UNIVERSITÀ DEGLI STUDI DI SALERNO
DIPARTIMENTO DI FARMACIA

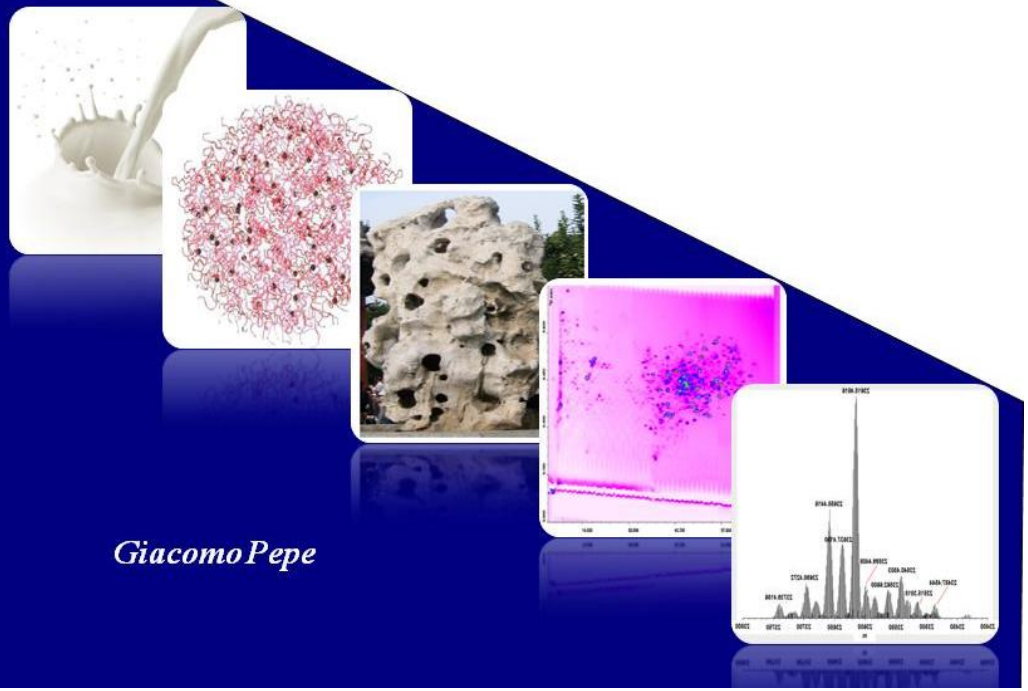
Dottorato di ricerca
in Scienze Farmaceutiche

Ciclo NS XIII- 2012-2015

Analysis and evaluation of potential nutraceutical milk and dairy products derived from the Centrale del latte di Salerno

Giacomo Pepe

Analysis and evaluation of potential nutraceutical milk and dairy products derived from the Centrale del latte di Salerno



Giacomo Pepe

Dipartimento di Farmacia
Via Giovanni Paolo II , 132-84084,
Fisciano, Salerno

UNIVERSITÀ DEGLI STUDI DI SALERNO

UNIVERSITÀ DEGLI STUDI DI SALERNO
Dipartimento di Farmacia

Dottorato di ricerca
in Scienze Farmaceutiche
Ciclo XIII NS – 2012-2015

Coordinatore: Chiar.mo Prof. *Gianluca Sbardella*

Analysis and evaluation of potential nutraceutical milk and dairy products derived from the Centrale del latte di Salerno

settore scientifico disciplinare di afferenza: CHIM/08

Dottorando

Dott. *Giacomo Pepe*

Tutore

Chiar.mo Prof.
Pietro Campiglia

Co-tutore
Chiar.mo Prof.
Gianluca Sbardella

Index

Chapter I:

Bovine milk, monolithic columns and comprehensive HPLC ..... - 1 -
1.1. Introduction ..... - 2 -
1.2. Lipids ..... - 3 -
1.2.1. Fatty acids ..... - 3 -
1.2.1.1. Saturated fatty acids ..... - 3 -
1.2.1.2 Unsaturated fatty acids ..... - 3 -
1.2.1.3. Conjugated linoleic acid (CLA) ..... - 4 -
1.2.1.4. Trans vaccenic acid ..... - 4 -
1.2.2. Phospholipids and glycosphingolipids ..... - 5 -
1.3. Minerals, vitamins and antioxidants ..... - 5 -
1.3.1. Calcium ..... - 5 -
1.3.2. Selenium ..... - 6 -
1.3.3. Iodine ..... - 6 -
1.3.4. Magnesium ..... - 6 -
1.3.5. Zinc ..... - 7 -
1.3.6. Vitamin E ..... - 7 -
1.3.7. Vitamin A ..... - 7 -
1.3.8. Folate ..... - 8 -
1.3.9. Riboflavin ..... - 8 -
1.3.10. Vitamin B12 ..... - 8 -
1.4. Proteins ..... - 9 -
1.4.1. Whey Proteins ..... - 9 -
1.4.1.1. α-Lactoalbumin ..... - 10 -
1.4.1.2. β-Lactoglobulin ..... - 11 -
1.4.1.3. Bovine Serum Albumin ..... - 11 -

1.4.1.4. Lactoferricin ..... - 11 -
1.4.1.5. Immunoglobulins ..... - 12 -
1.4.1.6. Lactoperoxidase ..... - 13 -
1.4.2. Caseins ..... - 13 -
1.4.2.1. α- and β-Casomorphins ..... - 14 -
1.4.2.2. Caseinphosphopeptides ..... - 14 -
1.5. Aim of work ..... - 15 -
1.6. Monolithic stationary phases ..... - 15 -
1.6.1. Preparation techniques for organic monolithic columns ..... - 18 -
1.6.1.1. Activation of capillary ..... - 19 -
1.6.1.2. Thermally and radiation polymerization ..... - 21 -
1.6.2. Monomers ..... - 23 -
1.6.3. Porogens ..... - 25 -
1.6.4. Crosslinking monomer ..... - 25 -
1.7. Comprehensive HPLC ..... - 28 -
1.7.1. Optimization of the LC × LC method ..... - 35 -
1.7.1.1. Optimization of the first dimension and mobile phase incompatibility ..... - 35 -
1.7.1.2. Peak focusing at the head of the secondary column ..... - 37 -
1.7.1.3. Optimization of the second dimension analysis time ..... - 37 -
1.8. References ..... - 39 -

Chapter II:

Susceptibility to denaturation of caseins in milk samples for improving protein conformational study and their identification ..... - 53 -
Abstract ..... - 54 -
Keywords ..... - 54 -
Abbreviations ..... - 54 -
2.1. Introduction ..... - 55 -
2.1.1. Aim of work ..... - 58 -

2.2. Materials and methods ..... - 58 -
2.2.1. Reagents and standards ..... - 58 -
2.2.2. Sampling and sample preparation ..... - 58 -
2.2.3. HPLC separation ..... - 59 -
2.2.4. Far-UV CD ..... - 60 -
2.2.5. Identification of caseins by MALDI-TOF-MS ..... - 60 -
2.3. Results and discussion ..... - 61 -
2.3.1. HPLC separation of purified casein fractions ..... - 61 -
2.3.2. Far-UV CD data of caseins ..... - 63 -
2.3.3. Identification of casein fraction variants ..... - 66 -
2.4. Conclusions ..... - 67 -
2.5. References ..... - 68 -

Chapter III:

Analysis of bovine milk caseins on organic monolithic columns: An integrated capillary liquid chromatography–high resolution mass spectrometry approach for the study of time-dependent casein degradation - 72 -
Abstract ..... - 73 -
Keywords ..... - 74 -
Abbreviations ..... - 74 -
3.1. Introduction ..... - 75 -
3.1.1. Aim of work ..... - 77 -
3.2. Materials and methods ..... - 77 -
3.2.1. Materials ..... - 77 -
3.2.2. Sample preparation ..... - 78 -
3.2.3. Preparation of the organic monolithic columns ..... - 78 -
3.2.4. Capillary HPLC–UV: chromatographic conditions ..... - 79 -
3.2.4.1. Analysis of standards ..... - 81 -
3.2.4.2. Analysis of casein fractions by Cap-HPLC–UV ..... - 82 -
3.2.4.3. Analysis of casein fractions in high resolution

(HR-Cap-HPLC–UV) ..... - 82 -
3.2.5. Mass spectrometry analysis: direct infusion ..... - 83 -
3.2.6. Analytical conditions in Capillary-HPLC–high-resolution-MS ..... - 84 -
3.3. Results and discussion ..... - 85 -
3.3.1. Preparation of the organic monolithic capillary columns ..... - 85 -
3.3.2. Analysis of casein standards present in bovine milk through Cap-HPLC–UV ..... - 86 -
3.3.3. High Resolution Mass Spectrometry of casein standards ..... - 89 -
3.3.4. Analysis of casein fraction in bovine milk ..... - 95 -
3.3.5. Cap-LC–HRMS analysis of week IV casein fractions ..... - 100 -
3.4. Conclusions ..... - 109 -
3.5. References ..... - 110 -

Chapter IV:

Evaluation of two sub-2 µm stationary phases, core-shell and totally porous monodisperse, in the second dimension of on-line comprehensive two dimensional liquid chromatography, a case study: separation of milk peptides after expiration date ..... - 117 -
Abstract ..... - 118 -
Keywords ..... - 118 -
Abbreviations ..... - 118 -
4.1. Introduction ..... - 120 -
4.1.1. Aim of work ..... - 122 -
4.2. Materials and methods ..... - 123 -
4.2.1. Chemicals ..... - 123 -
4.2.2. Sampling and sample preparation ..... - 123 -
4.2.3. Peptides isolation ..... - 124 -
4.2.4. Columns ..... - 124 -
4.2.4.1. Organic monolithic column ..... - 124 -
4.2.4.2. HPLC columns ..... - 125 -

4.2.5. Evaluation of column perfomances ..... - 125 -
4.2.5.1. Instrumentation ..... - 125 -
4.2.5.2. Chromatographic conditions: kinetic evaluation of monolithic column ..... - 126 -
4.2.5.3. Efficiency evaluation of sub-2 μm columns ..... - 126 -
4.2.6. Analysis of soluble fractions ..... - 127 -
4.2.6.1. Analysis of whey proteins by Cap-HPLC-UV ..... - 127 -
4.2.6.2. Analysis of whey proteins by Cap-LC-MS ..... - 128 -
4.2.7. 1D and LC × UHPLC analysis of peptides ..... - 128 -
4.2.7.1. Instrumentation ..... - 128 -
4.2.7.2. Chromatographic conditions: comparative high efficiency monodimensional (1D) analysis ..... - 129 -
4.2.7.3. Chromatographic conditions: LC × UHPLC (set-up 1 and 2) ..... - 129 -
4.2.8. Identification of peptides ..... - 130 -
4.3. Results and discussion ..... - 131 -
4.3.1. Analysis of whey proteins present in bovine milk ..... - 131 -
4.3.1.1. Kinetic and thermodynamic evaluation of monolithic column ..... - 131 -
4.3.1.2. Analysis of whey proteins in Cap-LC-UV and Cap-LC-HRMS ..... - 133 -
4.3.1.3. Cap-LC-UV analysis of whey proteins extracted from semi-skim milk after expiration date. ..... - 138 -
4.3.2. Analysis of peptides ..... - 139 -
4.3.2.1. Kinetic and thermodynamic evaluation of 2D columns ..... - 139 -
4.3.2.2. Preliminary search of chromatographic condition in both dimensions ..... - 143 -
4.3.2.3. Application of the LC × UHPLC approach to the separation of peptides of soluble fraction of milk ..... - 143 -
4.3.2.4. Performance evaluation and comparison of set-ups #1 and #2 ..... - 144 -
4.3.3. Identification of peptides by 1D high efficiency monodimensional-MS analysis ..... - 153 -
4.4. Conclusions ..... - 156 -

4.5. References ..... - 157 -

Chapter V:

5.1. Conclusions ..... - 163 -

Figure captions

Chapter I:

Figure 1.1. *Comparison between monolithic (left) and packed stationary phases (right).....* - 16 -

Figure 1.2. *SEM-image of the porous structure of a typical monolithic silica column (A), enlarged view of the entrance to a macropore or throughpore (B) and mesoporous structure of the skeleton (C).....* - 16 -

Figure 1.3. *Comparison of the efficiency of particle-packed and monolithic columns.....* - 18 -

Figure 1.4. *Activation of inner wall surface of fused silica capillary..* - 19 -

Figure 1.5. *Reaction scheme of silanating "Tentacle type".....* - 20 -

Figure 1.6. *Reaction scheme of derivatization "Grafting-on-to .....* - 21 -

Figure 1.7. *Decomposition reaction of azobisisobutyronitrile.....* - 21 -

Figure 1.8. *Comparison between monolithic polimer obtained by radiation (left) and thermally (right) polymerization.....* - 22 -

Figure 1.9. *Comparison between monolithic columns obtained by radiation (left) and thermally (right) polymerization. 1: Uracil; 2: Benzaldehyde; 3: Nitrobenzene; 4: Ethylbenzene; 5: Butylbenzene….* - 23 -

Figure 1.10. *Non-modulated peak α (a) and peak α modulated 5 times (b) .....* - 30 -

Figure 1.11. *Effects of modulation on 3 co-eluting peaks.....* - 30 -

Figure 1.12. *Quantitative procedure in LC × LC.....* - 31 -

Figure 1.13. *Peak intensity colour-scale for comprehensive HPLC analyses*..... - 32 -

Figure 1.14. *Data elaboration in comprehensive two-dimensional HPLC: (A) 1D non-modulated peak; (B) modulated 2D peaks; (C) fractions of modulated peaks, analyzed in 2 dimensions; (D) bidimensional data visualization (color); (E) bidimensional data visualization (grayscale); (F) three-dimensional visualization*..... - 32 -

Figure 1.15. *10-port two-position switching valve*..... - 33 -

Chapter II:

Figure 2.1. *(a) The nanocluster model for casein micelles. Casein monomers are thread-like, while the dark circles represent calcium phosphate nanoclusters. (b) The model of Horne – dual bonding model of casein micelles using casein monomers as indicated. Protein-protein interactions occur between hydrophobic regions (rectangular bars) while the protein hydrophilic regions (loops) bind to calcium phosphate clusters (triangles)*..... - 57 -

Figure 2.2. *HPLC chromatograms of casein fractions dissolved in: (A) water; (B) solution of 8 M urea and water/acetonitrile (70:30); (C) solution of 0.3% β-mercaptoethanol, water/acetonitrile (70:30); and (D) solution of 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol. I, κ-casein; II, α_{S2}-casein; III, α_{S1}-casein; and IV, β-casein. Peaks 1–4, κ-casein in different states of glycosylation; peaks 5 and 6: α_{S2}- and α_{S1}-casein, respectively; and peaks 7–10, β-casein variants (B, A1, A2, A3, respectively)*..... - 66 -

Chapter III:

Figure 3.1. *Sketch of 10-valve port used for load-trapping purposes..* - 83 -

Figure 3.2. *Chromatograms of casein standards on (A) a 27-cm long Protein-Cap-RP-Lauryl-γ-Monolithic column (270 mm × 0.250 mm, L. × I.D.); (B) on an ACE C4 column packed with 3 μm particles (150 mm × 0.300 mm, L. × I.D.); and (C) on a Jupiter C4 column packed with 5 μm particles (150 mm × 0.300 mm, L. × I.D.). Gradient time: 30 min. Commercially available casein standards were dissolved in water. UV detection at 214 nm. See Section 3.2.4.1 for full experimental details.....* - 88 -

Figure 3.3. *Mass spectra of α_S-CN (A) and β-CN (B) obtained through direct infusion of a 4.0 μM sample solution in MeCN/iso-propanol/H_2O/CF_3COOH 45/45/9.9/0.1 (v/v/v/v). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000–2500; maximum injection time: 100 ms; 1 microscan.....* - 90 -

Figure 3.4. *Expansion of mass spectrum of α_S-CN obtained through direct infusion of a 4.0 μM sample solution in MeCN/iso-propanol/ H_2O/CF_3COOH 45/45/9.9/0.1 (v/v/v/v). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000-2500; maximum injection time: 100ms; 1 microscan.....* - 91 -

Figure 3.5. *Expansion of mass spectrum of β-CN obtained through direct infusion of a 4.0 μM sample solution in MeCN/iso-propanol/ H_2O/CF_3COOH 45/45/9.9/0.1 (v/v/v/v). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000-2500; maximum injection time: 100ms; 1 microscan.....* - 91 -

Figure 3.6. *Deconvoluted spectra of αs-CN (A) and β-CN (B) obtained using the Xtract software. Monoisotopic masses are*

reported. Experimental conditions as in Figure 3.3..... - 92 -

Figure 3.7. *Deconvoluted spectra of α_S-CN (A) and β-CN (B) obtained using the Xtract software. Averaged molecular masses are reported, determined by matching the measured isotopic pattern to a calculated isotope pattern obtained from the elemental composition of an average hypothetical amino acid. Experimental conditions as in text (section 3.2.5.)*..... - 93 -

Figure 3.8. *Chromatographic profile of casein fraction extracted from basal whole, semiskim and skim milk and at weeks I–IV from expiry date. Column: Protein-Cap-RP-Lauryl-γ-Monolithic (27 cm × 0.250 mm, L. × I.D.). Elution: from 20% to 40% of mobile phase B with a 60-min gradient time; flow rate: 10 μL/min. Tcol = 60 °C. UV detection at 214 nm. See Section 3.2.4.2. for full experimental details*…… ..... - 99 -

Figure 3.9. *High-resolution (HR-Cap-LC) chromatogram of casein fraction of basal skim milk (A) and of casein fraction extracted at week IV from skim milk (B) using a 50-cm long Protein-Cap-RP-Lauryl-γ-Monolithic column (0.250 mm I.D.). Elution: 120-min gradient from 15% to 45% of mobile phase (B); flow rate: 7 μL/min. Tcol = 60 °C. UV detection at 214 nm. Full experimental details in Section 3.2.4.3*…… ..... - 100 -

Figure 3.10. *Total ion current chromatogram (Cap-LC–HRMS analysis) of casein fraction extracted at week IV from skim milk (B) using a 50-cm long protein-Cap-RP-lauryl-γ-monolithic column (0.250 mm I.D.). Elution: 60-min gradient from 15% to 45% of mobile phase (B); flow rate: 7 μL/min. Tcol = 60 °C. UV detection at 214 nm. LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass*

range: 1000–3000 amu. Full experimental details in Section 3.2.6..... - 101 -

Figure 3.11. *Mass spectra of peaks 1–4 in region 1 in TIC chromatogram reported in Figure 3.10. Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000–3000; maximum injection time: 100 ms for 1 microscan*..... - 102 -

Figure 3.12. *Mass spectrum of peak 6 of TIC chromatogram in Figure 3.10. (see text). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000-3000; maximum injection time: 100 ms for 1 microscan*..... - 106 -

Figure 3.13. *Mass spectrum of peak 5 in region 2 in TIC chromatogram reported in Figure 3.10. Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000–3000; maximum injection time: 100 ms for 1 microscan*..... - 107 -

Figure 3.14. *Range of exact molecular masses of α_{S1}-CN. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.13. using Xtract software*..... - 107 -

Figure 3.15. *Range of exact molecular masses of α_{S2}-CN. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.13. using Xtract software*..... - 108 -

Figure 3.16. *Range of exact molecular masses of κ-CN. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.13. (see text) using Xtract software*..... - 108 -

Chapter IV:

Figure 4.1. *Expansion of software window showing the ^{2}D continuous shifted gradient.....* - 130 -

Figure 4.2. *Chromatographic characterization and efficiency test of Protein-Cap-RP-Lauryl-γ-Monolithic column (250 × 0.250 mm L. × I.D.). (A): Φ: 3.00 μL/min, ΔP_c: 817 psi; (B) Φ: 2.00 μL/min, ΔP_c: 523 psi; Φ: 1.00 μL/min, ΔP_c: 287 psi.....* - 132 -

Figure 4.3. *Experimental van Deemter for n-pentyl-benzene, column: Protein-Cap-RP-Lauryl-γ-Monolithic column (250 × 0.250 mm, L. × I.D.). Mobile phase: ACN/H_2O; 60/40, v/v; η: 0.72 × 10^{-3} Pa s at 25 °C. Column oven: 25 °C; Detection: UV at 214 nm. Injection volume: 50 ηL.....* - 133 -

Figure 4.4. *Chromatographic profile of whey fraction. Column: Protein-Cap-RP-Lauryl-γ-Monolithic (250 × 0.250 mm, L. × I.D.); Pre-column: Protein-Cap-RP-Lauryl-γ-Monolithic (50 × 0.200 mm, L. × I.D.). Elution: from 10% to 35% of mobile phase B with a 65-min gradient time; flow rate: 10 μL/min. Tcol = 60 °C. UV detection at 214 nm. See Section 4.2.6.1. for full experimental details.....* - 134 -

Figure 4.5. *Total ion current of whey fraction using a 250 cm long protein-Cap-RP-Lauryl-γ-Monolithic column (0.250 mm I.D.). Elution: 70-min gradient from 15% to 45% of mobile phase (B); flow rate: 10 μL/min. Tcol = 60 °C. Exactive Orbitrap MS-ESI (+) detector. R = 10,000, mass range: 500-4000 amu. Full experimental details in Section 4.2.6.2.....* - 135 -

Figure 4.6. *Mass spectrum of peaks A in TIC chromatogram of whey protein. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum in Figure 4.5. using*

Xtract software. Averaged molecular masses are reported, determined by matching the measured isotopic pattern to a calculated isotope pattern obtained from the elemental composition of an average hypothetical amino acid..... - 136 -

Figure 4.7. *Mass spectrum of peaks B in TIC chromatogram of whey fraction. Deconvoluted spectra of BSA obtained using the Promass software*…..... - 136 -

Figure 4.8. *Mass spectrum of β-Lactoglobulin B. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum in Figure 4.5. using Xtract software*..... - 137 -

Figure 4.9. *Range of exact molecular masses of β-Lactoglobulin A. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum in Figure 4.5. using Xtract software*…..... - 137 -

Figure 4.10. *Chromatographic profile of whey fraction extracted from semi-skimed milk at weeks I-IV from expiry date. Column: Protein-Cap-RP-Lauryl-γ-Monolithic (25 cm × 0.250 mm, L. × I.D.). Elution: from 10% to 35% of mobile phase B with a 70-min gradient time; flow rate: 10 µL/min. Tcol = 60 °C. UV detection at 214 nm. See Section 4.2.6.1. for full experimental details*..... - 138 -

Figure 4.11. *Experimental van Deemter (A) and Popple plot (B) for ethylbenzene, columns: (■) Kinetex™ C18 50 × 3.0 mm, 1.7 µm, (●) Titan™ C18 50 × 3.0 mm, 1.9 µm, (▲) Kinetex™ 50 × 3.0 mm, 2.6 µm. Mobile phase: ACN/H_2O; 60/40, v/v; (viscosity given as (η): 0.59 × 10^{-3} Pa s at 35 °C). Column oven: 35 °C; Detection: UV at 214 nm, sampling rate: 80 points/s; resolution: 4.8 nm; no filter. Injection volume: 0.5 µL*..... - 141 -

Figure 4.12. *Comparison of isocratic elution on the Kinetex™ C18 50 × 3.0 mm, 1.7 μm (black line), Titan™ C18 50 × 3.0 mm, 1.7 μm (red dotted line). Column oven: 35 °C; Detection: UV at 214 nm, Sampling rate: 80 points/s; resolution: 4.8 nm; no filter. Injection volume: 0.5 μL. Sample: small molecule mixture. Results are expressed as retention factors (k) and theoretical plates per column (N).....* - 142 -

Figure 4.13. *Comparison of a fast gradient elution on the Kinetex™ C18 50 × 3.0 mm, 1.7 μm (red dotted line), Titan™ C18 50 × 3.0 mm, 1.9 μm (black line). Instrument: Waters UPLC; Detector: PDA; Cell: 500 nL; UV at 214 nm; Tcol: 40 °C; Vinj: 6.0 μL; Mobile phase: (A) H_2O/ACN 95/5 v/v + 0.1% v/v TFA; (B) ACN/H_2O 95/5 v/v + 0.1% v/v TFA; Flow rate: 1.5 mL/min. Sample: peptides < 3,000 Da fraction.....* - 142 -

Figure 4.14. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. ^{1}D column Discovery® 150 × 1.0 mm, 5 μm, flow rate 20 μL/min; column oven: 25 °C, injection volume 2 μL. ^{2}D column Titan™ C18 50 × 3.0 mm, 1.9 μm.; flow rate 2.5 mL/min. Modulation time 60 sec; column oven: 60 °C.....* - 149 -

Figure 4.15. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. ^{1}D column Discovery® 150 × 1.0 mm, 5 μm; flow rate 20 μL/min; column oven: 25 °C; injection volume 2 μL. ^{2}D column Kinetex™ C18 50 × 3.0 mm, 1.7 μm; flow rate 2.5 mL/min. Modulation time 60 sec; column oven: 60 °C.....* - 149 -

Figure 4.16. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. 1D column Discovery® 150 ×*

1.0 mm, 5 μm, flow rate 20 μL/min; column oven: 25 °C, injection volume 2 μL. 2D column Titan™ C18 50 × 3.0 mm, 1.9 μm; flow rate 2.5 mL/min. 2D Modulation time 45 s; column oven: 60 °C..... - 150 -

Figure 4.17. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. 1D column Discovery® 150 × 1.0 mm, μm; flow rate 20 μL/min; column oven: 25 °C; injection volume 2 μL. 2D column Kinetex™ C18 50 × 3.0 mm, 1.7 μm; flow rate 2.5 mL/min. Modulation time 45 s; column oven: 60 °C*..... - 150 -

Figure 4.18. *Three dimensional projection of 2D LC × UHPLC plot of peptides < 3,000 Da fraction obtained using in second dimension a (A) Titan™ C18 and (B) Kinetex™ C18 columns, respectively, with a modulation time of 45 s. The LC × LC data were visualized and elaborated in three dimensions using Chromsquare® ver. 1.5.01 software*..... - 151 -

Figure 4.19. *Chromatograms of high efficiency monodimensional comparative analysis of the peptides belonging to < 3,000 Da fraction. Titan™ C18 2 × 100 × 2.1 mm, 1.9 μm, n_c: 454 (A); Kinetex™ C18 100 + 50 × 2.1 mm, 1.7 μm, n_c: 490 (B)*..... - 152 -

Figure 4.20. *Total ion chromatograms of high efficiency monodimensional comparative analysis of the peptides < 3,000 Da fraction. Columns top: Titan™ C18 2 × 100 × 2.1 mm, 1.9 μm; bottom: Kinetex™ C18 100 + 50 × 2.1 mm, 1.7 μm*..... - 153 -

Table captions

Chapter I:

Table 1.1. *Representative performance data for a variety of packed and monolithic columns*..... - 27 -

Chapter II:

Table 2.1. *Precision (repeatability and reproducibility of analysis method) of peak retention times of caseins from whole bovine milk samples of CdLdS dissolved in denaturing solution consisting of 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol*..... - 62 -

Table 2.2. *Relative amounts of secondary structures present in casein different solutions*..... - 64 -

Table 2.3. *MALDI-TOF-MS data of casein fraction variants*..... - 67 -

Chapter III:

Table 3.1. *Exact measured monoisotopic molecular masses of peaks 1-4 reported in Figure 3.11. (see text). Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.11. using Xtract software*..... - 103 -

Table 3.2. *Summary of monoisotopic exact molecular masses obtained by the deconvolution of the high resolution mass spectrum of peak 5 (see Figure 3.13.) using Xtract software*..... - 105 -

Chapter IV:

Table 4.1. *Peak capacity values for comparative monodimensional analysis and for comprehensive LC × UHPLC set-ups #1 and #2, values are corrected for both orthogonality and undersampling effect*…..... - 145 -

Table 4.2. *Sequence and masses of identified milk peptides after four week from expiration date by 1D-LC-ESI-IT-TOF*..... - 154 -

CHAPTER I

Bovine milk, monolithic columns and comprehensive HPLC

1.1. Introduction

Milk has been described as nature's most complete food. However, both the traditional and contemporary view of the role of milk has been remarkably expanded beyond the horizon of nutritional subsistence of infants. Milk is more than a source of nutrients for any neonate of mammalian species, as well as for growth of children and nourishment of adult humans. Recent studies have shown that milk furnishes a broad range of biologically active compounds protecting neonates and adults against pathogens and illnesses, such as immunoglobulins, antibacterial peptides, antimicrobial proteins, oligosaccharides and lipids, besides many other components at lower concentrations (called "*minor*" components, but with considerable potential benefits).

