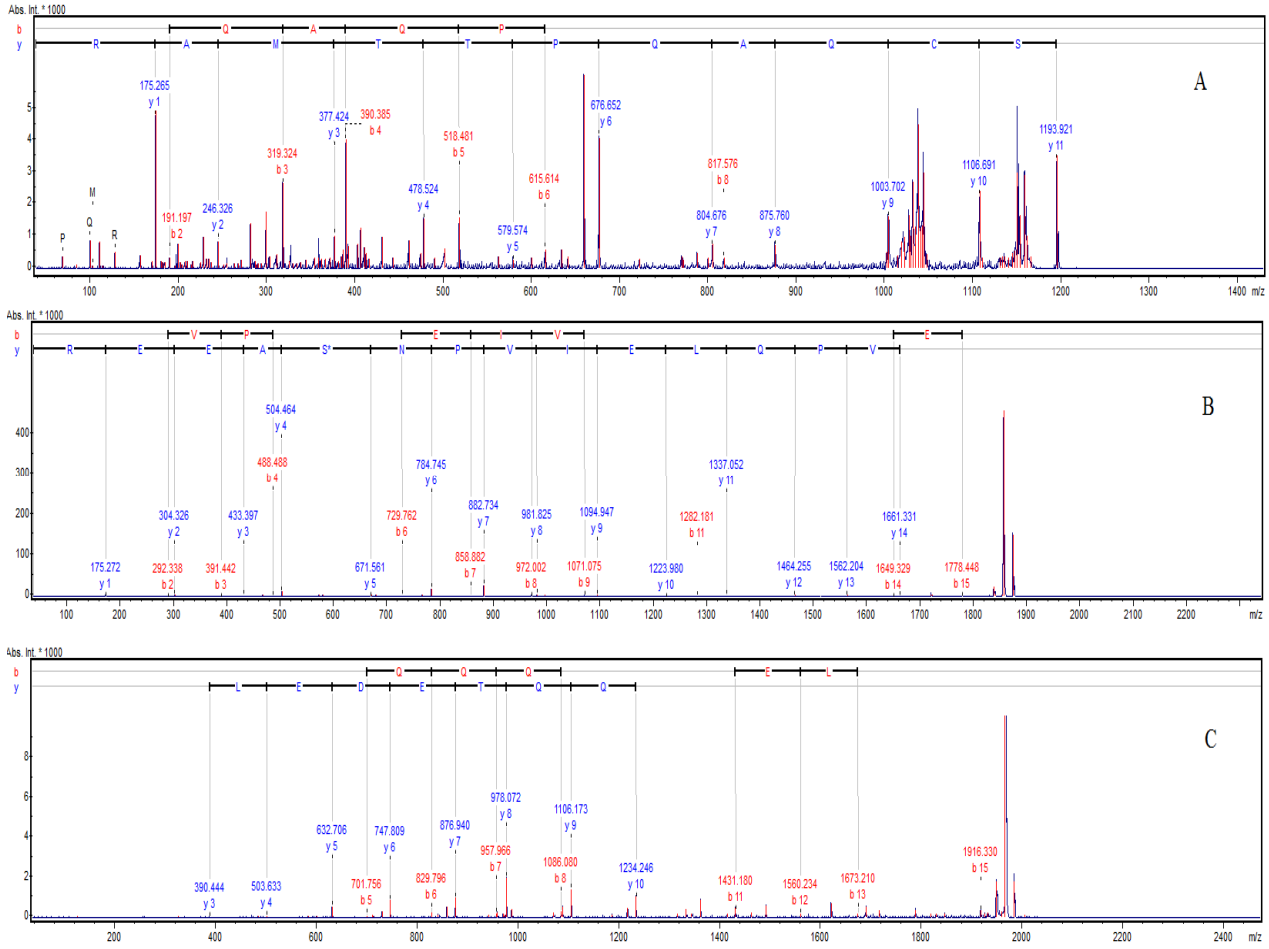

Fig. 5 Fragment mass spectra of chosen tryptic peptides: (A) κ-casein, parent ion 1193.70 Da, sequence of the identified peptide: (*S)CQAQPTTMAR; (B) α_{S1}-casein, parent ion 1952.08 Da, sequence of the identified peptide: YKVPQLEIVPN(*S)AEER; and (C) β-casein, parent ion 2061.97 Da, sequence of the identified peptide: FQ(*S)EEQQQTEDELQDK)

Conclusions

The presented study comprises valuable results concerning the retention mechanism and separation process of casein components with the use of various chromatographic stationary phases. It was stated that pH of the mobile phase and addition of a modifier to the mobile phase was a powerful parameter enabling to improve the final result of casein components separation with the use of HPLC. The best selectivity was obtained with the mobile phase containing trifluoroacetic acid, and diol-bonded silica stationary phase exhibited the highest application potential in advanced separation of casein fractions. The developed experimental method exploited off-line connection of HPLC system with MALDI mass spectrometer serving as a proper detector. This combination allowed for fast identification and detailed characterization of different components of casein (α_{s1}-, β-, κ-).

Notes and references

[a] *Department of the Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Interdisciplinary Centre of Modern Technologies, Nicolaus Copernicus University, 7 Gagarin St. PL 87-100 Torun, Poland. Fax: +48 56 6114837; Tel: +48 56 6114753; E-mail: bbusz@chem.umk.pl*

[b] *Laboratory of High Resolution Mass Spectrometry, Regional Laboratory of Physicochemical Analysis and Structural Research, Faculty of Chemistry, Jagiellonian University, Ingardena 3, PL 30-060 Krakow, Poland. E-mail: wojciech.piekoszewski@uj.edu.pl*

[c] *Toxicological and Pharmaceutical Analysis Group, Department of Analytical Chemistry, Faculty of Chemistry, Jagiellonian University, Ingardena 3, PL 30-060 Krakow, Poland.*

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