Among the many valuable constituents in milk, the high levels of calcium play an important role in the development, strength, and density of bones in children and in the prevention of osteoporosis in elderly people. Calcium is also beneficial in reducing cholesterol absorption, and in controlling body weight and blood pressure [1].

Bovine milk is a source of lipids, proteins, amino acids, vitamins and minerals. It contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other bioactive peptides. The lipids in milk are emulsified in globules coated with membranes. The proteins are in colloidal dispersions as micelles. The casein micelles occur as colloidal complexes of protein and salts, primarily calcium. Lactose and minerals are mainly in solution. Bovine milk composition has a dynamic nature, and the composition varies with stage of lactation, age, breed, nutrition, energy balance and health status of the udder. Milk contains many different types of fatty acids. All these components make milk a nutrient rich food item [2].

1.2. Lipids

1.2.1.Fatty acids

Milk contains about 33 g total lipid (fat)/L. Triacylglycerols, which account for about 95 % of the lipid fraction, are composed of fatty acids of different length (4-24 C-atoms) and saturation. Each triacylglycerol molecule is built with a fatty acid combination giving the molecule a liquid form at body temperature. Other milk lipids are diacylglycerol (about 2% of the lipid fraction), cholesterol (less than 0.5%), phospholipids (about 1%), and free fatty acids (FFA) accounting to less than 0.5% of total milk lipids. Increased levels of FFA in milk might result in off-flavours in milk and dairy products, and the free volatile short-chain fatty acids contribute to the characteristic flavours of ripened cheese [3].

1.2.1.1. Saturated fatty acids

More than half of the milk fatty acids are saturated, accounting to about 19 g/L whole milk. The specific health effects of individual fatty acids have been extensively studied. Butyric acid (4:0) is a wellknown modulator of gene function, and may also play a role in cancer prevention. Caprylic and capric acids (8:0 and 10:0) may have antiviral activities, and caprylic acid has been reported to delay tumour growth. Lauric acid (12:0) may have antiviral and antibacterial functions, and might act as an anti caries and anti plaqueagent. Interestingly, *Helicobacter pylori* can be eradicate by this fatty acid. Capric and lauric acid are, moreover addressed for inhibition of COX-I and COX-II. Finally, stearic acid (18:0) does not seem to increase serum cholesterol concentration, and is not atherogenic [4].

1.2.1.2. Unsaturated fatty acids

Oleic acid is the single unsaturated fatty acid with the highest concentration in milk accounting to about 8 g/litre whole milk. Accordingly milk and milk products

contribute substantially to the dietary intake of oleic acid in many countries. Oleic acid is considered to be favourable for health, since diets with high amounts of this monounsaturated fatty acid reduce plasma cholesterol, LDL-cholesterol as well triacylglycerol concentrations. Moreover, replacement of saturated fatty acids with cis unsaturated fatty acids reduces risk for coronary artery disease. Several studies also indicate a cancer protective effect of oleic acid, but the data are not fully persuasive. Milk fat is rich in oleic acid (about 25%) containing also a very high ratio oleic acid/polyunsaturated fatty acids. A diet rich in milk fat therefore may be helpful to increase this ratio in the total dietary fatty acids [5].

1.2.1.3.Conjugated linoleic acid (CLA)

Bovine milk, milk products and bovine meat are the main dietary sources of the cis-9, trans-11 isomer of conjugated linoleic acid. In most cases this isomer is the most abundant CLA-isomer in bovine milk. Minor amounts of other geometrical and positional isomers of CLA also occur in milk, with different biological effects. Milk content of cis-9, trans-11 CLA vary considerably, but may constitute about 0,6% of the fat fraction. The biochemical role of CLA effects in metabolism added to its reported anti-proliferative and pro-apoptotic effect on various types of cancer cell, makes CLA potential therapeutic agent in nutritional cancer therapy [6].

1.2.1.4. Trans vaccenic acid

The main trans 18:1 isomer in milk fat is vaccenic acid, (18:1, trans-11, VA), but trans double bounds in position 4 to 16 is also observed in low concentrations in milk fat. The amount of VA in milk fat may vary, constituting 1.7%, or 4-6% of the total fatty acid content. Typically, the concentration of VA may be about 2-4% when the cows are on fresh pasture and about 1-2% on indoor feeding. Normally, naturally increase in cis-9, trans-11 CLA in milk also results in increased

concentration of VA. VA has a double role in metabolism as it is both a trans fatty acid and a precursor for cis-9, trans-11 CLA [7].

1.2.2. Phospholipids and glycosphingolipids

Phospholipids and glycosphingolipids accounts to about 1% of total milk lipids. These lipids contain relatively larger quantities of polyunsaturated fatty acids than the triacylglycerols. They have different functional roles, such as cations binding, emulsions stabilization, enzymatic activity regulation on the globule surface, cell-cell interactions, differentiation, proliferation, immune recognition, transmembrane signaling, hormones receptors and growth factors. Gangliosides are one of these components found in milk. Gangliosides (with more than one sialicacid moiety) are mainly found in nerve tissues, and they have been demonstrated to play important roles in neonatal brain development, receptor functions, allergies, for bacterial toxins [8].

1.3. Minerals, vitamins and antioxidants

Milk contains many minerals, vitamins and antioxidants. The antioxidants have a role in prevention of oxidation of the milk, and they may also have protective effects in the milk-producing cell and for the udder. Most important antioxidants in milk are the mineral selenium and the vitamins E and A. As there are many compounds that may have antioxidative function in milk, measurement of total antioxidative capacity of milk may be a useful tool [9].

1.3.1. Calcium

The calcium concentration in bovine milk is about 1 g/L. Thus, daily intake of milk and milk products has a central role in securing calcium intake. In human nutrition adequate calcium intake is essential. Getting enough calcium in the diet gives

healthy bones and teeth, and it may also help prevent hypertension, decrease the risk of colon, breast cancer and kidney stones, improving, at the same time, weight control [10].

1.3.2. Selenium

Selenium concentrations in body fluids and tissues are directly related to selenium intake. Selenium is important in human health; it has a role in the immune- and antioxidant system and in DNA synthesis and repair. Selenium protects against many types of cancer [11]. There are evidences about selenium role in asthma protection, since low selenium intake usually worsen the asthma symptoms. Selenium deficiency has even been linked to adverse mood states. Selenium is also a component of enzymes involved in metabolism of thyroid hormone [12]. Recommended daily intake of selenium is 55 µg, and the optimal selenium concentration in bovine milk may be discussed. If milk contains about 50-100 µg selenium/L, it would be a good selenium source.

1.3.3. Iodine

Iodine is an essential component of the thyroid hormones. These hormones control the regulation of body metabolic rate, temperature regulation, reproduction and growth. The recommended iodine intake is 150 µg/d for adults. Accordingly, a daily intake of 0.5 litres milk with an average content of 160 µg iodine/L meets about 50% of the requirement. However, it is important to underline the great seasonal variation in iodine content of milk.

1.3.4. Magnesium

Magnesium is ubiquitous in foods, and milk is a good source, containing about 100 mg/L milk. Recommended intake is 400 mg/day for men and 310 mg/day for women. Magnesium has many functions in the body, participating in more than

300 biochemical reactions. Magnesium deficiency has been linked to atherosclerosis as results of oxidative stress [10].

1.3.5. Zinc

Zinc is an essential part of several enzymes and metalloproteins. Zinc has several functions in the body, in DNA repair, cell growth and replication, gene expression, protein and lipid metabolism, immune function and hormone activity [10]. Milk is a good zinc source; containing about 4 mg/L. Recommended intake is 8 and 11 mg/day for adult female and male. The bioavailability of zinc is better from milk than from vegetable food, and inclusion of milk in the diet may improve total bioavailability of zinc [13].

1.3.6. Vitamin E

Vitamin E concentration in milk is about 0,6 mg/L, but may increase 3–4 folds by proper feeding regimes. Recommended intake is 15 mg/day [10]. Vitamin E is not a single compound; it includes tocoferols and tocotrienols. In whole milk, alpha-tocopherol is the major form of vitamin E (> 85%); gamma-tocopherol and alpha-tocotrienol are present to a lesser extent (about 4% each of the total tocoferols and tocotrienols fraction) [14]. Observational studies indicate that high dietary intake of vitamin E are associated with decreased risk for cancer and coronary heart disease, and that vitamin E can stimulate T-cells and increase the immune defence system. Milk seems to be a food item favouring absorption and transportation of vitamin E from ingested food into the chylomicrons [15].

1.3.7. Vitamin A

Milk is a good source of retinoids, containing 280 µg/L. Recommended daily intake is 700–900 µg/da. Vitamin A has a role in vision, proper growth,

reproduction, immunity, cell differentiation, in maintaining healthy bones as well as skin and mucosal membranes [10].

1.3.8. Folate

Bovine milk contains 50 µg folate/L. Studies indicate that 5-methyl-tetrahydrofolate is the major folate form in milk. Recommended intake of folate is 400 µg/day for adults. Many scientists believe that folate deficiency is the most prevalent of all vitamin deficiencies. It is generally accepted that folate supplementation (400 µg/day) before conception and during the first weeks of pregnancy reduces the risk of neural tube defects. A higher total folate intake was associated with a decreased risk of incident hypertension, particularly in younger women [16].

1.3.9. Riboflavin

Milk is a good source of riboflavin containing 1.83 mg of the vitamin per liter milk [10]. Daily recommended intake is 1.1 and 1.3 mg for women and men, respectively. Riboflavin is part of two important coenzymes participating in a numerous metabolic pathways in the cell. It has a role in the antioxidant performance of glutathione peroxidase and DNA repair via the ribonucleotid reductase pathway.

1.3.10. Vitamin B12

Milk is also a good source of vitamin B12, being 4.4 µg/L. The daily recommendation is 2.4 µg [10]. Vitamin B12 is found only in animal foods, and plays a central role in folate and homocysteine metabolism, by transferring methyl groups. Vitamin B12 deficiency may cause megaloblasticanaemia and breakdown of the myelin sheath.

1.4. Proteins

Bovine milk contains about 32 g protein/L. The milk proteins have a high biological value, and milk is therefore a good source for essential amino acids. Milk proteins can exert a wide range of physiological activities, including enhancement of immune function, defense against pathogenic bacteria, viruses and yeasts, and development of the gut and its functions [17,18]. Besides the biologically active proteins naturally occurring in milk, a variety of bioactive peptides are encrypted within the sequence of milk proteins that are released upon suitable hydrolysis of the precursor. A wide range of bioactivities has been reported for milk protein components.

Bovine milk possesses a protein system constituted by two major families of proteins: caseins (insoluble) and whey proteins (soluble). Caseins (α_{S1}, α_{S2}, β, and κ) are the predominant phosphoproteins in the milk of ruminants, accounting for about 80% of total protein, while the whey proteins, representing approximately 20% of milk protein fraction, include β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, bovine lactoferrin, lactoperoxidase and other minor components. Different bioactivities have been associated with these proteins. In many cases, caseins and whey proteins act as precursors of bioactive peptides that are released, in the body, by enzymatic proteolysis during gastrointestinal digestion or during food processing. The biologically active peptides are of particular interest in food science and nutrition due to their physiological roles, including opioid-like features, as well as immunomodulant, antihypertensive, antimicrobial, antiviral and antioxidant activities [19,20].

1.4.1. Whey Proteins

The liquid part of milk, whey, has traditionally not received the same attention paid to source milk, probably because it is a by-product of cheese making, barely

considered an additive to animal feeding. Interestingly, Hippocrates already commended the health properties of whey in Ancient Greece. During the Middle Age, whey was considered not simply a medicine but also even as an aphrodisiac potion and a skin balm: it was in fact a regular component of salves and ointments to soothe burns, to inspire vitality and to cure various illnesses. Recent decades have witnessed an increased interest in whey protein products and their nutritional and active role upon human health has been clearly disclosed.

Whey proteins represent approximately 20% of milk protein fraction and include β-lactoglobulin, α-lactalbumin, immunoglobulins, bovine serum albumin, bovine lactoferrin, and lactoperoxidase, together with other minor components.The actual concentrations of whey proteins depend onthe type of whey (acid or sweet), the source of milk (bovine, caprine or ovine), the time of the year, the type of feed, the stage of lactation and the quality of processing.

1.4.1.1. α-Lactoalbumin

α-Lactoalbumin (α-LA) is, quantitatively, the second most important protein in whey, representing ca. 20% (*w/w*) of the total whey protein inventory, and is fully synthesized in the mammary gland [21]. It contains 123 amino acid residues with a molecular weight of 14,175 kDa and isoelectric point between 4.2 and 4.5. In aqueous solution, α-LA has a globular structure stabilized by four disulphide bonds, and, actually, three genetic variants (A, B, and C) have already been identified. This globular protein consists in a single polypeptide chain with eight cysteine residues, and it is physiologically important for its involvement in lactose synthesis [22].

1.4.1.2. β-Lactoglobulin

β-Lactoglobulin (β-Lg) is quantitatively a noncasein protein in bovine milk (58% *w/w*). It is a small, soluble, and globular protein, but its quaternary structure is pH dependant. At pH of 3.0 and above 8.0, β-Lg is a monomer molecule with a molecular weight of 18 kDa, while, at pH between 7.0 and 5.2, it is a stable dimer with molecular mass of about 36.7 kDa; at pH between 5.2 and 3.5 it is an octamer with molecular mass of 140 kDa. β- Lg is composed mainly of β-sheet motifs and consists of 162 aminoacid residues [23]. The high nutritional and functional value of β-Lg is widely recognized and has made this protein an ingredient of choice in the formulation of modern foods and beverages [24].

1.4.1.3. Bovine Serum Albumin

Bovine serum albumin (BSA) is not synthesized in the mammary gland, but appears in milk following passive leakage from the blood stream. It contains 582 aminoacid residues with a molecular weight of 66,267 kDa; it also possesses 17 intermolecular disulphide bridges and one thiol-group at residue 34 [22]. Because of its size, BSA can bind to free fatty acids and other lipids as well as flavour compounds a feature that is severely hampered upon denaturation. Its heat-induced gelation at pH 6.5 is initiated by an intermolecular thiol-disulphide interchange, similar to what happens with β-Lg [25].

1.4.1.4. Lactoferricin

Lactoferrin (LF-B), an iron-binding glycoprotein with a molecular weight of about 80 kDa (703 aminoacid), is mainly found in external secretions that include breast milk and saliva and in the secretory granules of neutrophils. In addition to its antimicrobial effects, it is well known to possess a variety of biological activities, like regulation of immune response, cells transcriptional activation and antiviral

properties [26-28]. The antimicrobial activity of bovine lactoferrin has been attributed to the bovine lactoferricin fragment (LfcinB), which, unlike the parental glycoprotein, displays no iron-binding capacity. In fact, the LfcinB is considered as the active domain responsible for antimicrobial activity of LF-B against a wide range of microorganisms. Lactoferricin is a cationic peptide produced by acid pepsin hydrolysis of mammalian lactoferrin [29] and consists of 25 aminoacid residues (FKCRRWQWRMKKLGAPSITCVRRAF), including two cysteine residues that create a disulfide bond linking the highly positively charged NH_2-terminal and the COOH-terminal regions of the peptide. LfcinB has a high content of asymmetrically clustered basic aminoacid residues, giving the peptide a net positive charge of 7.84 at pH 7.0 [30].

1.4.1.5. Immunoglobulins

Immunoglobulins (IG) constitute a complex group whose components are produced by B-lymphocytes; they significantly contribute to the whey protein content, exerting an important immunological function (especially in colostrums). These proteins are present in the serum and physiological fluids of all mammals, behaving as receptors, when attached to surfaces, as well as antibodies, when released in blood and lymph. IG are subject to postnatal transfer via colostrums, as the placenta does not permit passage of macromolecules [31]. In terms of quaternary structure, IG are either monomers or polymers of a four-chain molecule, consisting of two light polypeptide chains (with a molecular weight in the range 25,000 kDa) and two heavy chains (with molecular weight of 50,000-70,000 kDa). There are, however, three basic classes of IG: IGG, IGA and IGM, although IGG is often sub-divided into two subclasses: IGG1 and IGG2. Up to 80% (*w/w*) of all IG in milk or whey is represented by IGG [21] but, qualitatively, the family of IG found in bovine whey and colostrums includes IGA and secretory IGA, IGG1, IGG2 and IGG fragments, IGM, IGE, J-chain or components, and free secretory components.

1.4.1.6. Lactoperoxidase

Lactoperoxidase (LP) is present in a variety of animal secretions such as tears, saliva and milk. It is one of the most abundant enzymes in plain milk, representing 1% (*w/w*) of the total protein pool in whey [32]. The complete LP system (i.e. enzyme plus substrate) was originally characterized in milk by its activity, depending on many factors, e.g. animal species, breed and lactation cycle. Other members of the group of oxidoreductases include myeloperoxidase (present in neutrophils and monocytes), eosinophil peroxidase and thyroid peroxidase. Chemical sequencing unfolded a great degree of homology among them, which suggests a close evolutionary relationship for these enzymes. Peroxidases utilize hydrogen peroxide to oxidise thiocyanate to hypothiocyanate, and are active in a variety of anatomic locations [33].

1.4.2. Caseins

Caseins are currently the main source of milk derived biologically active peptides. Different peptides are released from the original sequence of the parent protein by enzymatic hydrolysis, during gastrointestinal digestion or cheese ripening. Casein-derived peptides are considered as highly prominent ingredients for health-promoting functional foods and pharmaceutical preparations. Casein has been considered a valuable amino acid supply source since ancient times. In the latter half of the 1970s, a variety of bioactive peptides has been isolated from a digestion of casein [34]. Caseins (α_{S1}, α_{S2}, β, and κ) are the predominant phosphoproteins in the milk of ruminants, accounting for about 80% of total protein.

1.4.2.1. α- and β-Casomorphins

β-Casomorphins (β-CMs) are a group of exogenous opioid-like peptides derived from the hydrolysis of β-casein and were first isolated from an enzymatic casein digest [35]. Their primary amino acid sequence is NH_2-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-COOH, located in bovine β-casein at positions 60–70. It has been reported that β-CMs reach significant level in the stomach because they are fairly resistant to proteolysis due to their proline-rich sequence [36]. α-Casomorphins (exorphins) have been isolated from peptic hydrolysates of α-casein fractions. In general, their structures differ considerably from those of β-casomorphins. Active fractions were shown to be a mixture of two separate peptides derived from α_1-casein fragments 90–95 and 90–96 [Arg_{90}-Tyr-Leu-Gly-Tyr-Leu_{95}-(Glu_{96})]. The N-terminal arginine residue was also reported to be essential for opioid activity. α- and β-Casomorphins may be produced by the enzymatic action of different proteases released from tumor cells [37]. Indeed, Hatzoglou et al. [38] have shown that five different casomorphins, α-casein fragments 90–95 and 90–96, β-Casomorphins 7 (BCM7) fragment 60–66, β-Casomorphins 5 (BCM5) fragment 60–64, and the morphiceptin, the amide of β-Casomorphins 4, exert antiproliferative action on T47D cells, blocking cells in G0/G1 phase.

1.4.2.2. Caseinphosphopeptides

Caseinphosphopeptides (CPPs) are a family of bioactive peptides derived from digestion of casein. Their name is due to their high content of phosphorylated sites, and they are characterized by the ability to bind and solubilize calcium [39]. This property is accounted for their anticancer activity against intestinal tumor HT-29 cells, by modulation of cell proliferation and apoptosis [40].

1.5. Aim of work

The main target of my PhD is aimed to the reutilization and valorization of potential nutraceutical milk and dairy products derived from the "Centrale del latte di Salerno". The research was focused on the analysis of bio-macromolecules recovered in by-products and waste derived from milk industry, by means of powerful analytical tools such as newly designed liquid chromatography platforms (monolithic columns and comprehensive two-dimensional liquid chromatography). In this regard, several matrices were considered. In particular, commercial milk samples stored at room temperature after expiration date were analyzed, in order to study the degradation of the protein fraction and identify biologically active peptides, potentially useful for the production of innovative value-added products and, simultaneously, reduce the amount of food waste and its environmental impact. Collected results showed how these techniques are the best strategies to identify and characterize new bioactive molecules, capable of modulating basal human physiological processes, that can be included in functional food and nutraceuticals.

1.6. Monolithic stationary phases

Whereas one of the main objectives of the analytical chemistry is to speed up the analysis; in order to fulfill these objectives without using extremely high pressures, monolithic stationary phases have been developed (Figure 1.1.). The term ''*monolith*'' refers to unibody structures, which consist of one single piece of porous material composed of interconnected repeating cells or channels [41]. The major advantage of monolithic supports is the fast mass transport between the monolithic support and the surrounding liquid [42,43].

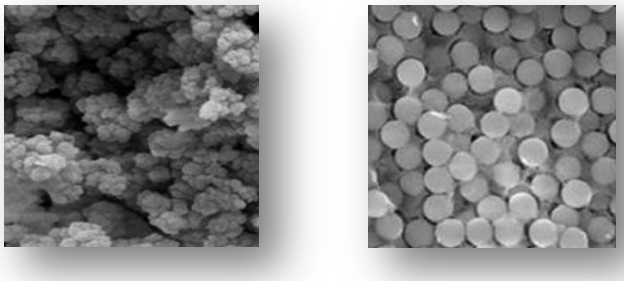


Figure 1.1. *Comparison between monolithic (left) and packed stationary phases (right).*

The porous rod fills totally the cylindrical volume of the column and possesses interconnected skeletons (diameter about 1.5-2.0 μm) and interconnected flow paths (through-pores, 10-12 nm) through the skeletons (Figure 1.2.).

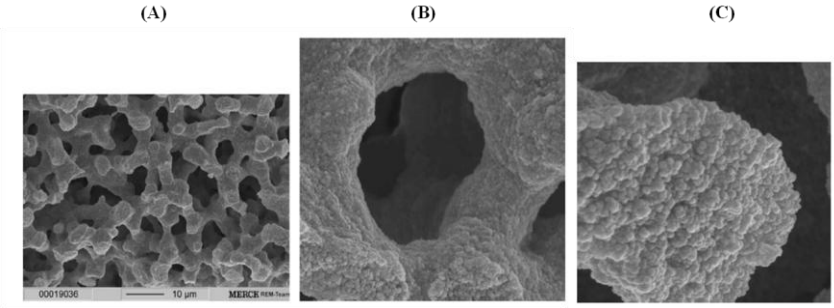


Figure 1.2. *SEM-image of the porous structure of a typical monolithic silica column (A), enlarged view of the entrance to a macropore or throughpore (B) and mesoporous structure of the skeleton (C).*

A monolithic column with small-sized skeletons and large through-pores can reduce the diffusion path length and flow resistance compared to a particle-packed column.

It is evident that a support structure which has a large (through-pore size)/(skeleton size) ratio, unattainable with a particle-packed column, can provide both high

permeability and high column efficiency. A wide range of monolithic columns, either capillary-sized or larger-sized, have been reported.

Depending on the nature of the monolithic support we can distinguish two major types of monoliths: organic polymer-based monoliths and silica-based monoliths [44].

Initially, monolithic columns were prepared from organic polymer-gel materials; high-speed separations of polypeptides and proteins were performed with these supports, but relatively low efficiency for small solutes compared with particles-packed columns was evidenced due to the presence of micropores in the polymer-gel structure. Moreover, at the presence of organic solvents, these early monoliths tend to swell and contract, thus causing dramatic effects for the efficiency of the separation and peak symmetry [45]. Monolithic silica columns, prepared by using a sol-gel process, have shown lower pressure drop and higher efficiency than particle-packed columns.

The advantage of this type of column consists of a high-speed separation of both small and large molecules This is allowed by a porosity up to 80%, which corresponds to 15% higher permeability than particle-packed columns.

Another advantage is the possibility to perform analyses with very high efficiency even at very high flow rates. This is due to the porous structure minimizing back pressures.

The efficiency of the separation of these columns does not significantly decrease when the flow rate is increased as it happens for the conventional particle-packed columns. This is clear looking at Figure 1.3., showing the van Deemter curve, obtained by plotting the height of the theoretical plate versus the linear velocity [46-48]. The efficiency of these columns is thus very similar to columns packed with 3 μm particles.

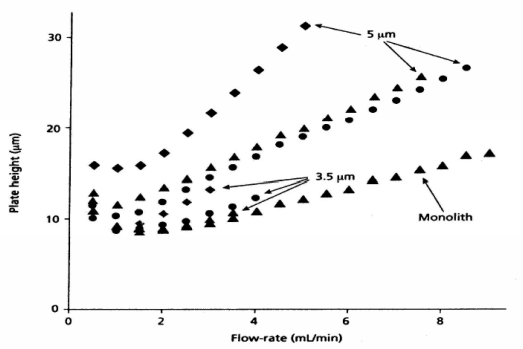


Figure 1.3. *Comparison of the efficiency of particle-packed and monolithic columns.*

Moreover, high flow rate (10 mL/min) are allowed with monoliths, leading to a reduced analysis time up to 70%. In the case of gradient analysis, the re-conditioning time between two analyses is also reduced.

However, the main drawback of using monolithic columns is their preparation, strongly limited by the length of the column. A straight monolithic column longer than 15 cm cannot be prepared without drawbacks [49,50]. Therefore, the monolithic silica columns (4-6 mm I.D.) for conventional HPLC are prepared in a mold capable of providing high-speed separation, leading to a reduction of the maximum number of theoretical plates The generation of a larger number of theoretical plates requires the use of a series of connected columns.

1.6.1. Preparation techniques for organic monolithic columns

The preparation of organic monolithic stationary phases for chromatographic columns is accomplished through an "*in situ*" heat- or γ-rays-induced process. Polymerization mixtures consist of acrylic monomers, cross-linker and porogens. Before undertaking the polymerization process a step of pretreatment of the capillary must be necessarily carried out, in order to homogeneously distribute the

monolith within the capillary. The synthesis of the monolithic support includes a series of very important steps which are listed below:

- ✓ Activation of the inner wall of the capillary through the step of "*Etching*" silanating "*New Tentacle Type*" and polymerization "*Grafting on to*".
- ✓ Filling of the capillaries with the polymerization mixture formed by the various components mixed in the right proportions.
- ✓ Polymerization by irradiation with γ-rays.
- ✓ Capillaries drying for uncured monomers and porogens removal.

1.6.1.1. Activation of capillary

The pretreatment of the capillary provides three distinct phases:

- ➢ "*Etching*":

  Transformation of siloxane groups present on the surface of the capillary in silanol groups (Figure 1.4.). Cifuentes et al. [51] proved that etching of columns with NaOH followed by leaching with HCl gives more reproducible surface treatment.

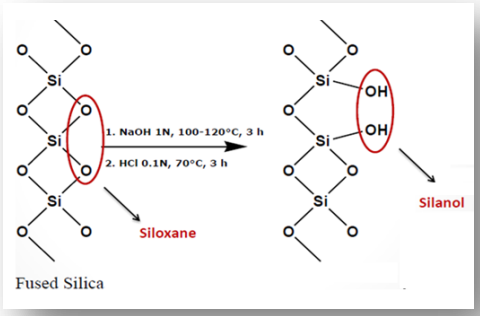


Figure 1.4. *Activation of inner wall surface of fused silica capillary.*

- Silanating step"*Tentacle type*"

  The capillary surface is usually modified with a bi-functional silanizing reagent such as vinyl silane, acrylate silane or methacrylate silane. The most common reagent used is 3-(trimethoxysilyl)propyl methacrylate (Figure 1.5.). This step allows the immobilization of the monolithic polymer on the inner wall of the capillary.

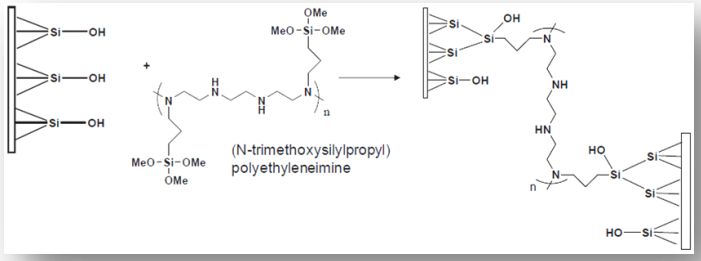


Figure 1.5. *Reaction scheme of silanating "Tentacle type".*

- Polymerization phase "*Grafting-on-to*"

  In this last phase of pre-treatment a solution of methacrylic anhydride is used in order to create a high number of unsaturated groups on which the polymerization process relies (Figure 1.6.).

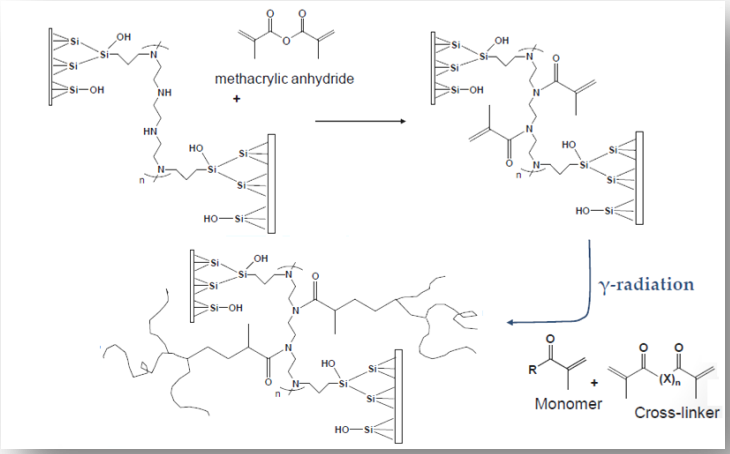


Figure 1.6. *Reaction scheme of derivatization "Grafting-on-to".*

1.6.1.2. Thermally and radiation polymerization

In case the polymerization is carried out through the use of heat (thermal polymerization), a compound ("*photoinitiator*" radical) is added to the polymerization mixture.

Azobisisobutyronitrile (AIBN) is quite popular. It decomposes while irradiated at 365 nm to afford free radicals [52-54]. Figure 1.7. shows the decomposition process involving AIBN, which, at high temperatures, releases N_2 and generates two radicals [55-57].

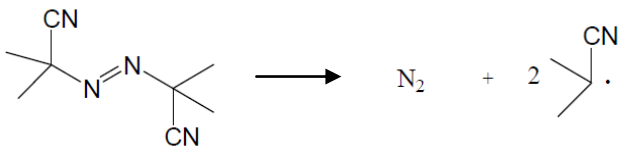


Figure 1.7. *Decomposition reaction of azobisisobutyronitrile.*

Thermally initiated free radical polymerization was the first method used for the preparation of rigid polymer-based monolith [58-60]. This process is very simple and its origin can be traced down to techniques typically applied in the preparation of porous beads by using suspension polymerization. This type of polymerization is generally treated in the literature as a "*clone*" of bulk polymerization in which each droplet of the dispersed phase containing monomer is an individual bulk reactor. Polymerizations initiated using high energy radiation such as γ-rays or electron beam belong to the group of "*exotic*" approaches to monoliths.

The major advantages of this method are no need for an initiator and thus absence of any functional groups at the chain ends, wide range of operating temperatures and wide choice of materials for containers, including stainless steel tubes.

The polymerization process triggered by γ rays allows a better distribution of the cross-linked structure for the entire length of the capillary, as is evident in Figure 1.8. in which a monolithic stationary phase obtained by a process of thermal polymerization is compared with a stationary phase obtained by polymerisation radiation. SEM images show that the monolith obtained by radical polymerization has a more homogeneous morphology.

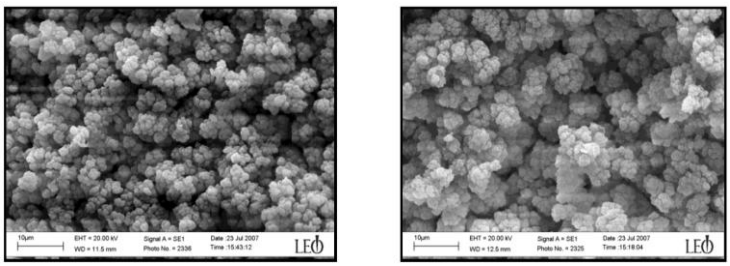
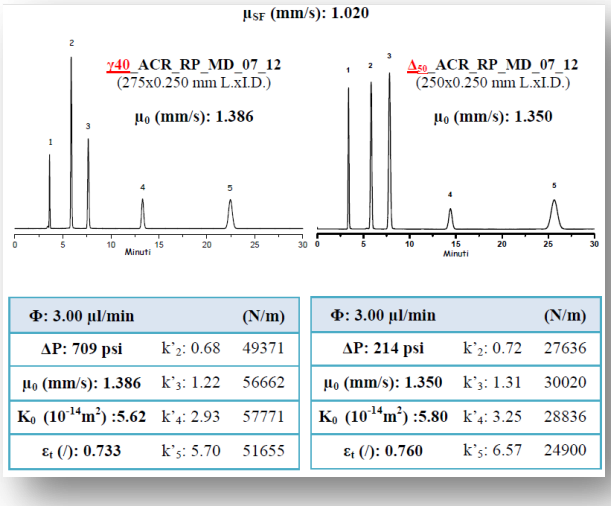


Figure 1.8. *Comparison between monolithic polimer obtained by radiation (left) and thermally (right) polymerization.*

This is why the photoinitiated monoliths exhibits about twice back pressure, thus indicating a difference in the porous structure (Figure 1.9.).



Φ: 3.00 μl/min		(N/m)
ΔP: 709 psi	k'2: 0.68	49371
μ0 (mm/s): 1.386	k'3: 1.22	56662
K0 (10^{-14}m^2) :5.62	k'4: 2.93	57771
εt (/): 0.733	k'5: 5.70	51655

Φ: 3.00 μl/min		(N/m)
ΔP: 214 psi	k'2: 0.72	27636
μ0 (mm/s): 1.350	k'3: 1.31	30020
K0 (10^{-14}m^2) :5.80	k'4: 3.25	28836
εt (/): 0.760	k'5: 6.57	24900

Figure 1.9. *Comparison between monolithic columns obtained by radiation (left) and thermally (right) polymerization. 1: Uracil; 2: Benzaldehyde; 3: Nitrobenzene; 4: Ethylbenzene; 5: Butylbenzene.*

Although use of gamma rays can provide secondary compounds, this process remains more advantageous than thermal polymerization. Pore size control by irradiation rate, lower operating temperatures and resulting freedom in low boiling solvents choice represent the main advantages of the technique.

1.6.2. Monomers

Various functional monomers can be applied in preparation of monoliths. Glycidyl methacrylate with a highly reactive epoxy ring is the most frequently used in the synthesis of methacrylate ester-based monoliths [61]. However, the effects of other methacrylate monomers on separation properties of monolithic columns, such as

C6- [62], C8- [63], C16- [64], C4-, C12-, C18-, and isobornyl- methacrylate monomers [65] has been investigated for the preparation monolithic stationary phases with modulated hydrophobicities. The column prepared with C12 alkylmethacrylate functional monomer has shown the highest efficiency; however, the C18-methacrylate monolithic column provided better permeability [66]. The column based on the lauryl methacrylate (LMA) monomer provided the best separation of peptidic fragments containing less than 20 amino acids in tryptic digests of cytochrome C [67]. Butyl methacrylate (BMA) and 2-hydroxy ethyl methacrylate monomers were polymerized with 1,3-butanediol dimethacrylate cross-linker to produce hydrophobic monoliths for proteins chromatographic separation [68].

The styrene–divinylbenzene co-polymer monolithic stationary phases generally show lower polarity than their polymethacrylate counterparts. Their hydrophobicity can be tuned by adding a methacrylate to the polymerization mixture. Addition of methacrylic acid in the polymerization mixture significantly improves the surface area of prepared monoliths up to 261 m^2/g in comparison to 0.1 m^2/g for the material prepared in absence of methacrylic acid. In this way increased resolution for small aromatic compounds was attained [69]. Incorporation of a methacrylate monomer to the polymerization mixture for preparation of poly(divinylbenzene-alkyl methacrylate) monolithic columns improved peak symmetry with respect to poly-(styrene-co-divinylbenzene) columns [70,71] and decreased the retention of aromatic compounds, probably because of decreased π–π interactions in presence of long hydrophobic alkyl moieties in the monolithic structure [72].

Another approach tested for tuning the polarity of monolithic stationary phases was mixing two different monomers in the polymerization mixture. Partial substitution (25%) of BMA by LMA produced columns with enhanced retention of neutral analytes, while the replacement of larger percentages (75%) resulted in columns with lower retentivity [73].

In spite of numerous reports on variation of properties of monolithic columns by varying the chemistry of functional monomers in the polymerization mixture, to the best of our knowledge the effects of hydrophobicity and other properties of functional monomers on the separation of small compounds and large biopolymers on monolithic stationary phases have not been systematically compared so far.

1.6.3. Porogens

The choice of pore-forming solvent or porogen is a key tool that may be used for the control of porous properties without changing the chemical composition of the final polymer. In general, larger pores are obtained using more macroporogen due to an earlier onset of phase separation. The porogenic solvent controls the porous properties of the monolith through the solvation of the polymer chains in the reaction medium during the early stages of the polymerization [74,75]. Infact, the space originally occupied by porogens contributes to the formation of pores inside monolith phase. During the polymerization step the solvent molecules are incorporated within the polymer and then be removed by washing, thereby leaving a series of interconnected channels between them which will represent the "*chromatographic bed*".

1.6.4. Crosslinking monomer

The porosity and the retentivity of the (poly)-methacrylate monolithic stationary phase can be changed by employing different alkyl dimethacrylate cross-linkers with various alkyl chain lengths between the two methacrylate units. Using 2-methyl-1,8-octanediol dimethacrylate as the cross-linker increased the proportion of mesopores in the final monolith, in comparison to the ethylene dimethacrylate (EDMA) cross-linker. The increased surface area in the mesopores improved the column efficiency [76].

Differently by temperature and porogenic solvent that modify only the porous properties of the resulting material, leaving unchanged its composition, variations in the monovinyl/divinyl monomer ratio also lead to materials with different compositions. A higher content of divinyl monomer implies in a direct manner a greater formation of more crosslinked polymers in the early stages of the polymerization process and therefore lead to earlier phase separation. Although this is similar to the effect of poor solvent, the nuclei are more crosslinked and since this crosslinking negatively affects their swelling with the monomers, they remain relatively small in size. The pre-globules can still capture the nuclei generated during the later stages of polymerization, but true coalescence does not occur. Since the final macroporous structure that results consists of smaller globules, it also has smaller voids. Thus, this approach is useful for the preparation of monoliths with very large surface areas [74,77].

Core-shell particles have a solid core surrounded by a porous outer layer, enabling the mobile phase to penetrate only the shell and not the core. Since larger particles are used, core-shell particles lead to reduced backpressure of the column in comparison to columns packed with porous particles. The porous layer structure, the larger diameter of core-shell particles and the open macropore structure of monolithic columns permit rapid separation of analytes at reasonable back pressure, while retaining good separation efficiency.

Table 1.1. *Representative performance data for a variety of packed and monolithic columns.*

Stationary Phase	Performance		K	Back pressure	Column dimensions	Ref.
	N (plates/m)	H (µm)				
			Particle packed columns			
Particle diameter						
5 µm	83,000	12.0	2.7	899 psi at 0.088 cm/s	33 cm × 50 µm I.D.	[78]
3 µm	110,000	9.1	0.9	Constant pressure of 200 kg/cm^2	100 cm × 200 µm I.D.	[79]
1.5 µm	209,000	2.4	0.2	23,000 psi at 0.145 cm/s	49.3 × 30 µm I.D.	[80]
1 µm	521,000	2.0	2.0	40,000 psi at 0.15 cm/s	46 × 30 µm I.D.	[81]
			Silica monoliths			
Domain size						
3.1 µm	186,000	5.4	1.4	377 psi at 2.0 mm/s	14.5 cm × 100 µm I.D.	[82]
2.6 µm	200,000	5.0	1.4	537 psi at 2.0 mm/s	15 cm × 100 µm I.D.	[82]
2.2 µm	210,000	4.8	1.4	653 psi at 2.0 mm/s	15 cm × 100 µm I.D.	[82]
			Organic monoliths			
Domain size						
N.A.	48,000	20.5	11.5	1740 psi at 6.4 mm/s	8 cm × 200 µm I.D.	[83]
N.A.	60,000	16.6	7.9	700 psi at 1.1 mm/s	16 cm × 75 µm I.D.	[84]
N.A.	83,200	12.0	0.04	3770 psi at 0.1 µL/min	13 cm × 100 µm I.D.	[77]

1.7. Comprehensive HPLC

Comprehensive two-dimensional liquid chromatography was at first introduced by Erni and Frei in 1978 [85], followed by Bushey and Jorgenson [86].

It has been stated that a two-dimensional separation should possess the following features [87,88]:

- All components in a sample mixture are subjected to two separations in which their displacement depends on different factors.
- Equal percentages (either 100% or lower) of all sample components pass through both columns and eventually reach the detector.
- Any two components separated in the first dimension must remain separated in the second dimension.
- The elution profiles from both dimensions are preserved.

A typical comprehensive two-dimensional HPLC separation is attained through the connection of two columns by means of an interface (usually a high pressure switching valve), which entraps specific quantities of first dimension eluate, and directs them onto a secondary column. This means that the first column effluent is divided into "*cuts*" which are transferred continuously to the second dimension by the interface. The type of interface depends on the methods used, although multiport valve arrangements have been the most frequently used.

Various comprehensive HPLC systems have been developed and proven to be effective both for the separation of complex sample components and in the resolution of a number of practical problems. In fact, the very different selectivities of the various LC modes enable the analysis of complex mixtures with minimal sample preparation. However, comprehensive HPLC techniques are complicated by the operational aspects of switching effectively from one operation step to

another, by data acquisition and interpretation issues. Therefore careful method optimization and several related practical aspects should be considered.

Since the introduction of two-dimensional chromatographic techniques, it has become clear that each separation step should ideally provide different selectivities as this maximizes the gain in peak capacity and hence the number of chromatographically resolvable components [85,89-91]. The complete reversal of component elution orders can be observed, for example, when normal- and reversed phase separations of non-homologous components are compared.

Because the system maintains its effectiveness it is necessary that the separation in both dimensions is controlled by parameters that are relatively independent characterizing each component peak emerging from a secondary column, the first identified by the position in the sequence of cuts from the first column and the second measured by the retention time in the secondary column. However, if the cut from the primary column is very broad (large Δt_r), then the component peaks emerging from the secondary column will have considerable uncertainty in the retention time for their migration through the primary column. The greater the "*cut*", the greater the degradation of the quality of information relative to each component's identity.

Broader cuts also incorporate more components and increase the likelihood of interference in subsequent separation steps. It is therefore clear that the separation of the components will improve with decreasing Δt_r. Murphy and co-workers have stated that to obtain a high 2D resolution, each peak in the first dimension should be sampled at least three-four times [92]. A modulation process is illustrated in Figure 1.10., where peak α is sampled (modulated) 5 times.

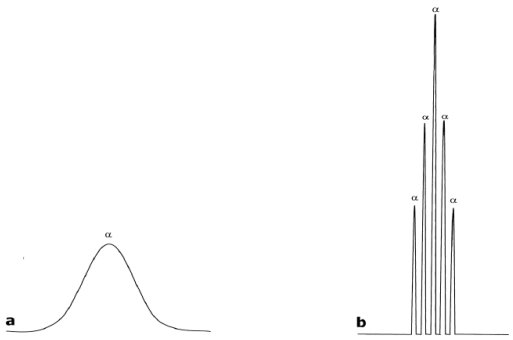


Figure 1.10. *Non-modulated peak α (a) and peak α modulated 5 times (b).*

The effectiveness of a two-dimensional separation, where a first dimension peak is unravelled in 3 compounds (α, β and γ), is illustrated in Figure 1.11. In this case the number of samplings over the first dimension peak is 3.

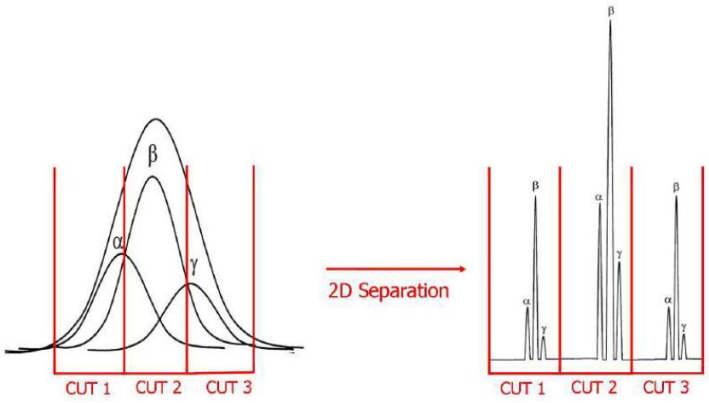


Figure 1.11. *Effects of modulation on 3 co-eluting peaks.*

For quantitation, the areas of all peaks relative to the same compound must be summed, as illustrated in Figure 1.12.

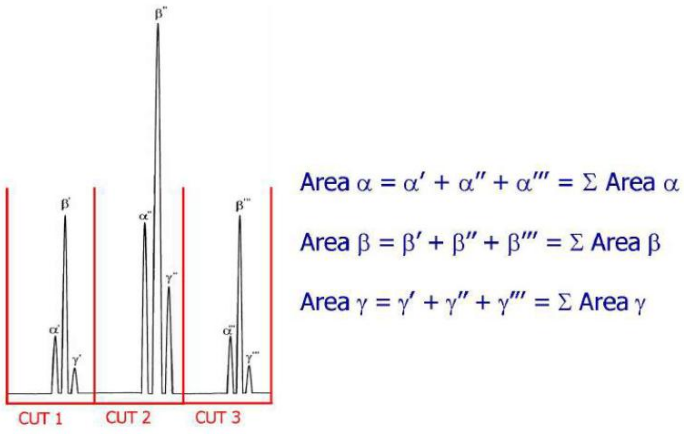


Figure 1.12. *Quantitative procedure in LC × LC.*

With regards to comprehensive LC data elaboration, the acquired data is commonly elaborated with dedicated software which constructs a matrix with rows corresponding to the duration of the 2nd dimension analysis and data columns covering all successive second dimension chromatograms. The result is a bidimensional contour plot, where each component is represented as an ellipse shaped peak, defined by double-axis retention time coordinates.

In fact, when create a three-dimensional chromatogram is created a third axis in the means of relative intensity is added. The color and dimension of each peak is related to the quantity of each compound present in the sample. Figure 1.13. provides an example of the colour-range from minimum to maximum intensity used for data visualization.

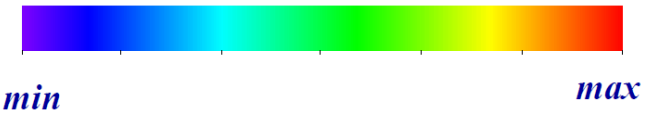


Figure 1.13. *Peak intensity colour-scale for comprehensive HPLC analyses.*

Figure 1.14. illustrates the schematic of comprehensive 2D-LC data elaboration and visualization.

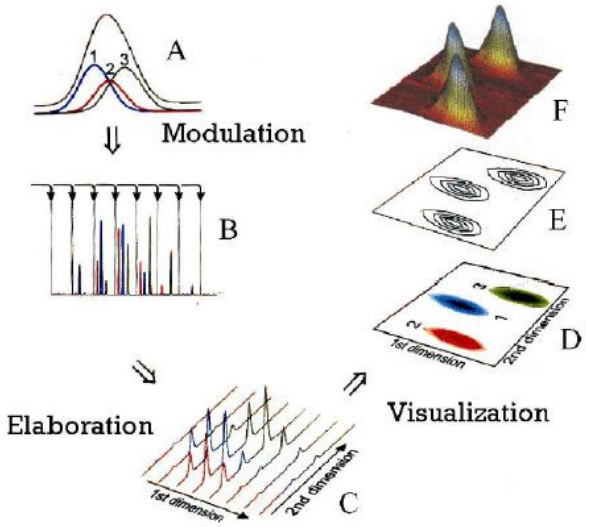


Figure 1.14. *Data elaboration in comprehensive two-dimensional HPLC: (A) 1D non-modulated peak; (B) modulated 2D peaks; (C) fractions of modulated peaks, analyzed in 2 dimensions; (D) bidimensional data visualization (color); (E) bidimensional data visualization (grayscale); (F) three-dimensional visualization.*

In the context of a two-dimensional system on-line coupling of two columns can be made in different ways, however, there are two main types of comprehensive 2D-

LC set-ups most used. The most common approach involves the use of a micro-bore LC column in the first and a conventional column (fast separation) in the second dimension. In this case, an 8-, 10- or 12-port valve equipped with two sample loops (or trapping columns) is used as an interface. In Figure 1.15. the schematic of a 2-position 10-port valve is illustrated.

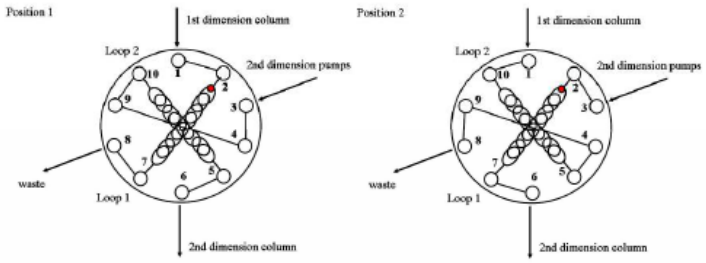


Figure 1.15. *10-port two-position switching valve.*

The principle on which is based the operation of this system is that the loops or trapping columns in the valve are filled alternately with the effluent of the first column and then a second pump forces the loop material onto a second column. The loop volume usually corresponds to the mobile phase quantity per modulation time eluting from the 1st D column.

The combination of a microbore column in the first dimension and a conventional column in the second dimension, connected by a multiport switching valve equipped with two sample loops, has been used for the comprehensive two-dimensional chromatography of proteins by Jorgenson and co-workers [86,93], of synthetic polymers by Schoenmakers [94] and of the oxygen heterocyclic fraction of cold-pressed citrus oils by Dugo et. al. [95]. A similar approach, based on the use of 2 trapping columns used instead of 2 loops, is described by Cacciola and co-workers [96].

The second approach foresees the use of a conventional LC column in the first and two conventional columns in the second dimension. One or two valves that allow transfers from the first column to two parallel secondary columns (without the use of storage loops) are used as interface. When a conventional column is used as a first-dimensional column, a different LC/LC interface has been developed, using two fast secondary columns in parallel rather than storage loops.

With this kind of interface, the mobile phase from the first column should have a very low strength so that analytes can be trapped at the head of the secondary columns during the loading step. Such a system has been used by Opiteck et al. [97] Wagner et al. [98] and Unger et al. [99] for the analysis of peptides and proteins. In most of the previously cited works, size-exclusion chromatography or ion exchange chromatography is used in one of the two dimensions, while reversed-phase LC is normally used in the other one.

Recently, a simple, automated 2D-LC system equipped with an electronically controlled, 12-port valve has been used for the analysis of aromatic amines and non-amines, operating both dimensions under comparable reversed-phase conditions [100]. In this case, the orthogonal separation was achieved by tuning the operating parameters, such as mobile-phase strength, temperature, and buffer strength, in conjunction with column selectivity.

The interfacing of normal-phase and reversed-phase systems is particularly difficult, due to the mobile phase immiscibility. The combination of normal (silica) and reversed (C18) phase HPLC in a comprehensive 2D-LC system was used for the analysis of alcohol ethoxylates [101]; the normal phase separation was run using aqueous solvents, so the mobile phases used in the two dimensions were miscible, resulting in the easy injection of the entire first-dimension effluent onto the second-dimension column. First fully comprehensive coupling of NP (Normal Phase) and RP (Reverse Phase), overcoming the difficulties related to solvents immiscibility,

was developed by Dugo et. al for the analyses of oxygen heterocyclic components of citrus essential oils [95].

1.7.1. Optimization of the LC × LC method

The complexity of LC × LC experiments regards greater attention for method development. In the experimental set-up, column dimensions and stationary phases, particle sizes, mobile phase compositions and flow-rates and second dimension injection volumes should be carefully defined in order to accomplish successful separations. The main challenges are related to the efficient coupling of columns and the preservation of mobile phase/column compatibility.

As aforementioned, the best results can be obtained when using two separation modes with totally different selectivities (orthogonal system). Through orthogonality, cross-information or synentropy existing between the two dimensions is minimized, resulting in maximum peak capacity and hence, high resolution [86,102]. Minimizing synentropy maximizes the efficiency and the information content, generating a key for complex sample analysis [100]. Of all LC × LC approaches, the combination of NP and RP is most probably the most orthogonal in nature.

1.7.1.1. Optimization of the first dimension and mobile phase incompatibility

In comprehensive LC, the selection of the mobile phases is of primary importance. The combination of two very different LC modes, can be hard since the eluent from the first separation dimension may not be compatible with the eluent of the second dimension. If a large volume of an incompatible eluent is transferred from the first to the secondary column, broadened and distorted peaks can be obtained and the solvent plug from the first chromatographic dimension can considerably alter the selectivity of the second dimension. If solvent incompatibility is unavoidable, the first dimension eluate isolation and suitable solvent changeover can be performed.

However, this can be complicated by the need of extra equipment, increased maintenance and, thus, longer sample treatment time. An alternative way is to use a microbore LC column in the first dimension. This approach offers several advantages [103,104]:

- the small column I.D. helps to ensure a minimum of dilution and provides flow rates that are compatible with the sample volume for the secondary column;
- the dead volumes of the system are minimized;
- there is no need for a preconcentration step at the head of the secondary column.

The low flow rate in the microbore column ensures sample volumes compatible with the secondary conventional column and permits the injection of a small volume onto the secondary column, making the transfer of incompatible solvents possible without peak shape deterioration or resolution losses [95,105].

The possible disadvantage could be the lower sample capacity of microbore LC columns. However, in LC × LC, a sensitivity enhancement can be obtained, if the formation of compressed solute bands at the head of the secondary column is achieved during the transfer from the first to the second dimension. In the case of a large injection volume, if the LC microcolumn is used as a highly efficient preseparation step, a limited decrease in efficiency due to a large injection volume can be tolerated.

Other drawbacks which may occur, are system peaks which originate from the solvent incompatibility, hence reducing the available chromatographic separation space in the second dimension, and the appearance of a solvent bump, due to the poor mixing of the solvents in the sample loop [105]. These factors can be accounted

for if a blank run is performed and the 2D contour plot can be constructed after background subtraction.

1.7.1.2. Peak focusing at the head of the secondary column

Distortion and multiplication of peaks may occur when an injected sample is dissolved in a solvent which is significantly stronger than the mobile phase [106]. The mobile phase used in the 1st D NP-LC separation is always stronger than then mobile phase at the head of the RP-secondary column and, therefore, peak distortion may occur. It is important that the solvents enable the trapping of analytes at the head of the 2nd D column. This can be achieved by selecting an "*weak*" first dimension solvent, or by initializing the 2nd D separation with a solvent of low strength. In this way the analytes in the 2nd column will be compressed in a narrow band.

1.7.1.3. Optimization of the second dimension analysis time

In LC × LC separations the total analysis time is the product of the second dimension analysis time and the total number of fractions injected onto the secondary column. Hence the primary limitation to the speed of the total analysis is the time of each 2nd D separation and it is obvious that fast analysis is required in the second dimension. When optimizing a 2D separation, it is fundamental that the sample fraction from the 1stD must be eluted before the next aliquot is injected onto the 2nd D column, thus avoiding wrap-around (peaks with retention times that exceed the modulation period); hence the sampling frequency depends on the retention time range of most and least retained components in the 2nd D.

Consequently, the most retained component must elute before the least retained component in the next second dimension separation. This may be difficult to attain when the differences in polarity and hydrophobicity of the components present in

the matrix are very large. In this case, isocratic conditions for rapid separations are insufficient and gradient elution should be preferred for 2nd D separations.

Murphy et al. [92] have demonstrated how changing the number of fractions collected across the width of a 1st D peak can affect resolution in two-dimensional separations, concluding that at least 3 (when the sampling is in phase) or 4 (if the sampling is maximally out of phase) fractions must be taken across the width of each 1st D peak, to avoid serious loss of information in the 2D separation due to under-sampling of 1st D peaks. This consideration further emphasizes the need for very fast second dimension separations in 2DLC. Moreover, short analysis times may be necessary if biological or other unstable samples degrade or change with time.

The high permeability and good mass-transfer characteristics of monolithic stationary phases make them ideally suited for use in the second dimension. In fact, the use of monolithic columns permits to work at high flow rates without loss of resolution, thus reducing the 2nd D analysis time. Moreover, successive gradient cycles with a very brief equilibration time can be performed. On the other hand, conventional short columns packed with 3.5 μm particles offer quite a good alternative: their efficiency versus velocity plots are flat and they can be operated at high flow rates.

Another possibility to speed up the 2D analysis time is to use high temperatures, as described by Stoll and co-workers [107]: the decreased viscosity of the eluent allowed a much higher linear velocity.

Sensitivity and dilution factors are important issues which must be considered from the point of view of analyte detectability. When more than one separation dimensions is utilized, a larger overall dilution of the original injected sample will occur. This has to be considered and samples with higher concentrations have to be injected into the 2D system.

1.8. References

[1] F.L. Schanbacher, R.S. Talhouk, F.A. Murray, L.I. Gherman, L.B. Willet, Milk-born bioactive peptides, *International Dairy Journal* 8 (**1998**) 393-403. http://www.sciencedirect.com/science/article/pii/S0958694698000624

[2] G.W. Smithers, F.J. Ballard, A.D. Copeland, K.J. De Silva, D.A. Dionysius, G.L. Francis, C. Goddard, P.A. Grieve, G.H. McIntosh, I.R. Mitchell, R.J. Pearce, G.O. Regester, New opportunities from the isolation and utilization of whey proteins, *Journal of Dairy Science* 79 (**1996**) 1454-1459. http://www.sciencedirect.com/science/article/pii/S0022030296765049

[3] R.G. Jensen, D.S. Newburg, Bovine milk lipids, *In Handbook of milk composition, Edited by: Jensen RG. Academic Press*, USA (**1995**) 543-557.

[4] A. Haug, A.T. Høstmark, O.M. Harstan, Bovine milk in human nutrition – a review, *Lipids in Health and Disease* 6 (**2007**) 25-41. http://www.biomedcentral.com/content/pdf/1476-511X-6-25.pdf

[5] P.M. Kris-Etherton, T.A. Pearson, Y. Wan, R.L. Hargrove, K. Moriarty, V. Fishell, T.D. Etherton, High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations, *The American Journal of Clinical Nutrition* 70 (**1999**) 1009-1015. http://www.ncbi.nlm.nih.gov/pubmed/10584045?dopt=Abstract

[6] K.W. Wahle, S.D. Heys, D. Rotondo, Conjugated linoleic acids: are they beneficial or detrimental to health?, *Progress in Lipid Research* 43 (**2004**) 553-587. http://www.ncbi.nlm.nih.gov/pubmed/15522764?dopt=Abstract

[7] J.K. Kay, T.R. Mackle, M.J. Auldist, N.A. Thomson, D.E. Bauman, Endogenous synthesis of cis-9, trans-11 conjugated linoleic acid in dairy cows fed fresh pasture, *Journal of Dairy Scienc*e 87 (**2004**) 369-3078. http://www.ncbi.nlm.nih.gov/pubmed/14762080?dopt=Abstract

[8] X.L. Pan, T. Izumi, Variation of the ganglioside compositions of human milk. Cow's milk and infant formulas, *Early Human Development* 57 (**2000**) 25-31. http://www.ncbi.nlm.nih.gov/pubmed/10690709?dopt=Abstract

[9] H. Lindmark-Månsson, B. Akesson, Antioxidative factors in milk, *British Journal of Nutrition* 84 (**2000**) S103-S110.

http://www.ncbi.nlm.nih.gov/pubmed/11242454?dopt=Abstract

[10] P. Insel, R.E. Turner, D. Ross, Nutrition Second edition, *American dietetic association*, Jones and Bartlett, USA (**2004**).

[11] M.F. Allam, R.A. Lucane, Selenium supplementation for asthma, *Cochrane Database of Systematic Reviews* (**2004**) CD003538.
http://www.ncbi.nlm.nih.gov/pubmed/15106206?dopt=Abstract

[12] M.P. Rayman, The argument for increasing selenium intake, *Proceedings of the Nutrition Society* 61 (**2002**) 203-215.
http://www.ncbi.nlm.nih.gov/pubmed/12133202?dopt=Abstract

[13] M. Hansen, B. Sandström, B. Lönnerdal, The effect of casein phosphopeptides on zinc and calcium absorption from high phytate infant diets assessed in rat pups and Caco-2 cells, *Pediatric Research* 40 (**1996**) 547-552.
http://www.ncbi.nlm.nih.gov/pubmed/8888281?dopt=Abstract

[14] S. Kaushik, R. Wander, S. Leonard, B. German, M.G. Traber, Removal of fat from cow's milk decreases the vitamin E contents of the resulting dairy products, *Lipids* 36 (**2001**) 73-78.
http://www.ncbi.nlm.nih.gov/pubmed/11214733?dopt=Abstract

[15] K. Hayes, A. Pronczuk, D. Perlman, Vitamin E in fortified cow milk uniquely enriches human plasma lipoproteins, *American Journal of Clinical Nutrition* 74 (**2001**) 211-218.
http://www.ncbi.nlm.nih.gov/pubmed/11470723?dopt=Abstract

[16] J.P. Forman, E.B. Rimm, M.J. Stampfer, G.C. Curhan, Folate intake and the risk of incident hypertension among US women, *Journal of the American Medical Association* 293 (**2005**) 320-329.
http://www.ncbi.nlm.nih.gov/pubmed/15657325?dopt=Abstract

[17] H.M. Korhonen, A. Pihlanto-Leppala, P. Rantamaki, T. Tupasela, Impact of processing on bioactive proteins and peptides, *Trends in Food Science & Technolog*y 8 (**1998**) 307-319.
http://www.sciencedirect.com/science/article/pii/S0924224498000545

[18] D.A. Clare, H.E. Swaisgood, Bioactive milk peptides: a prospectus, *Journal of Dairy Science* 83 (**2000**) 1187-1195.
http://www.ncbi.nlm.nih.gov/pubmed/10877382?dopt=Abstract

[19] I. Lòpez-Exposito, I. Recio, Protective effect of milk peptides: antibacterial and antitumor properties, *Advances in Experimental Medicine and Biology*, 606 (**2008**) 271-293.
http://www.ncbi.nlm.nih.gov/pubmed/18183934

[20] P.W. Parodi, A role for milk proteins and their peptides in cancer prevention, *Current Pharmaceutical Design* 13 (**2007**) 813-828.
http://www.ncbi.nlm.nih.gov/pubmed/17430183

[21] J.N. de Wit, Nutritional and functional characteristics of whey proteins in food products, *Journal of Dairy Science* 81(**1998**) 597-602.
http://www.journalofdairyscience.org/article/S0022-0302%2898%2975613-9/abstract

[22] K. Brew, F.J. Castellino, T.C. Vanaman, R.L. Hill, The complete amino acid sequence of bovine alpha-lactalbumin, *Journal of Biological Chemistry* 245 (**1970**) 4570-4582.
http://www.jbc.org/content/245/17/4570

[23] G. Kontopidis, C. Holt, L. Sawyer, Invited review: beta-lactoglobulin: binding properties, structure, and function, *Journal of Dairy Science* 87 (**2004**) 785-796.
http://www.ncbi.nlm.nih.gov/pubmed/15259212

[24] D.E.W. Chatterton, G. Smithers, P. Roupas, A. Brodkorb, Bioactivity of β-lactoglobulin and α-lactalbumin-Technological implications for processing, *International Dairy Journal* 16 (**2006**) 1229-1240.
http://www.sciencedirect.com/science/article/pii/S0958694606001439

[25] J.E. Kinsella, D.M. Whitehead, Proteins in whey: chemical, physical, and functional properties, *Advances in Food and Nutrition Research* 33 (**1989**) 343-438.
http://www.ncbi.nlm.nih.gov/pubmed/2697234

[26] J. Hauer, W. Voetsh, F.A. Anderer, Identification of a mannose-acetate-specific 87-kDa receptor responsible for human NK and LAK activity, *Immunology Letters* 42 (**1994**) 7-12.
http://www.sciencedirect.com/science/article/pii/0165247894900272

[27] T. Zagulski, P. Lipinski, A. Zagulska, S. Broniek, Z. Jarzabek, Lactoferrin can protect mice against a lethal dose of Escherichia coli in experimental infection in vivo, *British Journal of Experimental Pathology* 70 (**1989**) 697-704.

http://www.ncbi.nlm.nih.gov/pubmed/2690922

[28] J. He, P. Furmanski, Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA, *Nature* 373 (**1995**) 721-724.
http://www.ncbi.nlm.nih.gov/pubmed/7854459

[29] M. Tomita, W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin, *Journal of Dairy Science* 74 (**1991**) 4137-4142.
http://www.ncbi.nlm.nih.gov/pubmed/1787185

[30] W. Bellamy, M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase, M. Tomita, Identification of the bactericidal domain of lactoferrin, *Biochimica et Biophysica Acta* 1112 (**1992**) 130-136.
http://www.ncbi.nlm.nih.gov/pubmed/1599934

[31] H. Korhonen, P. Marnila, H. Gill, Milk immunoglobulins and complement factors, *British Journal of Nutrition* 84 (**2000**) S75-S80.
http://www.ncbi.nlm.nih.gov/pubmed/11242450

[32] B. Reiter, G. Harnuly, Lactoperoxidase antibacterial system, natural occurrence, biological functions and practical applications, *Journal of Food Protection* 47 (**1984**) 724-732.
http://www.ingentaconnect.com/content/iafp/jfp/1984/00000047/00000009/art00014

[33] P. Zapico, P. Gaya, M. Nunez, M. Medina, Activity of goat's milk lactoperoxidase system on Pseudomonas fluorescens and Escherichia coli at refrigeration temperatures, *Journal of Food Protection* 58 (**1995**) 1136-1138.
http://www.ingentaconnect.com/content/iafp/jfp/1995/00000058/00000010/art00015

[34] S.V. Silva, F.X. Malcata, Caseins as source of bioactive peptides, *International Dairy Journal* 15 (**2005**) 1-15.
http://www.sciencedirect.com/science/article/pii/S0958694604001050

[35] A. Henschen, F. Lottspeich, V. Brantl, H. Teschemacher, Novel opioid peptides derived from casein (β-casomorphins). II. Structure of active components from bovine casein peptone, *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie* 360 (**1979**) 1217-1224.
http://www.ncbi.nlm.nih.gov/pubmed/511111

[36] Z. Sun, Z. Zhang, X. Wang, R. Cade, Z. Elmir, M. Fregly, Relation of β-casomorphin to apnea in sudden infant death syndrome, *Peptides* 24 (**2003**) 937-943.
http://www.ncbi.nlm.nih.gov/pubmed/12948848

[37] A.K. Tandon, G.M. Clark, G.C. Chamness, J.M. Chirgwin, W.L. McGuire, Cathepsin D and prognosis in breast cancer, *New England Journal of Medicine* 322 (**1990**) 297-302.
http://www.nejm.org/doi/full/10.1056/NEJM199002013220504

[38] A. Hatzoglou, E. Bakogeorgou, C. Hatzoglou, P.M. Martin, E. Castanas, Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line, *European Journal of Pharmacology* 310 (**1996**) 217-223.
http://www.ncbi.nlm.nih.gov/pubmed/8884220

[39] R. Berrocal, S. Chanton, M.A. Juillerat, B. Pavillard, J.C. Scherz, R. Jost, Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to the solubilization of calcium, *Journal of Dairy Research* 56 (**1989**) 335-341.
http://www.ncbi.nlm.nih.gov/pubmed/2760299

[40] A. Zawadzki, Q. Liu, Y. Wang, A. Melander, B. Jeppsson, H. Thorlacius, Verapamil inhibits L-type calcium canne mediated apoptosis in human colon cancer cells, *Diseases of the Colon and Rectum* 51 (**2008**) 1696-1702.
http://www.ncbi.nlm.nih.gov/pubmed/18575938

[41] M.R. Buchmeiser, New ways to porous monolithic materials with uniform pore size distribution, *Angewandte Chemie International Edition* 40 (**2001**) 3795-3797.
http://onlinelibrary.wiley.com/doi/10.1002/15213773%2820011015%2940:20%3C3795::AID-ANIE3795%3E3.0.CO;2-5/full

[42] E. Klodzinska, D. Moravcova, P. Jandera, B. Buszewski, Monolithic continuous beds as a new generation of stationary phase for chromatographic and electro-driven separations, *Journal of Chromatography A* 1109 (**2006**) 51-59.
http://www.sciencedirect.com/science/article/pii/S0021967305024143

[43] A.-M. Siouffi, About the C term in the van Deemter's equation of plate height in monoliths, *Journal of Chromatography A* 1126 (**2006**) 86-94.
http://www.sciencedirect.com/science/article/pii/S0021967306010119

[44] M.A. Brook, Y. Chen, K. Guo, Z. Zhang, J.D. Brennan, Sugar-modified silanes: precursors for silica monoliths, *Journal of Materials Chemistry* 14 (**2004**) 1469-1479.
http://pubs.rsc.org/en/Content/ArticleLanding/2004/JM/B401278J#!divAbstract

[45] R.E. Majors, Developments in HPLC column packing design, *LC-GC LC column technology Supplement*, April (**2006)**.
http://connection.ebscohost.com/c/articles/20473014/developments-hplc-column-packing-design

[46] N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Hirao, N. Tanaka, Chromatographic characterization of macroporous monolithic silica prepared via sol-gel process, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 31 (**2001**) 273-279.
http://www.sciencedirect.com/science/article/pii/S0927775701006422

[47] S. Xie, R.W. Allington, F. Svec, J.M.J. Frechét, Rapid reversed-phase separation of proteins and peptides using optimized 'moulded' monolithic poly(styrene–co-divinylbenzene) columns, *Journal of Chromatography A* 865 (**1999**) 169-174.
http://www.sciencedirect.com/science/article/pii/S0021967399009814

[48] P. Zöllner, A. Leitner, D. Lubola, K. Kabrera, W. Linder, Application of a Chromolith SpeedROD RP-18e HPLC column: determination of Ochratoxin A in different wines by High-Performance Liquid Chromatography-Tandem Mass Spectrometry, *Chromatographia* 52 (**2000**) 818-820.
http://download.springer.com/static/pdf/240/art%253A10.1007%252FBF02491011.pdf?auth66=1422549777_244cd35d740905f1c232e6ff3de2eeeb&ext=.pdf

[49] J.L. Liao, R. Zhang, S. Hjerten, Continuous beds for standard and micro high-performance liquid chromatography, *Journal of Chromatography A* 586 (**1991**) 21-26.
http://www.sciencedirect.com/science/article/pii/002196739180020H

[50] N. Ishizuka, H. Kobayashi, H. Minakuchi, K. Nakanishi, K. Hirao, K. Hosoya, T. Ikegami, N. Tanaka, Monolithic silica columns for high-efficiency separations by high-performance liquid chromatography, *Journal of Chromatography A* 960 (**2002**) 85-86.
http://www.ncbi.nlm.nih.gov/pubmed/12150566

[51] A. Cifuentes, P. Canalejas, A. Ortega, J.C. Diez-Masa, Treatments of fused-silica capillaries and their influence on the electrophoretic characteristics of these columns before and after coating, *Journal of Chromatography A* 823 (**1998**) 561-571.
http://www.sciencedirect.com/science/article/pii/S0021967398002957

[52] C. Yu, F. Svec, J.M. Fréchet, Towards stationary phases for chromatography on a microchip: molded porous polymer monoliths prepared in capillaries by photoinitiated in situ polymerization as separation media for electrochromatography, *Electrophoresis* 21 (**2000**) 120-127.
http://www.ncbi.nlm.nih.gov/pubmed/10634478

[53] Y. Fintschenko, W.Y. Choi, S.M. Ngola, T.J. Shepodd, Chip electrochromatography of polycyclic aromatic hydrocarbons on an acrylate-based UV-initiated porous polymer monolith, *Fresenius' Journal of Analytical Chemistry* 371 (**2001**) 174-181.
http://www.ncbi.nlm.nih.gov/pubmed/11678188

[54] D.J. Throckmorton, T.J. Shepodd, A.K. Singh, Electrochromatography in microchips: reversed-phase separation of peptides and amino acids using photopatterned rigid polymer monoliths, *Analytical Chemistry* 74 (**2002**) 784-789.
http://www.ncbi.nlm.nih.gov/pubmed/11866058

[55] V. Augustin, G. Proczek, J. Dugay, S. Descroix, M.C. Hennion, Online preconcentration using monoliths in electrochromatography capillary format and microchips, *Journal of Separation Science* 30 (**2007**) 2858-2865.
http://www.ncbi.nlm.nih.gov/pubmed/17973277

[56] G. Proczek, V. Augustin, S. Descroix, M.C. Hennion, Integrated microdevice for preconcentration and separation of a wide variety of compounds by electrochromatography, *Electrophoresis* 30 (**2009**) 515-524.
http://www.ncbi.nlm.nih.gov/pubmed/19156759

[57] V. Augustin, A. Jardy, P. Gareil, M.C. Hennion, In situ synthesis of monolithic stationary phases for electrochromatographic separations: study of polymerization conditions, *Journal of Chromatography A* 1119 (**2006**) 80-87.
http://www.ncbi.nlm.nih.gov/pubmed/16549072

[58] S. Hjertén, J.L. Liao, R.J. Zhang, High-performance liquid chromatography on continuous polymer beds, *Journal of Chromatography A* 473 (**1989**) 273-275.
http://www.sciencedirect.com/science/article/pii/S0021967300913098

[59] T.B. Tennikova, M. Bleha, F. Svec, T.V. Almazova, B.G. Belenkii, High-performance membrane chromatography of proteins, a novel method of protein separation, *Journal of Chromatography A* 555 (**1991**) 97-107.
http://www.sciencedirect.com/science/article/pii/S0021967301871703

[60] F. Svec, J.M.J. Fréchet, Continuous rods of macroporous polymer as high-performance liquid chromatography separation media, *Analytical Chemistry* 64 (**1992**) 820-822.
http://pubs.acs.org/doi/abs/10.1021/ac00031a022

[61] M.R. Buchmeiser, Polymeric monolithic materials: syntheses, properties, functionalization and applications, *Polymer* 48 (**2007**) 2187-2198.
http://www.sciencedirect.com/science/article/pii/S003238610700184X

[62] T. Umemura, Y. Ueki, K. Tsunoda, A. Katakai, M. Tamada, H. Haraguchi, Preparation and characterization of methacrylate-based semi-micro monoliths for high-throughput bioanalysis, *Analytical and Bioanalytical Chemistry* 386 (**2006**) 566-571.
http://www.ncbi.nlm.nih.gov/pubmed/16685518

[63] X. Huang, Q. Wang, H. Yan, Y. Huang, B. Huang, Octyl-type monolithic columns of 530 μm i.d. for capillary liquid chromatography, *Journal of Chromatography A* 1062 (**2005**) 183-188.
http://cat.inist.fr/?aModele=afficheN&cpsidt=16421788

[64] Z. Jiang, N.W. Smith, P.D. Ferguson, M.R. Tailor, Preparation and characterization of long alkyl chain methacrylate-based monolithic column for capillary chromatography, *Journal of Biochemical and Biophysical Methods* 70 (**2007**) 39-45.
http://www.sciencedirect.com/science/article/pii/S0165022X0600159X

[65] B. Buszewski, M. Szumski, Study of bed homogenity of methacrylate-based monolithic columns for Micro-HPLC and CEC, *Chromatographia* 60 (**2004**) S261-S267.
http://link.springer.com/article/10.1365%2Fs10337-004-0295-8

[66] Y. Ueki, T. Umemura, Y. Iwashita, T. Odake, H. Haraguchi, K. Tsunoda, Preparation of low flow-resistant methacrylate based monolithic stationary phases of different hydrophobicity and the application to rapid reversed-phase liquid chromatographic separation of alkylbenzenes at high flow rate and elevated temperature, *Journal of Chromatography A* 1106 (**2006**) 106-111.

http://www.ncbi.nlm.nih.gov/pubmed/16443455

[67] S. Eeltink, L. Geiser, F. Svec, J.M.J. Fréchet, Optimization of the porous structure and polarity of polymethacrylate-based monolithic capillary columns for the LC-MS separation of enzymatic digests, *Journal of Separation Science* 30 (**2007**) 2814-2820.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2759379/

[68] P. Hemström, A. Nordborg, K. Irgum, F. Svec, J.M.J. Fréchet, Polymer-based monolithic microcolumns for hydrophobic interaction chromatography of proteins, *Journal of Separation Science* 29 (**2006**) 25-32.
http://onlinelibrary.wiley.com/doi/10.1002/jssc.200500239/abstract;jsessionid=CE3489D8750025BA6F4BACE1ED748880.f04t03

[69] A. Svobodová, T. Křižek, J. Ŝirc, P. Ŝálek, E. Tesařová, P. Coufal, K. Ŝtulìk, Monolithic columns based on a poly(styrene-divinylbenzene-methacrylic acid) copolymer for capillary liquid chromatography of small organic molecole, *Journal of Chromatography A* 1218 (**2011**) 1544-1547.
http://ac.elscdn.com/S0021967311001130/1s2.0S0021967311001130main.pdf?_tid=3c694130a86e11e48fdd00000aab0f26&acdnat=1422615394_55f04b52f54c61597658e35dd3dfc584

[70] L.D. Bowers, S. Pedigo, Solvent strength studies on polystyrene-divinylbenzene columns, *Journal of Chromatography A* 371 (**1986**) 243-251.
http://www.sciencedirect.com/science/article/pii/S0021967301947090

[71] B. Ells, Y. Wang, F.F. Cantwell, Influence of solvent uptake and swelling by poly(styrene–divinylbenzene) column packings on sample sorption rate and band broadening in reversed-phase liquid chromatography, *Journal of Chromatography A* 835 (**1999**) 3-18.
http://www.sciencedirect.com/science/article/pii/S002196739801070X

[72] H.-Y. Huang, Y.-J. Cheng, W.-L. Liu, Y.-F. Hsu, S. Lee, Poly(divinylbenzene-alkyl methacrylate) monolithic stationary phases in capillary electrochromatography, *Journal of Chromatography A* 1217 (**2010**) 5839-5847.
http://ac.elscdn.com/S0021967310009702/1s2.0S0021967310009702main.pdf?_tid=a2f45c72a86f11e4b4b100000aab0f27&acdnat=1422615995_fcb7003b13ad32dd6bb62ffc21ca3e50

[73] A. Canto-Mirapeix, J.M. Herrero-Martìnez, C. Mongay-Fernandez, E.F. Simo-Alfonso, CEC column behaviour of butyl and lauryl methacrylate monoliths prepared in non-aqueous media, *Electrophoresis* 30 (**2009**) 607-615.
http://onlinelibrary.wiley.com/doi/10.1002/elps.200800485/full

[74] C. Viklund, F. Svec, J.M.J. Fréchet, K. Irgum, Monolithic, "molded", porous materials with high flow characteristics for separations, catalysis, or solid-phase chemistry: control of porous properties during polymerization, *Chemistry of Materials* 8 (**1996**) 744-750.
http://pubs.acs.org/doi/abs/10.1021/cm950437j

[75] E.C. Peters, F. Svec, J.M.J. Fréchet, C. Viklund, K. Irgum, Control of porous properties and surface chemistry in "Molded" porous polymer monoliths prepared by polymerization in the presence of TEMPO, *Macromolecules* 32 (**1999**) 6377-6379.
http://pubs.acs.org/doi/pdf/10.1021/ma990538t

[76] Z.D. Xu, L.M. Yang, Q.Q. Wang, Different alkyl dimethacrylate mediated stearyl methacrylate monoliths for improving separation efficiency of typical alkylbenzenes and proteins, *Journal of Chromatography A* 1216 (**2009**) 3098-3106.
http://www.sciencedirect.com/science/article/pii/S0021967309001526

[77] B.P. Santora, M.R. Gagne, K.G. Moloy, N.S. Radu, Porogen and Cross-linking effects on the surface area, pore volume distribution, and morphology of macroporous polymers obtained by bulk polymerization, *Macromolecules* 34 (**2001**) 658-661.
http://pubs.acs.org/doi/pdf/10.1021/ma0004817

[78] R.T. Kennedy, J.W. Jorgenson, Preparation and evaluation of packed capillary liquid chromatography columns with inner diameters from 20 to 50 micrometers, *Analytical Chemistry* 61 (**1989**) 1128-1135.
http://pubs.acs.org/doi/abs/10.1021/ac00185a016

[79] Y. Hirata, K. Jinno, High resolution liquid chromatography with a packed micro glass capillary column, *Journal of High Resolution Chromatography* 6 (**1983**) 196-199.
http://onlinelibrary.wiley.com/doi/10.1002/jhrc.1240060407/abstract;jsessionid=4B44172A6FD98F900284175E040BFDDD.f02t01

[80] J.S. Mellors, J.W. Jorgenson, Use of 1.5-microm porous ethyl-bridged hybrid particles as a stationary-phase support for reversed-phase ultrahigh-pressure liquid chromatography, *Analytical Chemistry* 76 (**2004**) 5441-5450.
http://www.ncbi.nlm.nih.gov/pubmed/15362905

[81] J.E. MacNair, K.D. Patel, J.W. Jorgenson, Ultrahigh pressure reversed-phase capillary liquid chromatography: Isocratic and gradient elution using columns packed with 1.0-micron particles, *Analytical Chemistry* 71 (**1999**) 700-708.
http://www.ncbi.nlm.nih.gov/pubmed/9989386

[82] T. Hara, H. Kobayashi, T. Ikegami, K. Nakanishi, N. Tanaka, Performance of monolithic silica capillary columns with increased phase ratios and small-sized domains, *Analytical Chemistry* 78 (**2006**) 7632-7642.
http://www.ncbi.nlm.nih.gov/pubmed/17105153

[83] A. Greiderer, L. Trojer, C.W. Huck, G.K. Bonn, Influence of the polymerisation time on the porous and chromatographic properties of monolithic poly(1,2-bis(p-vinylphenyl))ethane capillary columns, *Journal of Chromatography A* 1216 (**2009**) 7747-7754.
http://www.sciencedirect.com/science/article/pii/S0021967309013405

[84] Y. Li, H.D. Tolley, M.L. Lee, Preparation of monoliths from single crosslinking monomers for reversed-phase capillary chromatography of small molecules, *Journal of Chromatography A* 1218 (**2011**) 1399-1408.
http://www.sciencedirect.com/science/article/pii/S0021967311000756

[85] F. Erni, R.W. Frei, Two-dimensional column liquid chromatographic technique for resolution of complex mixtures, *Journal of Chromatography A* 149 (**1978**) 561-569.
http://www.sciencedirect.com/science/article/pii/S0021967300810110

[86] M.M. Bushey, J.W. Jorgenson, Automated instrumentation for comprehensive two-dimensional high-performance liquid chromatography of proteins, *Analytical Chemistry* 62 (**1990**) 161-167.
http://www.ncbi.nlm.nih.gov/pubmed/2310013

[87] J.C. Giddings, Two-dimensional separations: concept and promise, *Analytical Chemistry* 56 (**1984**) 1258A-1260A.
http://www.ncbi.nlm.nih.gov/pubmed/6507850

[88] P. Schoenmakers, P. Marriott, J. Beens, Nomenclature and conventions in comprehensive multidimensional chromatography, LC-GC Europe 16 (**2003**) 335-339.
https://cifea.milaulas.com/pluginfile.php/94/mod_resource/content/1/Coupling_Matters_Proof_V2_1_.pdf

[89] P.J. Slonecker, X. Li, H.T. Ridgway, J.G. Dorsey, Informational orthogonality of two-dimensional chromatographic separations, *Analytical Chemistry* 68 (**1996**) 682-689.
http://www.ncbi.nlm.nih.gov/pubmed/8999742

[90] J.C. Giddings, Sample dimensionality: a predictor of order-disorder in component peak distribution in multidimensional separation, *Journal of Chromatography A* 703 (**1995**) 3-15.
http://www.sciencedirect.com/science/article/pii/002196739500249M7

[91] Z. Liu, D.G. Patterson Jr., M.L. Lee, Geometric approach to factor analysis for the estimation of orthogonality and practical peak capacity in comprehensive two-dimensional separations, *Analytical Chemistry* 67 (**1995**) 3840-3845.
http://cat.inist.fr/?aModele=afficheN&cpsidt=3701757

[92] R.E. Murphy, M.R. Schure, J.P. Foley, Effect of sampling rate on resolution in comprehensive two-dimensional liquid chromatography, *Analytical Chemistry* 70 (**1998**) 1585-1594.
http://cat.inist.fr/?aModele=afficheN&cpsidt=2225757

[93] G.J. Opiteck, K.C. Lewis, J.W. Jorgenson, R.J. Anderegg, Comprehensive on-line LC/LC/MS of proteins, *Analytical Chemistry* 69 (**1997**) 1518-1524.
http://www.ncbi.nlm.nih.gov/pubmed/9109352

[94] A. van der Horst, P.J. Schoenmakers, Comprehensive two-dimensional liquid chromatography of polymers, *Journal of Chromatography A* 1000 (**2003**) 693-709.
http://www.sciencedirect.com/science/article/pii/S0021967303004953

[95] P. Dugo, O. Favoino, R. Luppino, G. Dugo, L. Mondello, Comprehensive two-dimensional normal-phase (adsorption)-reversed-phase liquid chromatography, *Analytical Chemistry* 76 (**2004**) 2525-2530.
http://europepmc.org/abstract/MED/15117193

[96] F. Cacciola, P. Jandera, E. Blahova, L. Mondello, Two-dimensional and serial column reversed-phase separation of phenolic antioxidants on octadecyl-,

polyethyleneglycol-, and pentafluorophenylpropyl-silica columns, *Journal of Separation Science* 29 (**2006**) 555-566.
http://www.ncbi.nlm.nih.gov/pubmed/16583694

[97] G.J. Opiteck, J.W. Jorgenson, R.J. Anderegg, Two-dimensional SEC/RPLC coupled to mass spectrometry for the analysis of peptides, *Analytical Chemistry* 69 (**1997**) 2283-2291.
http://www.ncbi.nlm.nih.gov/pubmed/9212702

[98] K. Wagner, K. Racaityte, K.K. Unger, T. Miliotis, L.E. Edholm, R. Bischoff, G. Marko-Varga, Protein mapping by two-dimensional high performance liquid chromatography, *Journal of Chromatography A* 893 (**2000**) 293-305.
http://www.sciencedirect.com/science/article/pii/S0021967300007366

[99] K.K. Unger, K. Racaityte, K. Wagner, T. Miliotis, L.E. Edholm, R. Bischoff, G. Marko-Varga, Is multidimensional high performance liquid chromatography (HPLC) an alternative in protein analysis to 2D gel electrophoresis?, *Journal of High Resolution Chromatography* 23 (**2000**) 259-265.
http://onlinelibrary.wiley.com/doi/10.1002/%28SICI%2915214168%2820000301%2923:3%3C259::AID-JHRC259%3E3.0.CO;2-V/pdf

[100] C.J. Venkatramani, Y. Zelechonok, An automated orthogonal two-dimensional liquid chromatograph, *Analytical Chemistry* 75 (**2003**) 3484-3494.
http://www.ncbi.nlm.nih.gov/pubmed/14570201

[101] R.E. Murphy, M.R. Schure, J.P. Foley, One-and two-dimensional chromatographic analysis of alcohol ethoxylates, *Analytical Chemistry* 70 (**1998**) 4353-4360.
http://cat.inist.fr/?aModele=afficheN&cpsidt=2437706

[102] C.J. Venkatramani, X. Jingzheng, J.B. Phillips, Separation orthogonality in temperature-programmed comprehensive two-dimensional gas chromatography, *Analytical Chemistry* 68 (**1996**) 1486-1492.
http://www.ncbi.nlm.nih.gov/pubmed/21619112

[103] A.P. Köhne, T. Welsch, Coupling of a microbore column with a column packed with non-porous particles for fast comprehensive two-dimensional high-performance liquid chromatography, *Journal of Chromatography A* 845 (**1999**) 463-469.
http://www.sciencedirect.com/science/article/pii/S002196739900206X

[104] H.J. Cortes, Developments in multidimensional separation systems, *Journal of Chromatography A* 626 (**1992**) 3-23.
http://www.sciencedirect.com/science/article/pii/002196739285324M

[105] I. François, A. de Villiers, P. Sandra, Considerations on the possibilities and limitations of comprehensive normal phase-reversed phase liquid chromatography, *Journal of Separation Science* 29 (**2006)** 492-498.
http://www.ncbi.nlm.nih.gov/pubmed/16583687

[106] N.E. Hoffman, S.-L. Pan, A.M. Rustum, Injection of eluites in solvents stronger than the mobile phase in reversed-phase liquid chromatography, *Journal of Chromatography A* 465 (**1989**) 189-200.
http://www.sciencedirect.com/science/article/pii/S0021967301926573

[107] D.R. Stoll, J.D. Cohen, Fast, comprehensive online two-dimensional high performance liquid chromatography through the use of high temperature ultra-fast gradient elution reversed-phase liquid chromatography, *Journal of Chromatography A* 1122 (**2006**) 123-137.
http://www.sciencedirect.com/science/article/pii/S0021967306008521

CHAPTER II

Susceptibility to denaturation of caseins in milk samples for improving protein conformational study and their identification

Abstract

Caseins are phosphoproteins kept together by non-covalent interactions to form a highly stabilized dispersion in milk. This study intended to evaluate the different effects of denaturing solvents and solutions on caseins in order to optimize the chromatographic resolution for a better identification of individual casein fractions. The caseins were obtained from bovine skimmed milks by precipitation at pH 4.3, and the proteins were dissolved in water and three different solutions. The casein separation was performed by reversed-phase high-performance liquid chromatography. Each casein was identified by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. The best result was achieved by treating the caseins with solution D.

Keywords

Bovine milk, caseins, denaturation, chromatography, mass spectrometry.

Abbreviations

Centrale del Latte di Salerno, CdLdS; High-Performance Liquid Chromatography, HPLC; Matrix-Assisted Laser Desorption Ionization-Time of Flight, MALDI-TOF; Trifluoroacetic Acid, TFA.

2.1. Introduction

Caseins (α_{S1}, α_{S2}, β, and κ) are the predominant phosphoproteins in the milk of ruminants, accounting for about 80% of total protein, and play an important nutritional role since they are rich in essential amino acids. The heterogeneity of these proteins depends on multiple factors, mainly genetics, environment and breeding strategies, which represent the most prominent sources of variability in the milk protein content [1-3]. The quantitative relationships between caseins and the sources of their variability represent an issue of crucial importance in the dairy industry as the natural variations in milk protein concentration can markedly affect the yield of the cheesemaking processes, thus causing a direct and significant economic impact on the producers. A wide number of methods have been developed to identify and quantify milk proteins with special regard to caseins; many of these methods rely on spectroscopic [4,5] and chromatographic techniques [6,7]. The latter, in particular, provide high accuracy and precision, allow isolation of the single casein fractions [7-10], and are more commonly coupled with other techniques such as mass spectrometry for finer analysis [11]. A major restrain limiting the wide application of chromatography to real-time process control, however, is constituted by the sample preparation steps, usually represented by pre-fractionation procedures like acid precipitation or solvent extraction, which affect the result reliability and are quite time-consuming [12].

Caseins are phosphate-containing proteins that occur as micelles in the native form which are held together by non-covalent interactions and appear as a highly stabilized dispersion in milk [13].

Calcium also plays a important role in the formation of micelles. Calcium is associated with the micelle as a combination of the micellar calcium (directly bound to caseins *via* their phosphorylated residues) and colloidal calcium (associated with the micelle but not directly bound to the casein polypeptide) [14].

There are several evidences about the role of the carboxyl groups of glutamate and aspartate residues at phosphate clusters as additional binding sites for calcium ions, since measured calcium concentration is usually higher than predicted form the number of phosphate groups within a molecule [15]. Holt delineated the casein micelle as a tangled web of flexible casein networks forming a gel-like structure with micro-granules of colloidal calcium phosphate through the casein phosphate center, and the C-terminal region of κ-casein extends to form a hairy layer. The two main features of this model are the cementing role of colloidal calcium phosphate and the surface location of hairy layer of κ-casein. Numerous models for the structure of casein micelles have been proposed in the past three decades. Recently the dual bonding model of Horne [16], which fits into the category of internal structure models, was proposed. This model suggests that the proteins in casein micelles are bound together by a balanced contribution of the attractive hydrophobic interactions and the electrostatic repulsions. Hydrophobic interaction is the driving force for the formation of casein micelles, while electrostatic repulsions are limiting the growth of polymers or, in other words, defining the degree of polymerization. The conformation of α_{S1}- and β-caseins when they are adsorbed at hydrophobic interfaces form a train-loop-train and a tail-train structure respectively and both caseins polymerize or self-associate, by hydrophobic interactions, as shown in Figure 2.1.A. Accordingly, the self-association of caseins makes it possible for polymerization to occur. Calcium phosphate nanoclusters, or CCP, are considered to be one of the linkages between casein micelles and neutralizing agents of the negative charge of the phosphoserine residues by binding to those residues; consequently, electrostatic repulsion is reduced, and the hydrophobic interaction between caseins is still dominant, resulting in increased proteins associations [17]. Unlike other caseins, κ-caseins can only interact hydrophobically and acts as a propagation terminator, due to lack of phosphoserine

cluster, for calcium binding, and of hydrophobic region, for chain eongation. The dual bonding model for the casein micelle structure is shown in Figure 2.1.B.

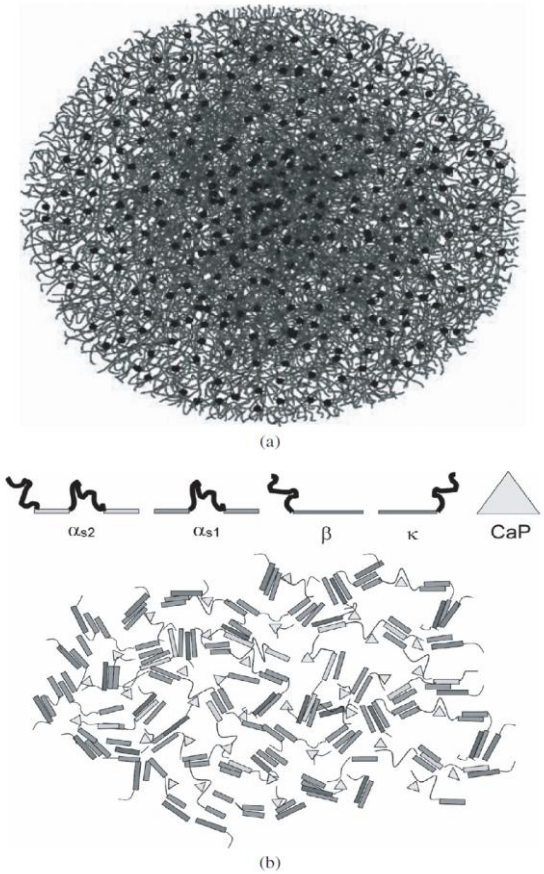


Figure 2.1. *(a) The nanocluster model for casein micelles. Casein monomers are thread-like, while the dark circles represent calcium phosphate nanoclusters. (b) The model of Horne – dual bonding model of casein micelles using casein monomers as indicated. Protein-protein interactions occur between hydrophobic regions (rectangular bars) while the protein hydrophilic regions (loops) bind to calcium phosphate clusters (triangles).*

2.1.1. Aim of work

The main goal of this work was to study the different effects of denaturing solvents and solutions on the structural conformation of caseins in order to optimize the chromatographic resolution for a better identification of its individual fractions.

2.2. Materials and methods

2.2.1. Reagents and standards

All chemicals and reagents were analytical-reagent or HPLC grade. Purified α_S- (85%), β- (90%) and κ- (80%) casein fractions, 3,5-dimethoxy-4-hydroxycinnamic acid (99%), water, acetonitrile, ethanol, acetone, sodium citrate and 2-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris-HCl buffer was purchased from Lonza (Rockland, ME, USA). Acetic acid (glacial) was purchased from Carlo Erba (Milano, Italy). Trifluoroacetic acid was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Urea was purchased from AppliChem GmbH (Darmstadt, Germany).

2.2.2. Sampling and sample preparation

Commercial samples of whole bovine milk from Centrale del Latte di Salerno (Salerno, Campania, Italy) were analyzed; samples were preserved frozen at -20 °C until analysis. Prior to analysis, samples were thawed overnight at room temperature: skimmed milks were prepared by separating the fat from whole milks by centrifugation at 4000 × *g* for 15 min at 4 °C. The caseins were obtained from skimmed milks by precipitation at pH 4.3 (20 °C) by the addition of acetic acid (10% *v/v*). The acidified milks were centrifuged for 15 min at 3000 × *g* (20 °C) to recover precipitated caseins. Caseins were dispersed in ethanol, precipitated again and centrifuged for 10 min at 3000 × *g* (20 °C). In order to eliminate the residual fat, caseins were washed with acetone and left to dry in a fume hood at room

temperature. The powder thus obtained was dissolved in a mixture of water and acetonitrile (70:30 *v/v*). The solution was filtered through a 0.45 µm pore cellulose membrane; subsequently, a 10 mL aliquot of the solution was lyophilized for 24 h. Finally, the residue of casein powder was stored at 4 °C until the beginning of the analysis [8].

A 10 mg aliquot of purified proteins was dissolved in: water (solution A); 8 M urea in water/acetonitrile (70:30 *v/v*) (solution B); 0.3% (*v/v*) β-mercaptoethanol in water/acetonitrile (70:30 *v/v*) (solution C); 8 M urea in 165 mM Tris-HCl, 44 mM sodium citrate and 0.3% β-mercaptoethanol (solution D). Prior to the addition of caseins, solutions B, C and D were incubated for one hour at room temperature. Then, caseins were dissolved until there was no noticeable foaming production.

2.2.3. HPLC separation

The chromatographic system used to perform the analyses consisted of a LC-20 AT pump (Shimadzu, Milan, Italy), a manual injector model P/N 7725i (Rheodyne, Cotati, CA, USA), equipped with a 20 µL loop, and a model UV/VIS SPD-20A detector; it was operated by means of the LC real Time Analysis Software (Shimadzu). The chromatographic separation was performed in reversed-phase mode [18] using a Vydac C4 column (250 × 4.6 mm, L. × I.D., 300 Å-sized pores, 5 µm-sized particles; Dionex, Mechelen, Belgium), kept at room temperature; the detection wavelength was 220 nm. The analyses were carried out applying a binary gradient profile to the mobile phase composition. Eluent A was HPLC-grade water containing 0.1% (*v/v*) TFA and eluent B was HPLC-grade acetonitrile containing 0.1% (*v/v*) TFA. The gradient elution program was run at a constant flow rate of 0.7 mL/min and was set as follows: 0–40 min linear gradient from 30% B to 50% B; 40–42 min linear gradient from 50% B to 100% B; 42–43 min isocratic elution 100% B; 43–46 min linear gradient from100% B to 30% B, followed by a 5 min isocratic elution at the initial conditions. For each analysis a 20 µL aliquot of each

standard and sample solution was injected. The total duration of a single run, including column re-equilibration, was 51 min.

2.2.4. Far-UV CD

Far-UV CD spectra were obtained by a Jasco J-810 polarimeter using 4.2 M casein solution. Spectra were recorded in the 190–260 nm range, using a cylindrical quartz cuvette of path length 1 mm. The scan parameters were: wavelength interval, 190–260 nm; scan rate, 50 nm/min; step size, 0.1 nm; bandwidth, 1 nm; averaging time, 2s. Spectra shown are averages of 4 consecutive scans, corrected by subtracting the baseline scan of the appropriate buffer and subjected to noise reduction.

2.2.5. Identification of caseins by MALDI-TOF-MS

A mixture of analyte and matrix solution [10 mg/mL 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in ACN:H_2O 1:1 (*v/v*), containing 0.1% (*v/v*) TFA] was applied to the sample plate and dried at room temperature. Experiments were performed using a MALDI-MX micro (Waters, Milford Massachusetts, USA). Positively charged ions were analyzed in linear mode, using delayed extraction. Typically, 100 shots were averaged to improve the signal-to-noise ratio. Mass spectra were acquired over the m/z 3000–40000 range by shooting the laser at random positions on the target spot. Data were acquired and processed using Masslynx software (Waters-Micromass). Horse heart myoglobin was used to calibrate the instrument (average molecular mass 16,951.5 Da); all masses are reported as average masses.

2.3. Results and discussion

2.3.1. HPLC separation of purified casein fractions

Casein fractions (α_{S1}, α_{S2}, β, and κ) were identified by comparison of their chromatographic retention times with those of commercial standards and data from other studies [18] (Figure 2.2.A.). As shown in Figure 2.2.B., the presence of urea did not led to a significant improvement of casein chromatographic profile: while resolution of fraction I was improved, the peak of fraction IV was not anymore detectable. Figure 2.2.C. shows the influence of β-mercaptoethanol in the separation of bovine caseins. An increase in resolution was obtained for fraction I; interestingly, fraction IV was well resolved again. However, the best chromatographic separation was achieved with solution D, an additional group of peaks (7-10) was evidenced, which could be attributed to the complete separation of caseins, with significant improvement of fractions III and IV resolution (Figure 2.2.D.). This chromatogram showed a good similarity with that obtained by Bordin et al. [7], thus enabling a fairly straightforward confirmation of peak identity. These patterns clearly demonstrated the success of the denaturing treatment. Such method of preparation, which provides the use of 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol, revealed great repeatability and reproducibility (Table 2.1.).

Table 2.1. *Precision (repeatability and reproducibility of analysis method) of peak retention times of caseins from whole bovine milk samples of CdLdS dissolved in denaturing solution consisting of 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol.*

Peak	Repeatability[¥]	Reproducibility	
	Retention time RSD (%)	*Retention time RSD[*] (%)*	*Retention time RSD[#] (%)*
1	0.30	0.29	0.19
2	0.44	0.26	0.09
3	0.33	0.16	0.08
4	0.38	0.23	0.15
5	0.28	0.21	0.21
6	0.22	0.11	0.16
7	0.25	0.10	0.10
8	0.08	0.04	0.20
9	0.33	0.16	0.08
10	0.22	0.11	0.16

*Solution D: 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol; RSD, relative standard deviation (%); [¥]four aliquots of precipitated bovine casein; *four aliquots of the same sample analyzed after 4 days; [#]sample prepared ex novo and analyzed after 5 days.*

A similar pre-treatment of casein fractions with the same denaturing solution was previously performed by Bonizzi et al. [19] on the whole milk sample. For the first time in this work the incubation of sample with the denaturing solution was performed directly on the caseins previously extracted from the organic matrix. This treatment allowed the separation of different casein sub-fractions and the identification of each component of casein portion. The main difference from the

method proposed by Bonizzi was the complete resolution of fraction III, characterized by the presence of four peaks corresponding to the variants of β-CN, which differ in few amino acid residues [20]. The improvement of the chromatographic profile is probably due to the direct interaction of casein powder with urea and β-mercaptoethanol. This pre-treatment of caseins allows a separation similar to that obtained in a study by Bordin et al. [7], in which guanidine-HCl, as denaturing reagent, and dithiothreitol, as reducing agent, were used.

2.3.2. Far-UV CD data of caseins

Table 2.2. shows the far-UV CD data of solutions A, B, C and D. In water caseins assumed a predominant β-sheet conformation while the presence of urea led to an increase of both α-helix and random coil conformations. This conformational change is caused by the increase in solubility of hydrophobic amino acids, making proteins unfold and altering their three-dimensional structure [21,22]. Infact, there are two possible mechanisms of action of urea: an "indirect mechanism" in which urea is presumed to disrupt the structure of water, thus making hydrophobic groups more readily solvated [23-26]; a "direct mechanism" in which urea interacts either directly with the protein backbone, via hydrogen bonds and other electrostatic interactions, or directly with the amino acids through more favorable van der Waals attractions as compared with water [27,28]; or both, thus causing the protein to swell, and then denature. Although urea caused an evident conformational change in caseins, no significant improvement of protein chromatographic profile was revealed. Differently, β-mercaptoethanol seemed to decrease the rate of β-sheet and α-helix conformations, promoting the random coil conformation of caseins. This conformational variation is caused by the reduction of casein disulfide bonds: infact, β-mercaptoethanol is capable of maintaining monothiols completely in the reduced state and of reducing disulfides quantitatively [29], causing an important improvement of protein chromatographic profile. In conclusion, as observed

previously, β-mercaptoethanol, a disulfide reducing agent, covalently interacts with specific protein functional groups. Urea, acting through generalized solvent changes or non-specific interactions with the protein, can alter protein folding. Two different reagents in combination, urea and β-mercaptoethanol, were used to unfold the caseins. The CD-spectrum of caseins dissolved in this denaturing solution, showed a significant increase in the α-helix conformation and a decrease in the rate of β-sheet, while random coil conformation remained unchanged. This was caused by disruption of casein hydrogen bonds by urea and by the reduction of casein disulfide bonds by β-mercaptoethanol.

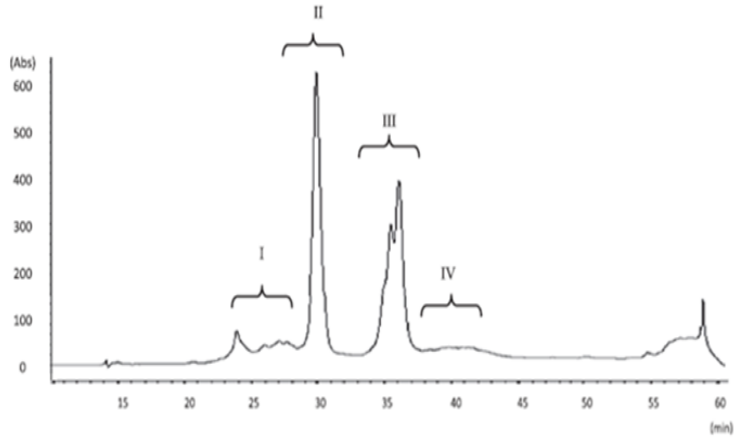
Table 2.2. *Relative amounts[¥] of secondary structures present in casein different solutions.*

Caseins	**Helix 1**	**Helix 2**	**Strand 1**	**Strand 2**	**Turns**	**Unordered**
Solution A	0.00	25.25	6.80	9.00	41.30	17.70
Solution B	20.15	20.20	0.00	6.90	25.75	26.95
Solution C	10.95	14.50	2.85	6.30	28.85	36.50
Solution D	39.70	26.25	0.35	0.90	17.20	15.60

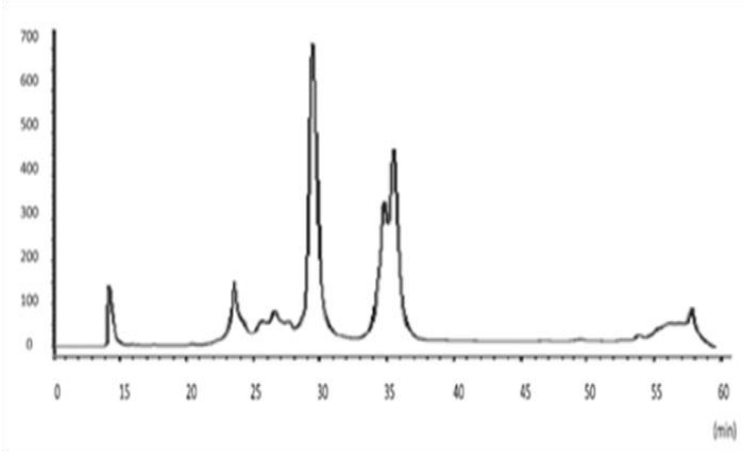
[¥]*The values of structural conformation are expressed in %.*

Solution A: water; Solution B: 8M urea and water/acetonitrile (70:30); Solution C: 0.3% β-mercaptoethanol, water/acetonitrile (70:30); Solution D: 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol.

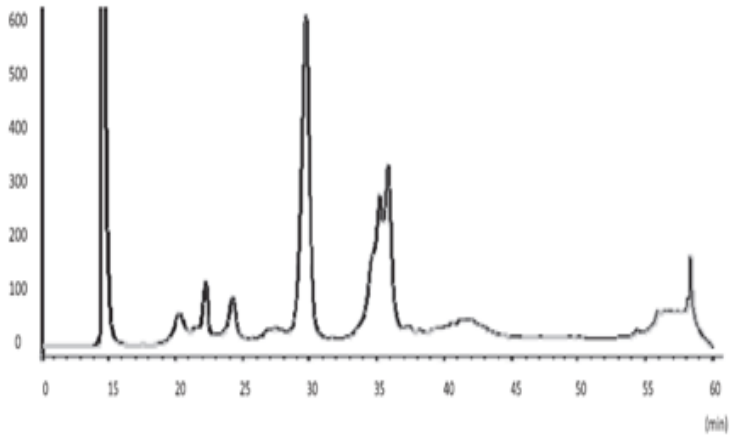
A)


II
III
I
IV
(Abs)
600
500
400
300
200
100
0
15
20
25
30
35
40
45
50
55
60
(min)

B)


700
600
500
400
300
200
100
0
0
15
20
25
30
35
40
45
50
55
60
(min)

C)


600
500
400
300
200
100
0
0
15
20
25
30
35
40
45
50
55
60
(min)

D)

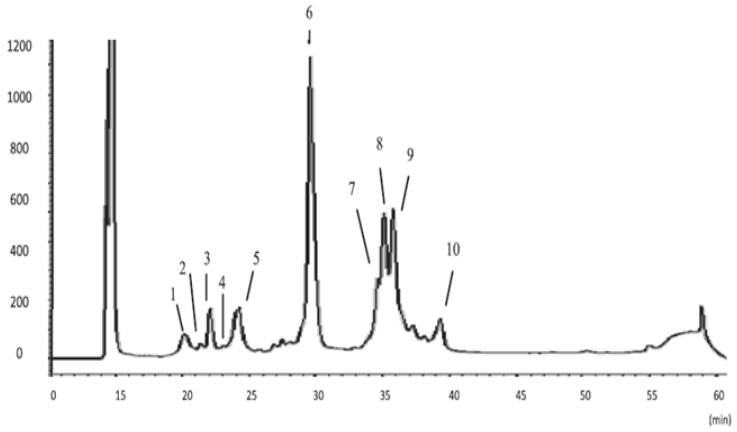


Figure 2.2. *HPLC chromatograms of casein fractions dissolved in: (A) water; (B) solution of 8M urea and water/acetonitrile (70:30); (C) solution of 0.3% β-mercaptoethanol, water/acetonitrile (70:30); and (D) solution of 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol. I, κ-casein; II, α_{S2}-casein; III, α_{S1}-casein; and IV, β-casein. Peaks 1–4, κ-casein in different states of glycosylation; peaks 5 and 6: α_{S2}- and α_{S1}-casein, respectively; and peaks 7–10, β-casein variants (B, A1, A2, A3, respectively).*

2.3.3. Identification of casein fraction variants

The HPLC peaks (1-10) arising from solution D were identified by comparing chromatographic and MALDI-TOF MS data with those reported in previous works [14] (Table 2.3.). Our results allowed the identification of peaks 1-4 with k-casein in different states of glycosylation. Peaks 5 and 6 could be assigned to α_{S2}- and α_{S1}-casein, respectively. Peaks 7-10 were attributed to the separation of β-casein variants (B, A1, A2, A3) [20].

Table 2.3. *MALDI-TOF-MS data of casein fraction variants.*

Molecular Mass of caseins [#]				
Peak	**k-CN**	**α_{s2}-CN**	**α_{s1}-CN**	**β-CN**
1	20,559			
2	19,441			
3	19,040			
4	19,189			
5		26,074		
6			23,615	
7				23,982
8				23,994
9				23,143
10				23,250

[#] *Values are the mean of molecular mass expressed in dalton*

2.4.Conclusions

Pinpointing the composition of the casein fractions is of pivotal importance to determine the specific biochemical and biological properties and possible dietary and dairy applications of milk. The aim of this study was to evaluate the denaturing effects of different solvents and solutions for improving casein protein purification and identification. The best chromatographic separation was achieved with the solution of 8M urea, 165mM Tris-HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol, allowing the identification and quantification of its individual fractions.

2.5. References

[1] E. Jakob, Z. Puhan, Technological properties of milk as influenced by genetic polymorphism of milk proteins: a review, *International Dairy Journal* 2 (**1992**) 157-178.
http://www.sciencedirect.com/science/article/pii/095869469290014D

[2] A. Summer, P. Franceschi, A. Bollini, P. Formaggioni, F. Tosi, P. Mariani, Seasonal variations of milk characteristics and cheesemaking losses in the manufacture of Parmigiano-Reggiano cheese, *Veterinary Research Communications* 27 (**2003**) 663-666.
http://link.springer.com/article/10.1023%2FB%3AVERC.0000014242.10523.81

[3] A.T.M. Van Knegsel, H. Van den Brand, J. Dijkstra, S. Tamminga, B. Kemp, Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle, *Reproduction Nutrition Development* 45 (**2005**) 665-668.
http://www.ncbi.nlm.nih.gov/pubmed/16285910

[4] M.F. Laporte, P. Paquin, Near-infrared analysis of fat, protein, and casein in cow's milk, *Journal of Agricultural and Food Chemistry* 47 (**1999**) 2600-2605.
http://pubs.acs.org/doi/abs/10.1021/jf980929r

[5] J. Belloque, M. Ramos, Determination of the casein content in bovine milk by 31P-NMR, *Journal of Dairy Research* 69 (**2002**) 411-418.
http://www.ncbi.nlm.nih.gov/pubmed/12369412

[6] I.M.P.L.V.O. Ferriera, E. Mendes, M.A. Ferriera, HPLC/UV analysis of proteins in dairy products using a hydrophobic interaction chromatographic column, *Analytical sciences* 17 (**2001**) 499-501.
http://www.ncbi.nlm.nih.gov/pubmed/11990565

[7] G. Bordin, F.C. Raposo, B. de la Calle, A.R. Rodriguez, Identification and quantification of major bovine milk proteins by liquid chromatography, *Journal of Chromatography A* 928 (**2001**) 63-76.
http://www.sciencedirect.com/science/article/pii/S0021967301010974

[8] A.C.A. Veloso, N. Teixeira, I.M.P.L.V.O. Ferriera, Separation and quantification of the major casein fractions by reverse-phase high-performance

liquid chromatography and urea–polyacrylamide gel electrophoresis: Detection of milk adulterations, *Journal of Chromatography A* 967 (**2002**) 209-218.
http://www.sciencedirect.com/science/article/pii/S0021967302007872

[9] F. Leiber, D. Nigg, C. Kunz, M.R.L. Scheeder, H.R. Wettstein, M. Kreuzer, Protein composition, plasmin activity and cheesemaking properties of cows' milk produced at two altitudes from hay of lowland and high-alpine origins, *Journal of Dairy Research* 72 (**2005**) 65-74.
http://www.ncbi.nlm.nih.gov/pubmed/15747733

[10] V. Bonfatti, L. Grigoletto, A. Cecchinato, L. Gallo, P. Carnier, Validation of a new reversed-phase high-performance liquid chromatography method for separation and quantification of bovine milk protein genetic variants, *Journal of Chromatography A* 1195 (**2008**) 101-106.
http://www.sciencedirect.com/science/article/pii/S0021967308007930

[11] M. Careri, A. Mangia, Analysis of food proteins and peptides by chromatography and mass spectrometry, *Journal of Chromatography A* 1000 (**2003**) 609-635.
http://www.sciencedirect.com/science/article/pii/S0021967303002371

[12] R. Karoui, J. De Baerdemaeker, A review of the analytical methods coupled with chemometric tools for the determination of the quality and identity of dairy products, *Food Chemistry* 102 (**2007**) 621-640.
http://www.sciencedirect.com/science/article/pii/S0308814606004353

[13] S.F. Ausar, I.D. Bianco, R.G. Badini, L.F. Castagna, N.M. Modesti, C.A. Landa, D.M. Beltramo, Characterization of casein micelle precipitation by chitosans, *Journal of Dairy Science* 84 (**2001**) 361-369.
http://www.ncbi.nlm.nih.gov/pubmed/11233020

[14] H.M. Farrell Jr., R. Jimenez-Flores, G.T. Bleck, E.M. Brown, J.E. Butler, L.K. Creamer, C.M. Hollar, K.F. Ng-Kwai-Hang, H.E. Swaisgood, Nomenclature of the proteins of cows' milk-sixth revision, *Journal of Dairy Science* 87 (**2004**) 1641-1674.
http://www.ncbi.nlm.nih.gov/pubmed/15453478

[15] C. Holt, Structure and stability of bovine casein micelles, *Advances in Protein Chemistry* 43 (**1992**) 63-151.
http://www.ncbi.nlm.nih.gov/pubmed/1442324

[16] D.S. Horne, Casein interactions: casting light on the black boxes, the structure in dairy products, *International Dairy Journal* 8 (**1998**) 171-177.
http://www.sciencedirect.com/science/article/pii/S0958694698000405

[17] D.S. Horne, Casein structure, self-assembly and gelation, *Current Opinion in Colloid & Interface Science* 7 (**2002**) 456-461.
http://www.sciencedirect.com/science/article/pii/S1359029402000821

[18] S. Visser, C.J. Slangen, H.S. Rollema, Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography, *Journal of Chromatography* 548 (**1991**) 361-370.
http://www.ncbi.nlm.nih.gov/pubmed/1939434

[19] I. Bonizzi, J.N. Buffoni, M. Feligini, Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context, *Journal of Chromatography A* 1216 (**2009**) 165-168.
http://www.sciencedirect.com/science/article/pii/S0021967308020268

[20] F. Grosclaude, M.F. Mahe, J.C. Mercier, B. Ribadeau-Dumas, Structure primaire de la caséine α_{S1} et de la caséine β bovines, *European Journal of Biochemistry* 40 (**1973**) 323-324.
http://onlinelibrary.wiley.com/doi/10.1111/j.14321033.1973.tb03199.x/abstract;jsessionid=FCD9DD07A89E86ACFD2D1D48D5D7D886.f01t01

[21] B. Bennion, V. Daggett, The molecular basis for the chemical denaturation of proteins by urea, *Proceedings of the National Academy of Sciences* 100 (**2003**) 5142-5147.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC154312/

[22] R. Dwek, Z. Luz, M. Shores, Opposite effect of urea and some of its derivatives on water structure, *The Journal of Physical Chemistry* 74 (**1970**) 2230-2232.
http://pubs.acs.org/doi/abs/10.1021/j100909a037

[23] H. Frank, F. Franks, Structural approach to the solvent power of water for hydrocarbons; urea as a structure breaker, *The Journal of Chemical Physics* 48 (**1968**) 4746-4757.
http://scitation.aip.org/content/aip/journal/jcp/48/10/10.1063/1.1668057

[24] E. Finer, F. Franks, M. Tait, Nuclear magnetic resonance studies of aqueous urea solutions, *Journal of the American Chemical Society* 94 (**1972**) 4424-4429.
http://pubs.acs.org/doi/abs/10.1021/ja00768a004

[25] G.G. Hammes, P.R. Schimmel, Water-urea and water-urea-polyethylene glycol interactions, *Journal of the American Chemical Society* 89 (**1967**) 442-446. http://pubs.acs.org/doi/abs/10.1021/ja00978a050

[26] D. Wetlaufer, S. Malik, L. Stoller, R. Coffin, Nonpolar group participation in the denaturation of proteins by urea and guanidinium salts. Model compound studies, *Journal of the American Chemical Society* 86 (**1964**) 508-514. http://pubs.acs.org/doi/abs/10.1021/ja01057a045

[27] D. Robinson, W. Jencks, Effect of compounds of urea-guanidinium class on activity coefficient of acetyltetraglycine ethyl ester and related compounds, *Journal of the American Chemical Society* 87 (**1965**) 2462-2469. http://pubs.acs.org/doi/pdf/10.1021/ja01089a028

[28] A. Wallqvist, D.G. Covell, D. Thirumalai, Hydrophobic interactions in aqueous urea solutions with implications for the mechanism of protein denaturation, *Journal of the American Chemical Society* 120 (**1998**) 427-428. http://pubs.acs.org/doi/pdf/10.1021/ja972053v

[29] A. Beis, A. Lazou, Removal of artifactual bands associated with the presence of 2-mercaptoethanol in two-dimensional polyacrylamide gel electrophoresis, *Analytical Biochemistry* 190 (**1990**) 57-59. http://www.ncbi.nlm.nih.gov/pubmed/2285145

CHAPTER III

Analysis of bovine milk caseins on organic monolithic columns: an integrated capillary liquid chromatography–high resolution mass spectrometry approach for the study of time-dependent casein degradation

Abstract

Casein proteins constitute approximately 80% of the proteins present in bovine milk and account for many of its nutritional and technological properties. The analysis of the casein fraction in commercially available pasteurized milk and the study of its time-dependent degradation is of considerable interest in the agro-food industry. Here we present new analytical methods for the study of caseins in fresh and expired bovine milk, based on the use of lab-made capillary organic monolithic columns. An integrated capillary high performance liquid chromatography and high-resolution mass spectrometry (Cap-LC–HRMS) approach was developed, exploiting the excellent resolution, permeability and biocompatibility of organic monoliths, which is easily adaptable to the analysis of intact proteins. The resolution obtained on the lab-made Protein-Cap-RP-Lauryl-γ-Monolithic column (270 mm × 0.250 mm, length × internal diameter, L. × I.D.) in the analysis of commercial standard caseins (α_S-CN, β-CN and κ-CN) through Cap-HPLC–UV was compared to the one observe using two packed capillary C4 columns, the ACE C4 (3 μm, 150 mm × 0.300 mm, L. × I.D.) and the Jupiter C4 column (5 μm, 150 mm × 0.300 mm, L. × I.D.). Thanks to the higher resolution observed, the monolithic capillary column was chosen for the following degradation studies of casein fractions extracted from bovine milk 1–4 weeks after expiry date. The comparison of the UV chromatographic profiles of skim, semi-skim and whole milk showed a major stability of whole milk towards time-dependent degradation of caseins, which was further sustained by high-resolution analysis on a 50 cm long monolithic column using a 120 min time gradient. Contemporarily, the exact monoisotopic and average molecular masses of intact α_S-CN and β-CN protein standards were obtained through high resolution mass spectrometry and used for casein identification in Cap-LC–HRMS analysis. Finally, the proteolytic degradation of β-CN in skim milk and the contemporary formation of low-molecular-weight proteose-peptones with exact monoisotopic mass between

9,444.0989 Da and 14,098.9861 Da was confirmed through the deconvolution of high resolution mass spectra and literature data.

Keywords

Caseins, high-resolution mass spectrometry, organic monoliths, bovine milk, capillary liquid chromatography.

Abbreviations

Capillary Electrokinetic Chromatography, CEC; Capillary-High Performance Liquid Chromatography–Ultraviolet, Cap-HPLC–UV; Capillary-High Performance Liquid Chromatography–High Resolution Mass Spectrometry, Cap-HPLC–HRMS; Capillary-Liquid Chromatography–High Resolution Mass Spectrometry, Cap-LC–HRMS, Centrale del Latte di Salerno, CdLdS; 2,2'-diphenyl-1-picrylhydrazyl radical, DPPH; Electrospray ionization, ESI; Gas Chromatography, GC; High-Performance Liquid Chromatography, HPLC; High Resolution Mass Spectrometry, HRMS; 1,6-Hexanediol Dimethacrylate, HDDMA; Lauryl Methacrylate, LMA; Liquid Chromatography, LC; Liquid Chromatography–Mass Spectrometry, LC–MS; Loading Pump, L.P.; Mass Spectrometry, MS; micro Pump, μ.P.; Proteose-Peptones, PP; Solid Phase Extraction, SPE; Solid Phase Microextraction, SPME; Stir Bar Sorptive Extraction, SBSE; Total Ion Current, TIC; Trifluoroacetic Acid, TFA.

3.1. Introduction

Bovine milk proteins have been widely studied in the last years due to their strict relationship with the nutritional and technological properties of milk. Caseins account for nearly 80% of total milk protein, corresponding to approximately 3–3.5% (*w/v*), while whey fraction proteins constitute the remaining 20%. More than 60 genetic variants of caseins have been identified in bovine milk by Farrell et al. in 2004 [1]. The composition of the casein fraction strongly influences both nutritional and technological properties of bovine milk, playing a major role, for example in the cheese-making procedures [1]. In this scenario, the development of efficient analytical methods for the separation, identification and quantification of variants of milk proteins within single protein fractions appears to be fundamental for both safety and marketing reasons. It is well documented that the digestion of milk proteins can yield physiologically important bioactive peptides [2,3]. These peptides are inactive within the sequence of the parent protein, but can be released during fermentation, food processing and gastrointestinal digestion, as discussed by Korhonen and Pihlanto in 2006 [4]. Bioactive peptides are obtained by *in vitro* and *in vivo* enzymatic proteolysis of caseins. These peptides have been reported to exert, amongst others, anti-hypertensive, opioid/anti-opioid, anti-microbial and immunomodulatory activities [5,6]. Bovine milk contains a number of potent immunoregulatory peptides that affect the immune system via cellular functions, leading to great interest towards the immunomodulatory properties of casein hydrolysates [6–11]. Therefore, the quantification of relative ratios for milk protein fractions, and their enzymatic and degradation products, can become important in the biological quality assessment of bovine milk, as well as in future possible uses of these compounds in nutraceutics.

A variety of methods have been described to analyze milk fractions: electrophoretic techniques [12,13], HPLC based on ion-exchange [14], hydrophobic interactions [15]

and reversed-phase methods [16–20], and, more recently, capillary electrophoresis and capillary zone electrophoresis [21–24], as well as mass spectrometry [25,26]. In particular, high-performance liquid chromatography allows rapid and automated analysis, characterized by good separations, high resolution, accuracy and reproducibility. The use of capillary columns, easy interfaceable to mass spectrometry, is particularly promising in the field of protein analysis. Amongst capillary columns, monolithic supports can become an excellent tool in the analytical laboratory, either in the field of separation or in the early stage of sample preparation, thanks to their particular structure. Monoliths are prepared by means of a polymerization process from either organic polymers, such as polymethacrylates and polystyrenes, or from inorganic polymers, such as silica. Monoliths possess a bimodal pore structure consisting of macropores, which form a dense network of pores, and mesopores, responsible for the fine porous structure of the column. The final structure, containing both mesopores and macropores, is responsible for the major chromatographic features of monolithic columns. When compared to conventional particle packed columns, monolithic columns exhibit lower pressure drop along with higher separation efficiency, since they do not contain, unlike packed columns, void volumes [27]. They can be used in HPLC, GC, CEC, as well as in sample preparation techniques such as SPE, SPME and SBSE. Up to date, monolithic columns have been employed for the analysis of different analytical matrixes, especially of biological interest, including food products. Few analytical methods can be, on the other hand, found in literature using monoliths in milk analysis. Although, monolithic columns have been occasionally applied to milk analysis to quantify melamine, penicillin antibiotics, cephalosporin antibiotics, sulfonamides, minor proteins (milk-lactoferrin, lactoperoxidase), no data are available on caseins so far [28].

Given our previous experience in the preparation of monolithic columns [29] to be used, amongst others, in protein analysis [30], lab-made organic columns of varying

length were prepared and used in the investigation of caseins and their degradation process.

3.1.1. Aim of work

The primary aim of this work was to compare HPLC conventional and monolithic columns for milk casein analysis attempting to improve separation, identification and future quantification of the single casein fractions and its time-dependent degradation to bioactive peptides. Parallel investigations were focused on searching new LC and LC–MS techniques, based on both packed and monolithic columns, allowing a better separation of commercially available bovine milk samples caseinic fraction. Secondarily, high resolution mass spectra were obtained starting from casein standards through direct infusion. Finally, knowledge acquired through Capillary HPLC–UV separations and mass spectrometry of casein standards were used in an integrated Capillary-HPLC–high-resolution-MS analysis of precipitated casein fractions obtained from samples of commercial whole, skim and semi-skim milk at 1–4 weeks after expiry date.

3.2. Materials and methods

3.2.1. Materials

Fused-silica capillary tubings of 0.250 mm I.D. and 0.375 mm O.D. with a polyimide outer coating were purchased from Polymicro Technologies (Phoenix, AZ, USA). All chemicals and reagents were of HPLC grade. Purified α_S-CN (85%), β-CN (90%) and κ-CN (80%) casein fractions, as well as acetonitrile, TFA, 3-(trimethoxysilyl)propyl methacrylate, DPPH, LMA, HDDMA, tert-butyl alcohol, β-mercaptoethanol, Tris–HCl and 1,4-butanediol were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium citrate was from Carlo Erba (Milano, Italy). Tris–HCl buffer was purchased from Lonza (Rockland, ME, USA), while

trifluoroacetic acid was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Urea was from AppliChem GmbH (Darmstadt, Germany). Batches of whole milk, partially skim milk and skim milk were provided from CdLdS (Salerno, Campania, Italy).

3.2.2. Sample preparation

High purity water was used for the preparation of samples and mobile phase solutions. Protein standards were dissolved in water (0.1 mg/mL) prior to injection into the capillary or nano-system.

Commercial samples of skimmed milk from Centrale del latte di Salerno were preserved at room temperature and the corresponding caseins were extracted at different weeks (weeks I–IV) after expiration date. The caseins were obtained following a previously reported procedure [31]. Briefly, skimmed milks were prepared by centrifugation at 4000 × *g* for 15 min at 4 °C and caseins were obtained by the addition of acetic acid (10% *v/v*) at −20 °C. The casein powder was dissolved in aqueous acetonitrile (30% *v/v*) and the solution was then filtered through a 0.45 μm membrane, frozen at −80 °C and lyophilized for 24 h. A solution of 8M urea, 165mM Tris–HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol was prepared for the dissolution of extracted lyophilized casein samples (see section 2.2.2.).

Caseins were dissolved in the denaturing solution until there was no residual precipitate.

3.2.3. Preparation of the organic monolithic columns

After etching the inner wall surface with 1 M NaOH and 0.1 N HCl, a 10-m-long fused silica capillary (0.250 mm I.D., 0.375 mm O.D.) was silanized through reaction with a solution of 3-(trimethoxysilyl)propyl methacrylate (50%, *v/v* in toluene) containing 2,2'-diphenyl-1-picrylhydrazyl radical (0.005% *w/v*) at 110 °C

for 6 h. When the desired capillary geometries were obtained from the single pre-treated long capillary, the polymerization steps took place. A mixture of monomers (0.328 mL) consisting of 78.3 *wt* % of LMA and 21.7 *wt* % of HDDMA was dissolved in a porogenic solvent (0.672 mL) to obtain the polymerization mixture. The porogenic solvent consisted of a binary mixture of 65.0% tert-butyl alcohol and 35.0% 1,4-butanediol (reported % are *w/v*). The polymerization mixture was degassed by sparging with helium and thus introduced into the pre-treated capillary using a low argon pressure. The ends of each capillary column were finally sealed and the filled capillaries was placed inside a ^{60}Co Gammacell (1.17 and 1.33 MeV) and irradiated at a temperature of 25 °C with a total dose of 40 kGy, at a rate of about 2 kGy/h. After the polymerization was completed, the monolithic columns were washed with a volume of acetone equal to that of 50 column-dead-volumes, using an HPLC pump under constant pressure (10 MPa), to remove residual unreacted monomers and porogenic solvents. The column was characterized in terms of efficiency and permeability using a set of small molecule probes (benzaldehyde, nitrobenzene, toluene, ethylbenzene, butylbenzene) eluted with CH_3CN/H_2O, 60/40 (*v/v*) as mobile phase at different flow rates and a T of 25 °C. Permeability, $K_0 = 7.01 \times 10^{-14}$ m^2; total porosity $\varepsilon_T = 0.72$; efficiency, $H_{min} = 10.5$ μm (N/m = 95,000) at optimum liner velocity $\mu_{opt} = 0.50$ mm/s (solute: benzaldehyde) (see section 4.3.1.1.).

3.2.4. Capillary HPLC–UV: chromatographic conditions

Liquid chromatography was performed using two different capillary and nano-systems. In standard Cap-HPLC–UV applications, a Micro/Capillary/Nano-HPLC system (Model UltiMate LPG-3000, Dionex, Sunnyvale, CA, USA) was used, equipped with two low-pressure gradient micro-pumps (Model DGP-3600M), a vacuum degasser (Model DG-1210), a micro-autosampler (Model WPS-3000TPL)

equipped with a 5 μL loop, and a UV-detector (Model VWD-3100, cell 20 nL). The integrated column oven (Model FLM-3100) (5–85 °C temperature range) is provided with a micro-column switching unit and a flow-splitting device (flow splitter 1:6, 1:100 or 1:1000 for micro-, capillary or nano-analysis, respectively). A second chromatographic system, Dionex UltiMate 3000 RSLC nano-LC (Amsterdam, NL), was used for high resolution Cap-HPLC–UV and Cap-LC–HRMS applications.

The pumping system comprises two different pumps: a binary rapid separation capillary flow pump (NCS-3500RS UltiMate 3000) with a ternary loading pump and two 10-ports micro-switching valves for capillary flow ranges, and a binary rapid separation nano-flow pump (NCP-3200RS UltiMate 3000). The apparatus also includes a thermostatted column compartment, a VWD-3400RS UltiMate 3000 rapid separation four channel variable wavelength detector with a 7.0 nL Z-shaped flow cell and a WPS-3000TPLRS UltiMate 3000 thermostatted rapid separation pulled loop well plate sampler for injection of low-sample volumes (a 5 μL loop was used in the current study). Injection volume in all cases was 1.0 μL. The UV detector was set at a time constant of 0.10 s and a data collection rate of 40 Hz. UV detection was performed at 214 nm. Chromatographic data were collected and processed with Chromeleon 6.8 (Dionex, Sunnyvale, CA, USA).

In this study, both packed and lab-made monolithic columns [29,30] were investigated. An ACE C4 column (Advanced Chromatography Technologies, UK) and a Jupiter C4 column (Phenomenex Inc., Torrance, CA, USA), packed with 3 and 5 μm particles, respectively, were used. Both columns present a porosity of 300 Å and a geometry of 150 mm × 0.300 mm, length × internal diameter, L. × I.D. Two protein-Cap-RP-lauryl-γ-monolithic columns were also used, based on the same lab-made organic monolith, having two different lengths of 27 cm and 50 cm, respectively, and an internal diameter of 0.250 mm. A third Protein-Cap-RP-

Lauryl-γ-Monolithic column (50 mm × 0.250 mm, L. × I.D.) was used for trapping purposes.

The mobile phases A and B consisted of 95/5 water/acetonitrile (*v/v*) and 5/95 water/acetonitrile (*v/v*), respectively. Both mobile phases were buffered with 0.1% *v/v* of TFA. A 10-port switching valve connected to a loading pump, a micro-pump, a trap column (50 mm length) and a separation column (270 mm or 500 mm length) was used to performed injection in load-trapping mode. As shown in detail in Figure 3.1., the sample is introduced onto the loading column (valve in position 10_1) using the L.P. with mobile phase A (unless otherwise indicated), delivered for 5 min, transferred to the separation column (valve in position 1_2) using the μ.P. with a linear gradient of mobile phase B, followed by column regeneration and equilibration.

3.2.4.1. Analysis of standards

Purified α_S-CN, β-CN and κ-CN were eluted in trapping mode, both on the monolithic and on the packed columns, using a 30 min linear gradient. The gradient composition was optimized for each column. In particular, on the monolithic column a gradient of mobile phase B from 15% to 45% was used, at a flow rate of 10 μL/min, while the column temperature was set at 60 °C. The two packed columns were flushed at a lower flow rate of 3 μL/min, while the column temperature was set to 30 °C for stability issues of the packed columns. The gradient composition was also optimized according to the characteristics of each column, using a 15% to 50% gradient of mobile phase B on the ACE C4 column, and a 15% to 70% variation of mobile phase B on the Jupiter C4 column.

The analysis of casein standards was performed on the UltiMate LPG-3000 system.

3.2.4.2. Analysis of casein fractions by Cap-HPLC–UV

The degradation of casein fractions at different weeks (I–IV) from expiry date was investigated by injecting the caseins fractions on a 27-cm-long lab-made monolithic column. The chromatographic system used was the UltiMate LPG-3000 instrument. Casein fractions were analyzed in trapping mode, using the lab-made monolithic capillary column and a 60-min gradient elution from 20% to 40% of eluting mobile phase (B). The column temperature was set at 60 °C while the flow rate was 10 μL/min.

3.2.4.3. Analysis of casein fractions in high resolution (HR-Cap-HPLC–UV)

The system used for high-resolution chromatography was the UltiMate 3000 RSLC nano-LC system. Analysis was performed in trapping mode, using a 50-cm long Protein-Cap-RP-Lauryl-γ-Monolithic column (500 mm × 0.250 mm, L. × I.D.). Load-trapping injection mode of the samples was performed using a 10-port switching valve connected to a loading pump, a micro-pump, a trap column (50 mm length) and a separation column (50 cm long). The sample is introduced into the loading column (valve in position 10_1) using the L.P. with water/methanol 98/2 (*v/v*) + 0.1% (*v/v*) TFA, at a flow rate of 10 μL/min, delivered for 5 min. It is then transferred to the separation column (valve in position 1_2) using the μ.P. with a linear gradient of mobile phase B at a flow rate of 7 μL/min (see Figure 3.1.).

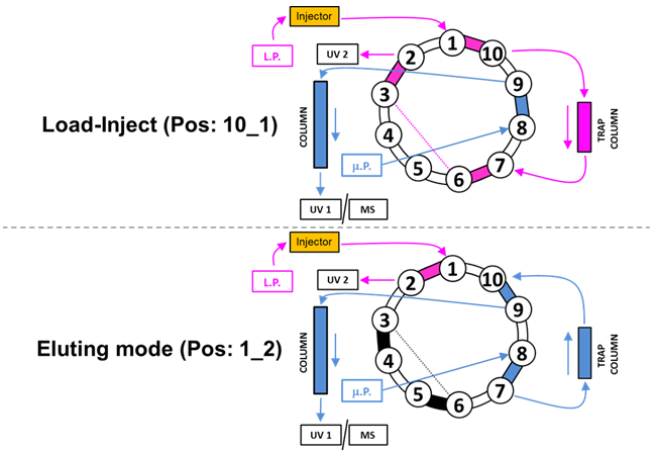


Figure 3.1. *Sketch of 10-valve port used for load-trapping purposes.*

Finally, suitable column regeneration and equilibration times were included before performing a second analysis. A 120-min gradient elution from 15% to 45% of eluting mobile phase (B) was used in HR-Cap-HPLC. The column temperature was set at 60 °C in all experiments.

3.2.5. Mass spectrometry analysis: direct infusion

MS analysis was performed with an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, CA, USA): a hybrid system combining the LTQ linear ion trap mass spectrometer and the Orbitrap mass analyzer. Ions are generated in the Standard Electrospray Source, trapped in the LTQ mass analyzer, ejected from the LTQ and collected in a C-shaped ion trap from which they are passed and analyzed into the Orbitrap.

ESI was employed at the flow rate of 10.00 µL/min with a 50 µm connecting fused-silica capillary and a 150 µm × 50 µm × 50 cm (O.D. × I.D. × L.) fused-silica emitter with an outlet tip of 20 µm I.D. (New Objective Woburn, MA, USA). The instrument was operated in positive ESI mode with a sheath gas flow of 5 units, an

auxiliary gas of 5 units, a spare gas of 0 units, a spray voltage of 3.0 kV, a capillary voltage of 40 V, a capillary temperature of 275 °C and a tube lens voltage of 245 V. A single microscan was accumulated with a maximum injection time of 100 ms. The MS parameters were optimized in the range of m/z 500–2500 by infusing a solution of 8.0 μM cytochrome C in water/acetonitrile/trifluoroacetic acid (80/20/0.05, *v/v/v*), at resolution a of 30,000–100,000 with a syringe pump at a flow rate of 10.00 μL/min. Mass calibration was accomplished with the LTQ FT-hybrid positive ion mode calibration solution (Calmix). Mass spectra were collected and processed using Xcalibur software and deconvoluted mass spectra were obtained by using Xtract. After the indication of the mass range, mass resolution and signal to noise criteria for deconvolution, Xtract was used to obtain the monoisotopic and averaged intact molecular masses from the signals of the multiply charged protein ions observed in the mass spectrum with and without isotopic resolution, respectively. Mass spectra of (commercial) α_S-CN and β-CN were obtained by direct infusion of a 4.0 μM solution of each protein in solution containing MeCN/iso-propanol/water/CF_3COOH 45/45/9.9/0.1, *v/v/v/v*. An Orbitrap resolving power of 60,000 was chosen as it provided the optimal resolution of the isotopic clusters.

3.2.6. Analytical conditions in Capillary-HPLC–high-resolution-MS

Cap-LC–HRMS analysis was performed using a 50-cm long Protein-Cap-RP-Lauryl-γ-Monolithic column (0.250 mm I.D.) and a trapping column (Protein-Cap-RP-Lauryl-γ-Monolithic column, 50 mm × 0.250 mm, L. × I.D.). Load-trapping injection mode was performed using a 10-port switching valve, as described earlier. The sample is introduced onto the loading column (valve in position 10_1) using the L.P. with water/methanol 98/2 (*v/v*) + 0.1% (*v/v*) TFA, at a flow rate of 10 μL/min, delivered for 5 min. It is then transferred to the separation column (valve in position 1_2) using the μ.P. with a linear gradient of mobile phase B at a flow

rate of 7 μL/min (see Figure 3.1.). Finally, suitable column regeneration and equilibration times were included before performing a second analysis. A 60-min gradient elution from 15% to 45% of eluting mobile phase (B) was used to in Cap-LC–MS. The column temperature was set at 60 °C in all experiments.

Mass spectrometry detection was performed on an LTQ Orbitrap, set at a mass range of 1000–3000 m/z. The instrument was operated at a resolution of 60,000 in positive ESI mode with a sheath gas flow of 5 units, an auxiliary gas of 5 units and a spare gas of 0 units, while spray voltage was set at 3.0 kV, capillary voltage at 40 V, capillary temperature at 275 °C and tube lens voltage at 245 V. Each single microscan was accumulated with a maximum injection time of 100 ms. Mass chromatograms were recorded in total ion current, within 1000 and 3000 amu. The signal-to-noise (s/n) ratios of the response were calculated on the unsmoothed mass spectra, with reference to the base peak in the mass spectrum.

3.3. Results and discussion

3.3.1. Preparation of the organic monolithic capillary columns

The monolithic columns described in this paper were prepared according to an already described patented procedure [29]. A two step procedure was developed, including an initial pre-treatment of the activated capillaries with 3-(trimethoxysilyl)propyl methacrylate, necessary to ensure immobilization of the growing polymer monolith on the capillary wall, and a polymerization step where a mixture of monomer, cross-linker and porogenic solvent was used to fill the capillaries. Finally, the polymerization was performed using a total dose of 40 kGy of γ-rays at 25 °C.

3.3.2. Analysis of casein standards present in bovine milk through Cap-HPLC–UV

Important results have been obtained in the last twenty years in the analysis of bovine milk, as an ever growing interest in foodomics, combined with the use of advanced hyphenated LC–MS techniques, has allowed the separation and identification of both major and minor proteins present in milk composition. Intact proteins have been traditionally analyzed on packed columns based on alkylated, wide-pore (300 Å or higher) silica particles. Many drawbacks, however, are associated with this type of stationary phase, including the irreversible adsorption of biomolecules on the unreacted silanols surface, often generating slow adsorption/desorption equilibria and poor peak shape. Furthermore, the need for efficiency has led to the use of smaller particles, which can generate considerably high backpressure values, severely limiting the use of (very) long columns at high flow rates and thus very-high resolution analysis. As shown in previous studies [32–37], organic monolithic columns are an excellent, highly promising alternative in the analysis of intact proteins as they provide excellent selectivity and good chemical passivation. Given their higher permeability, monolithic columns are characterized by lower backpressure values. They can, thus, be used at elevated flow-rates, leading to faster gradients without loss in resolution. Finally, extra-long columns can be used in high-resolution experiments, as the backpressure generated is compatible with currently available instrumentation.

A comparison between packed and monolithic capillary columns was performed to evaluate their selectivity towards the caseins typically present in bovine milk and to study the elution conditions suitable for the analysis of lyophilized caseins extracted from commercially available milk samples. Optimization of the chromatographic process can provide valuable information on the protein profile of the casein standards, which can then be used to study the stability of the casein fraction in bovine milk, after 1–4 weeks from expiry date.

In order to obtain the necessary preliminary data prior to method development, three different commercially available casein standards were eluted on two packed C4 columns (ACE and Jupiter) and on a lab-made monolithic column (Protein-Cap-RP-Lauryl-γ-Monolithic) [29,30]. Using a 30 min gradient elution, the three casein standards (α_S-CN, β-CN and γ-CN) were successfully eluted on the monolithic column with good selectivity and at different retention times, which is an excellent pre-requisite for the separation of these major proteins in milk. The quality of the resolution achieved on the monolithic column was excellent with regards to the differentiation between major peaks and minor peaks present in the considered standards. The efficiency and the overall resolution during the separation of caseins on the monolithic stationary phase were found to improve with higher column temperatures, which led to the use of a column oven temperature of 60 °C in all the chromatographic runs. The particulate stationary phases, on the other hand, showed limited thermal stability at the same temperature, and thus the operating temperature was set at 30 °C when using the two packed columns. After optimization of the elution conditions for each column, the monolithic column displayed higher selectivity, resulting in a higher resolution when compared to the two packed capillary column, as can be seen in Figure 3.2. In particular, in Figure 3.2.A., the number of peaks obtained on the monolithic column was higher for all three standards, compared to the ACE C4 column (packed with 3 μm particles, 150 mm × 0.300 mm, L. × I.D.; see Figure 3.2.B.) and to the Jupiter C4 column (packed with 5 μm particles, 150 mm × 0.300 mm, L. × I.D.; see Figure 3.2.C.). Although all three columns showed different elution profiles, proving in many ways complementary, the monolithic column displayed a different selectivity with regards to the different isoforms and/or modified versions of each protein present in the standard, as well as a better chromatographic peak profile. In particular, the selectivity shown towards the casein standards was clearly visible from the comparison of the chromatograms obtained for β-CN on the three

capillary columns, with the monolithic column clearly resolving peaks that were co-eluted on the two packed capillary columns. The chromatogram of κ-CN standard (Figure 3.2.A.) on the monolithic column not only presents the three well known main peaks, representing already reported glycosylated and unglycosylated variants of isoforms A and B [16,18,20,38], but also an important number of minor peaks which were undisclosed with the two packed columns (Figure 3.2.B. and C).

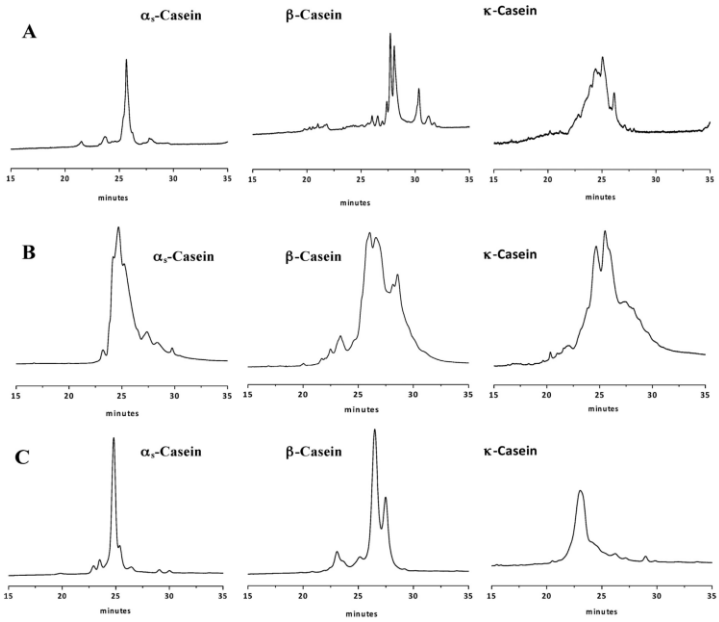


Figure 3.2. *Chromatograms of casein standards on (A) a 27-cm long Protein-Cap-RP-Lauryl-γ-Monolithic column (270 mm × 0.250 mm, L. × I.D.); (B) on an ACE C4 column packed with 3 μm particles (150 mm × 0.300 mm, L. × I.D.); and (C) on a Jupiter C4 column packed with 5 μm particles (150 mm × 0.300 mm, L. × I.D.). Gradient time: 30 min. Commercially available casein standards were dissolved in water. UV detection at 214 nm. See Section 3.2.4.1 for full experimental details.*

This can become a crucial point in LC–MS applications, with the optimization of the chromatographic process providing a supplementary tool in the characterization of the genetic pool of each protein group.

These preliminary results were encouraging for the future development of analytical methods for the analysis of bovine milk casein fractions on the Protein-Cap-RP-Lauryl-γ-Monolithic column.

3.3.3. High Resolution Mass Spectrometry of casein standards

The characterization of casein standards was integrated by high resolution mass spectrometry. A similar study is now being performed in our lab for intact proteins present in the whey fraction and will be the topic of future papers. Recently, interesting studies have been reported in literature on the analysis of whey protein standards and of β-CN standards using both top-down and bottom-up approaches [39,40]. In the present study, intact proteins were analyzed to obtain the exact masses of α_S-CN and β-CN, data that were later used for protein identification in LC–MS experiments.

The results obtained using hybrid LTQ Orbitrap mass spectrometry were excellent both in terms of resolution and quality and are, at the best of our knowledge, some of the few high resolution spectra obtained for these two bovine proteins. Using hybrid instrument LTQ Orbitrap, we were able to carry out high quality analysis, key requirement for the identification and characterization of intact proteins isoforms. Multiply charged signals were observed in all experiments. The Orbitrap mass analyzer was capable of resolving the isotopic clusters of both casein standards as shown in Figure 3.3.A. and B for α_S-CN and β-CN, respectively. The high resolution achieved during the analysis can be appreciated in more detail when looking at the expanded spectra shown in Figures 3.4. and 3.5.

In particular, by zooming on z= 14 for α_S-CN and z= 17 for β-CN, respectively (see Figures 3.4. and 3.5.), a very good isotopic resolution was evidenced. Intact protein

MS data revealed the presence of at least three highly abundant protein isoforms in each spectrum. The signals related to multiply charged ions were further deconvoluted into spectra of the uncharged protein isoforms using the Xtract software.

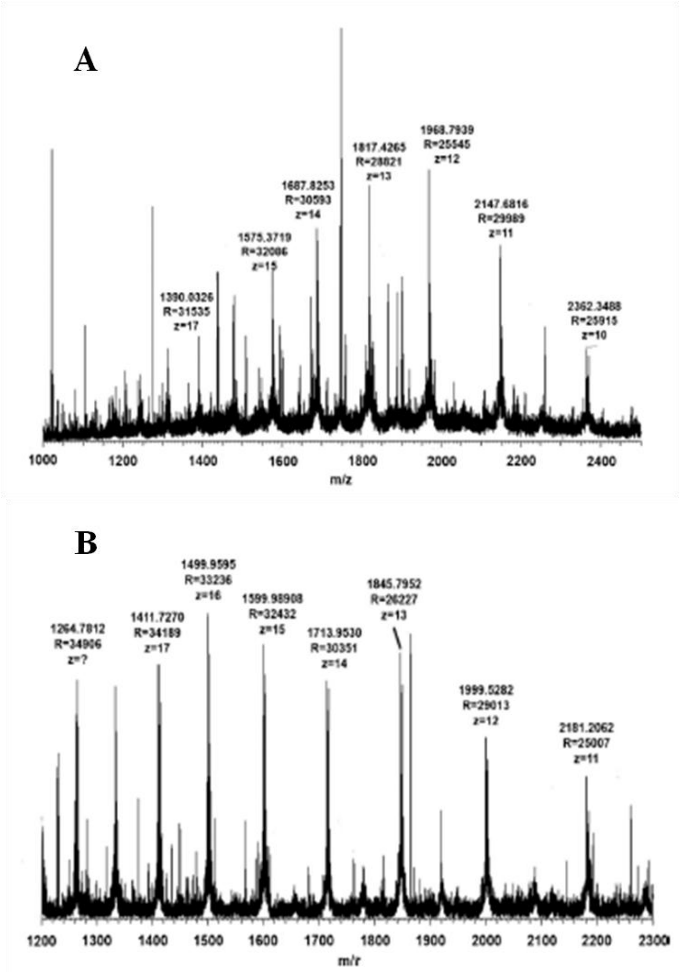


Figure 3.3. *Mass spectra of α_S-CN (A) and β-CN (B) obtained through direct infusion of a 4.0 μM sample solution in MeCN/iso-propanol/H_2O/CF_3COOH 45/45/9.9/0.1 (v/v/v/v). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000–2500; maximum injection time: 100 ms; 1 microscan.*

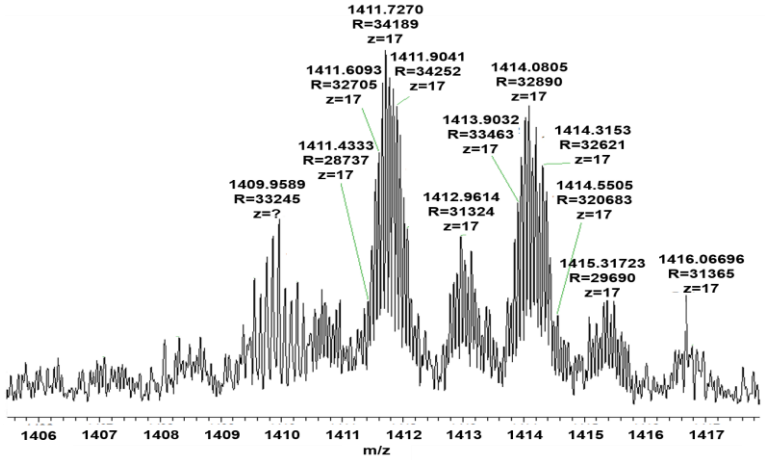


Figure 3.4. *Expansion of mass spectrum of α_S-CN obtained through direct infusion of a 4.0 µM sample solution in MeCN/iso-propanol/ H_2O/CF_3COOH 45/45/9.9/0.1 (v/v/v/v). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000-2500; maximum injection time: 100ms; 1 microscan.*

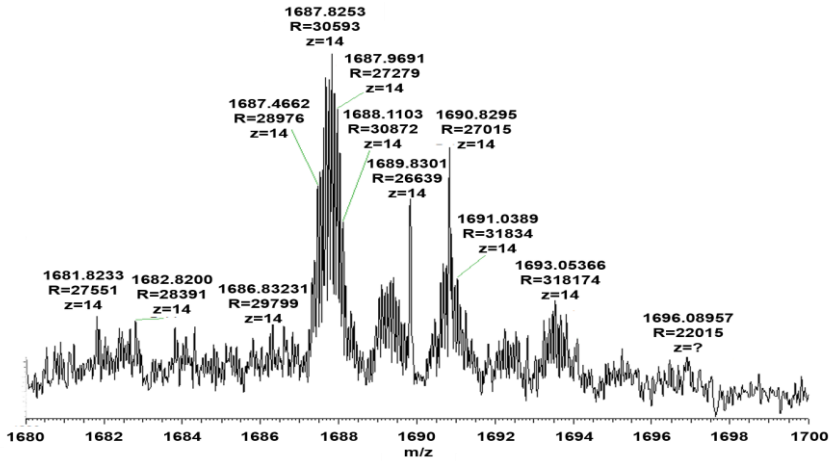


Figure 3.5. *Expansion of mass spectrum of β-CN obtained through direct infusion of a 4.0 µM sample solution in MeCN/iso-propanol/ H_2O/CF_3COOH 45/45/9.9/0.1 (v/v/v/v). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000-2500; maximum injection time: 100ms; 1 microscan.*

Monoisotopic masses are reported in Figure 3.6., while average molecular masses, determined by matching the measured isotopic pattern to a calculated isotope pattern, obtained from the elemental composition of an average hypothetical amino acid, are reported in Figure 3.7.

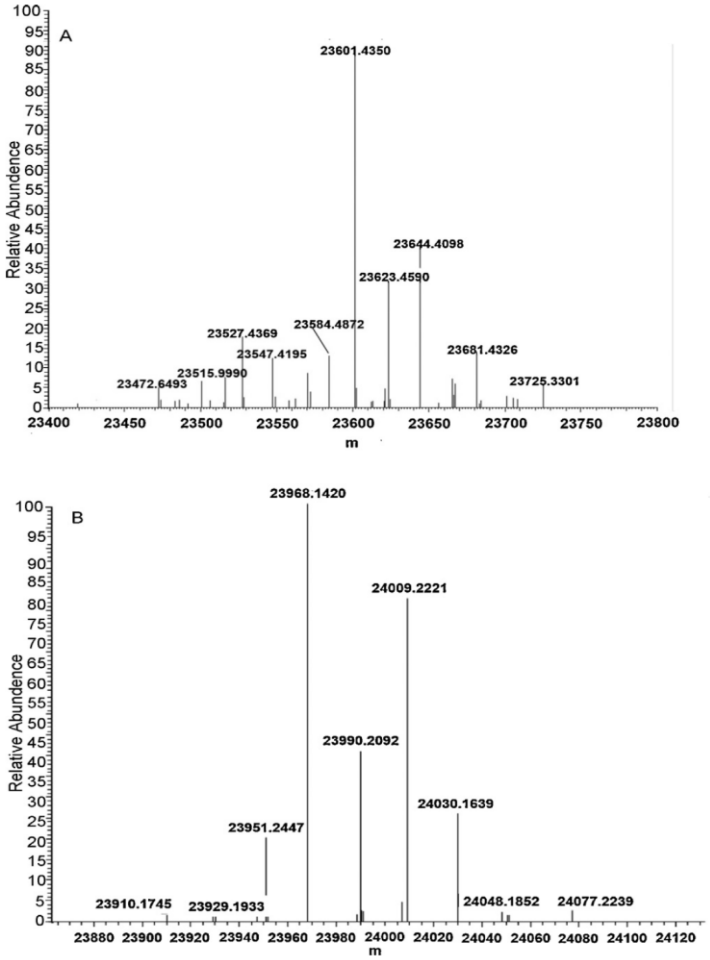


Figure 3.6. *Deconvoluted spectra of α_S-CN (A) and β-CN (B) obtained using the Xtract software. Monoisotopic masses are reported. Experimental conditions as in Figure 3.3.*

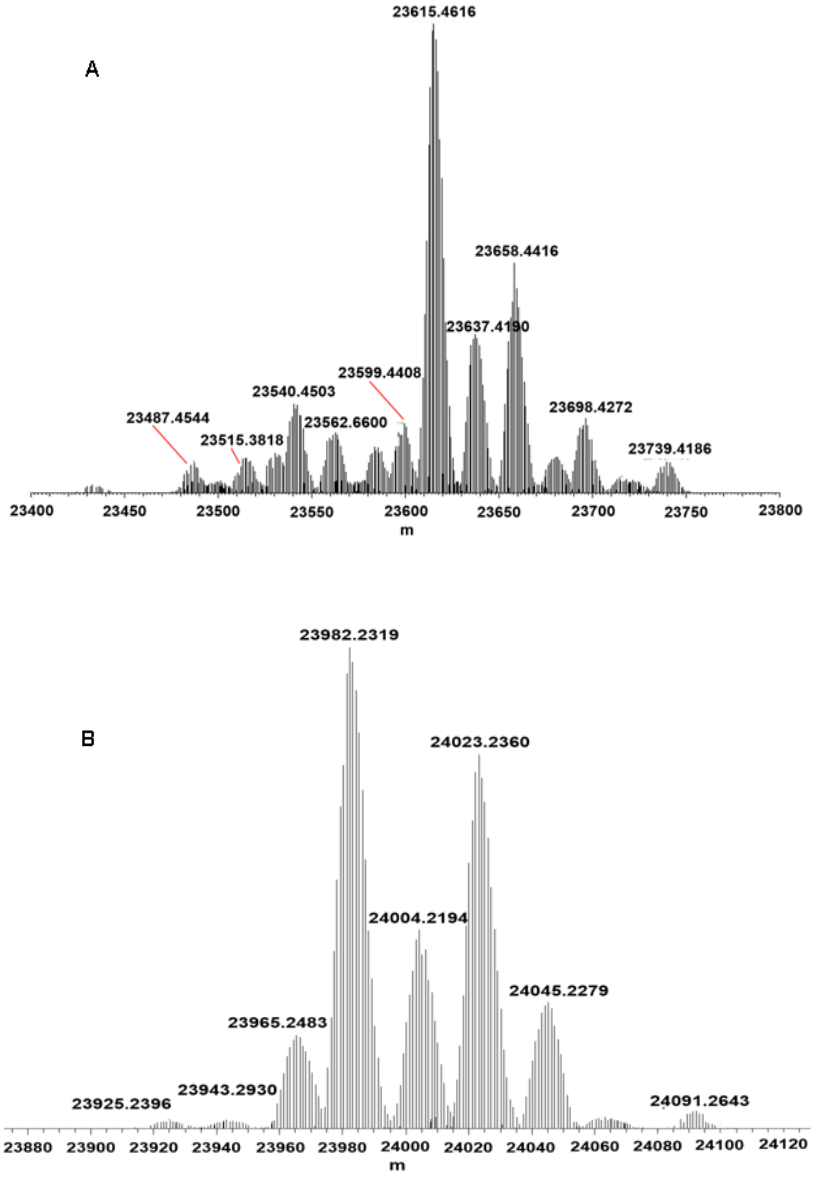


Figure 3.7. *Deconvoluted spectra of α_S-CN (A) and β-CN (B) obtained using the Xtract software. Averaged molecular masses are reported, determined by matching the measured isotopic pattern to a calculated isotope pattern obtained from the elemental composition of an average hypothetical amino acid. Experimental conditions as in text (section 3.2.5).*

As can be seen in Figure 3.7., the deconvoluted results detected nine average molecular masses corresponding to nine different β-CN variants, in agreement with the masses reported in literature [1,39]. In detail, three averaged molecular masses (23,982.2319 Da, 24,023.2360 Da and 24,091.2643 Da, see Figure 3.7.B.) corresponded to the theoretical and experimental masses reported in 2009 by Pâsa-Tolìc and collaborators for variants A2, A1 and B of β-CN respectively [39]. For example, the protein with Mr = 23,982.2319 Da was identified as isoform A2 of β-CN containing 5 phosphorylation sites.

Monoisotopic molecular masses of α_S-CN variants were in the range of 23,472.6493–23,725.3301 Da, as shown in Figure 3.6.A., while averaged values were in the 23,487.4544–23,739.4186 Da range (Figure 3.7.A.). These values are normally reported in literature for variants B and C of α_{S1}-CN [1]. We were unable to identify spectral values corresponding to α_{S2}-CN variants in the casein standard sample commercially available, which limited our high-resolution analysis to α_{S1}-CN. Pâsa-Tolìc and co-workers reported two masses of 25,226.9975 Da and 25,306.1167 Da for the α_{S2}-CN variants bearing 11 and 12 phosphorylations, respectively [39]. This is probably due to the fact that the authors in Ref. [39] reported only LC–HRMS data on α_{S2}-CN, found as a secondary product present in the β-CN standard. The mass range observed in our experiments corresponds to the molecular masses reported by Bonizzi [20] and Farrell [1] for α_{S1}-CN, although the data reported in literature are not derived from high-resolution spectra and often refer to average masses. Unfortunately, a high resolution spectrum of κ-CN could not be produced given the high variety of phosphorylation and glycosylation degrees present in the variants of the intact protein. To the purpose, chromatographic retention times obtained through LC–UV, together with literature data, were used for identification of κ-CN within the casein fraction in the Cap-LC–MS studies.

3.3.4. Analysis of casein fraction in bovine milk

Although many researchers have studied the chromatographic profile of the casein fraction, both as such and after high temperature treatment, the effect of multi-week storage at room temperature has never been investigated so far.

The removal of expired milk from the local vendors as well as its final disposal is an issue of major interest for many milk producers. The recovered milk is often used in the first week after its expiry to obtain the whey fraction which, after the necessary treatments, is used in the health care and food industries. However, a great amount of potentially active ingredients is lost during this cycle. In particular, it was observed that many bioactive peptides can be obtained following the natural degradation of caseins, operated by bacteria present in expired milk, such as plasmin and other proteolytic enzymes [10].

The recovery and the reutilization of caseins and casein-derived peptides to be used in the food industry and in the production of dietary supplements would be extremely cost-effective. To the purpose, an analysis of the degradation process of the casein fraction becomes increasingly interesting, especially if appropriate methods for the identification and the quantification of relative ratios of protein fraction and their enzymatic and degradation products can be successfully established. The present investigation aims at developing new Cap-HPLC methods based on lab-made organic monolithic columns to study the time-dependent degradation/transformation of caseins.

The first step of the investigation consisted in obtaining casein samples extracted at different weeks (weeks I–IV) after expiry date. Commercial samples of skimmed milk were preserved at room temperature and the corresponding caseins were extracted, following a previously reported procedure [31]. Caseins were then dissolved in a solution of 8M urea, 165mM Tris–HCl, 44mM sodium citrate and 0.3% β-mercaptoethanol, prior to injection into the capillary monolithic column. A gradient elution from 20% to 40% of mobile phase B (acetonitrile/water, 95/5, *v/v*

+ 0.1% *v/v* TFA) was used to elute the caseins from the column. In Figure 3.8. the chromatographic profile of the casein extract deriving from whole, semi-skim and skim milk at different weeks are shown. The chromatographic profile of basal milk corresponds to the casein fraction precipitated from milk before its expiry date, when the degradation of caseins due to milk acidity and bacteria is at the lowest degree. Comparing basal milk with the casein fraction from whole milk at week I and week II after expiration, a very similar profile was obtained, although the peaks eluted at approximately 60 min, belonging to β-CN, were not observed at samples of week I and week II. Degradation of β-CN was even more evident when samples of week III and week IV were tested, showing that after the first two weeks the protein undergoes complete degradation and fragmentation. The peptide fraction which elutes earlier in the chromatogram, is not very different on the three first chromatograms. As previously described κ-CN was eluted first, followed by α_S-CN and then β-CN, in accordance with literature data [16–18,20,38]. After 3 weeks from expiry date, the chromatographic profile of the casein fraction in whole milk changes significantly, as shown at the top of Figure 3.8. (in violet and black). Peptides deriving from the major proteins account for the differences observed, as there is an increment of peaks with lower retention times and a contemporary loss of peaks in the area of the chromatogram between 50 and 60 min, where the different isoforms of β-CN are eluted.

The results obtained for whole milk were then compared to those deriving from the analysis of casein fractions extracted from skim and semi-skim milk. As with whole milk, the peaks eluted at approximately 60 min, when analyzing caseins extracted prior to expiration (basal sample), are not present in the chromatograms of weeks I–IV. Furthermore, skim milk appears to have a more substantial variation between the chromatogram of basal milk and the ones registered for samples obtained at later stages during the milk storage. In particular, an important degradation and fragmentation of caseins occurs already at week I after expiry

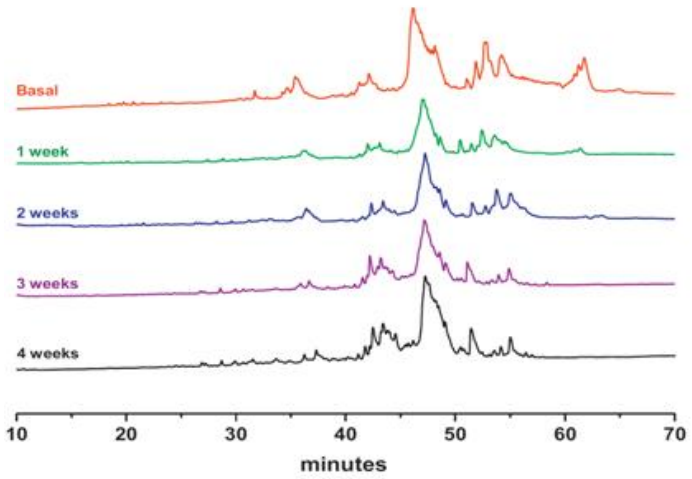
(green line), while at week II (blue line) the chromatographic profile is very different from the one observed in the case of basal milk (red line). The peptide fraction that elutes earlier in the chromatogram is considerably richer in the case of casein samples extracted from skim milk compared to the ones extracted from whole milk. On the other hand, semi-skimmed milk shows an intermediate situation, displaying a lower degree of fragmentation if compared to skim milk, without reaching the stability of whole milk.

The degradation of the casein fraction present in skim milk is easily observed performing a high-resolution Cap-HPLC analysis. In order to obtain the required extra resolution, a combination of a 50 cm long monolithic column (I.D. of 0.250 mm) with a longer 120 min gradient elution was used. This approach allowed a good resolution during the elution of both the casein fraction from basal skim milk and of the one obtained after casein extraction at week IV after expiry date, as depicted in Figure 3.9. As observed earlier, peaks eluted between 75 and 90 min in basal skim milk chromatogram (Figure 3.9.A.), are either not present or have a much lower concentration when analyzing samples from week IV (Figure 3.9.B.). The degradation of these proteins, some of which could be identified as variants of β-CN (after comparison with protein standards) leads to a further simplified chromatographic profile at retention times higher than approximately 80 min. Furthermore, at retention times comprised between 60 and 78 min, corresponding to a region of the chromatogram where the α_S-CN isoforms are eluted, the complexity of the analysis is higher for caseins from week IV than for caseins from basal skim milk.

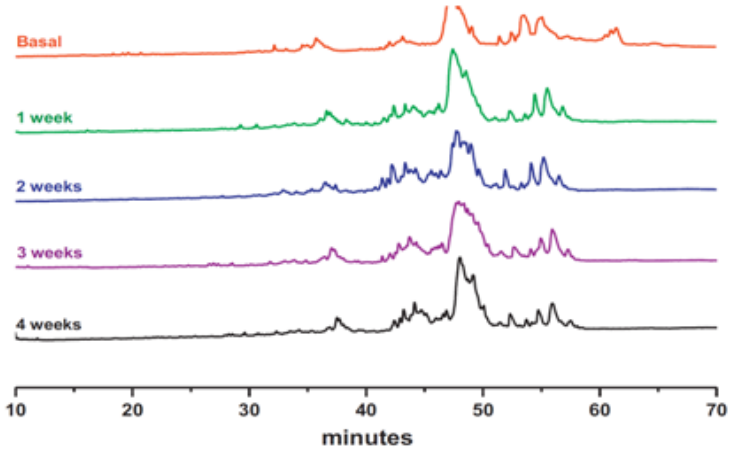
At this point, we hypothesize a possible stabilizing role exerted by the lipidic fraction in inhibiting casein degradation in whole milk, effect which is not possible in skim milk and which only partially occurs in semi-skimmed milk. This phenomenon provides an interesting starting point for further investigation on the role of the lipidic fraction in milk stabilization. It should be noted, in fact, that

lipids undergo an important degradation leading to higher acidity of expired milk. Nevertheless, the presence of lipids probably plays a stabilizing role for the casein micelles, showing that further indepth studies of the lipidomic profile of bovine milk are necessary to better understand their importance in the constitution of the complex emulsion represented by bovine milk.

Whole Milk



Semi-Skim Milk



Skim Milk

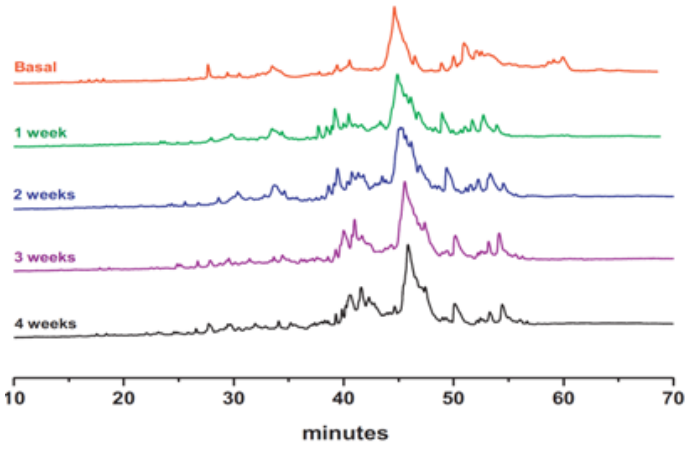


Figure 3.8. *Chromatographic profile of casein fraction extracted from basal whole, semi-skim and skim milk and at weeks I–IV from expiry date. Column: Protein-Cap-RP-Lauryl-γ-Monolithic (27 cm × 0.250 mm, L. × I.D.). Elution: from 20% to 40% of mobile phase B with a 60-min gradient time; flow rate: 10 µL/min. Tcol = 60 °C. UV detection at 214 nm. See Section 3.2.4.2 for full experimental details.*

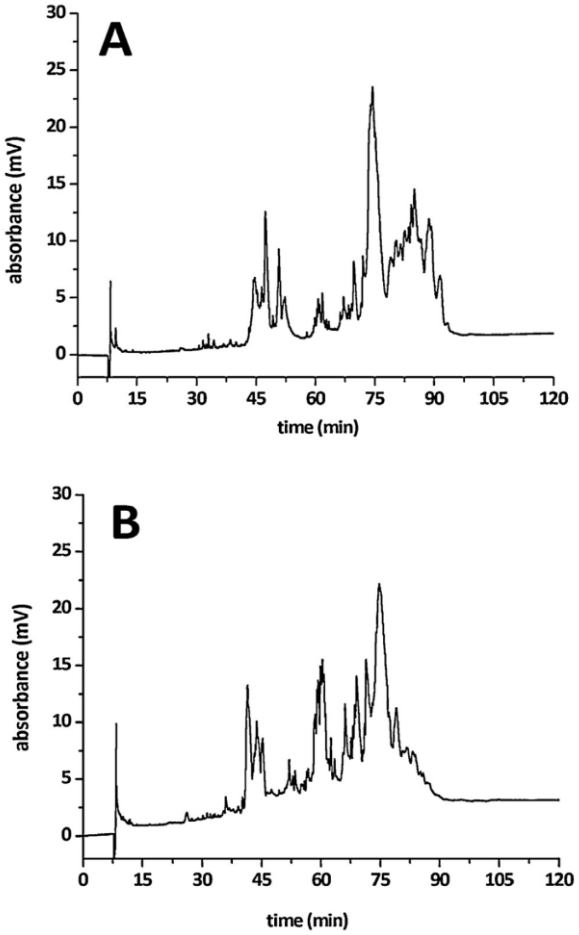


Figure 3.9. *High-resolution (HR-Cap-LC) chromatogram of casein fraction of basal skim milk (A) and of casein fraction extracted at week IV from skim milk (B) using a 50-cm long Protein-Cap-RP-Lauryl-γ-Monolithic column (0.250 mm I.D.). Elution: 120-min gradient from 15% to 45% of mobile phase (B); flow rate: 7 μL/min. Tcol = 60 °C. UV detection at 214 nm. Full experimental details in Section 3.2.4.3.*

3.3.5. Cap-LC–HRMS analysis of week IV casein fractions

Figure 3.6. shows the total ion current chromatograms obtained through Cap-LC–HRMS during the analysis of week IV caseins deriving from skim milk. A 60 min

gradient elution from 15% to 45% of mobile phase B (acetonitrile/water 95/5, *v/v*, 0.1%, *v/v*, TFA) was operated on the 50-cm long Protein-Cap-RP-Lauryl-γ-Monolithic column (0.250 mm I.D.) at a flow rate of 7 µL/min (see Figure 3.10.).

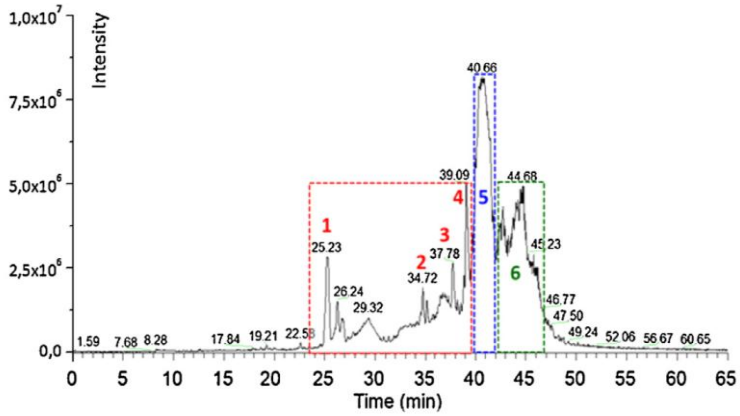


Figure 3.10. *Total ion current chromatogram (Cap-LC–HRMS analysis) of casein fraction extracted at week IV from skim milk (B) using a 50-cm long protein-Cap-RP-lauryl-γ-monolithic column (0.250 mm I.D.). Elution: 60-min gradient from 15% to 45% of mobile phase (B); flow rate: 7 µL/min. Tcol = 60 °C. UV detection at 214 nm. LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000–3000 amu. Full experimental details in Section 3.2.6.*

Three main regions were identified in the TIC chromatogram: a first one at retention times comprised between 24 and 39.5 min approximately (indicated in red, peaks 1–4), a second one comprised between 39.5 and 42 min (indicated in blue, peak 5) and a third one characterized by peaks eluting between 42 and 47 min approximately (in green, peak 6).

Mass spectra of main peaks identified within region 1 are shown in Figure 3.11., together with most significant mass/charge (m/z) values and charges (z) observed.

Xtract high resolution deconvolution of spectra of peaks 1–4 reported in Figure 3.11., lead to exact measured monoisotopic molecular masses in the range of Mr = 9444.0989–14,098.9861 Da.

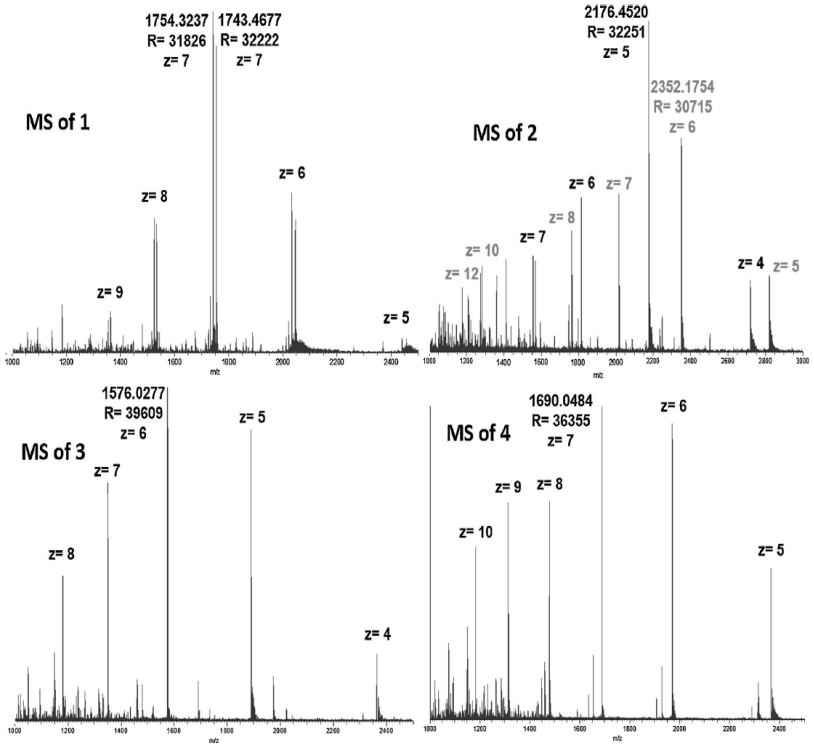


Figure 3.11. *Mass spectra of peaks 1–4 in region 1 in TIC chromatogram reported in Figure 3.10. Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000–3000; maximum injection time: 100 ms for 1 microscan.*

In more detail, at least six principal exact masses were detected, as reported in Table 3.1.

Table 3.1. *Exact measured monoisotopic molecular masses of peaks 1-4 reported in Figure 3.11. (see text). Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.11. using Xtract software.*

Mass Spectrum	Measured M_r (Da)	
1	**12189.2012**	**12265.1659**
2	**10871.1993**	**14098.9861**
3	**9444.0989**	
4	**11816.2338**	

The peaks eluted between 24 and 39.5 min were not present when analysing caseins of basal skim milk, as shown during the degradation studies (see Figures 3.8. and 3.9.), and were likewise absent during Cap-HPLC–MS analysis of the same fraction. Furthermore, peaks imputable to β-CN were missing in the third region of the chromatogram in Figure 3.10. during the analysis of caseins extracted from expired (week IV) skim milk, as also shown in degradation studies in section 3.3.4. These peaks were, on the other hand, present in the analysis of caseins extracted from basal skim milk, as observed both through Cap-HPLC–UV (Figures 3.8. and 3.9.) and Cap-LC–MS.

The mass spectrum of peak 6 confirmed the lower concentration of β-CN in the week IV sample (see Figure 3.12.).

A time- and temperature-dependent denaturation of β-CN has already been reported in literature, with different authors describing the formation of proteose–

peptones from the C-terminal fraction of the protein [41–44]. As early as 1979, Eigel showed that fragments with approximately the correct molecular weights and similar electrophoretic mobilities to γ_1-A2, γ_2-A2 and γ_3-A are formed by *in vitro* proteolysis of β-CN-A2 promoted by bovine plasmin [45]. Later, Andrews was able to prove that some of the smaller proteins present in the PP fraction of milk derive from the N-terminal of β-CN after proteolysis, suggesting that the remaining C-terminal constitute the earlier reported γ caseins [46].

Mass ranges present in literature for both the PPs deriving from the N-terminal and proteins deriving from the C-terminal of β-CN are similar to the mass range of peaks 1–4 present in region 1. In fact, PP component 5 (deriving from N-terminal), isolated from bovine milk, has molecular weight values within the range of 12,000 Da -13,500 Da, as showed by sedimentation equilibrium, dodecylsulphate/polyacrylamide gel electrophoresis and gel filtration in urea-containing buffers [46], while proteins of molecular weight around 9900 could also derive from β-CN, although deeper structural and analytical investigations were necessary. It seems possible that these proteins derive from PP component 8. From the limited and timeworn literature data, it is thus possible to conclude that peaks 1–4 derive from the degradation of β-CN. De Noni et al. more recently investigated the quantification of PP from both raw and pasteurized milk by HPLC [47], obtaining a similar chromatographic profile to the one observed in our lab, with the PP fraction eluted before the major caseins. The combined use of HPLC and MS allowed the identification of the PP deriving from the degradation of β-CN: molecular masses reported by this group in 2007 are included in the mass range experimentally observed in our studies.

Finally, the second region of Figure 3.10. was investigated. The mass spectrum of peak 5 is depicted in Figure 3.13. This spectrum allowed the identification of α_{S1}-CN (see Figure 3.14. for deconvoluted spectra), thanks to the exact masses obtained through direct infusion of standards (see section 3.3.2.). The exact

monoisotopic molecular masses registered were comprised in the region of 22,224.7167 Da and 23,884.9178 Da. Similarly, α_{S2}-CN was present in this second region, with exact monoisotopic molecular masses in the range of 25,125.7136 Da–26,846.1767 Da (see Table 3.2. and Figure 3.15.), corresponding to theoretical data contained in the range of 25,226–26,074 Da [1,20,39]. Finally, we were also able to identify κ-CN isoforms (Figure 3.16.), with experimental exact monoisotopic molecular masses comprised between 18,934.5082 Da and 19,974.2953 Da, very similar to previously reported data [1,20]. These results further demonstrate that these three proteins and their various isoforms are stable at room temperature after long period of times (4 weeks from expiry date) and do not undergo the proteolytic degradation displayed by β-CN. However, further studies need to be performed in this field to deeper understand the degradation mechanisms taking place and the reason for the pronounced instability of β-CN in comparison to the other caseins present in bovine milk. Finally, the early eluting minor proteins in expired skim milk need to be further characterized in order to obtain a complete identification with the already reported PP present in literature. Future studies should aim at fully understanding their origin as well as their potential role in milk, in terms of safety, nutraceutical and cheese-making properties.

Table 3.2. *Summary of monoisotopic exact molecular masses obtained by the deconvolution of the high resolution mass spectrum of peak 5 (see Figure 3.13.) using Xtract software.*

Protein	Range of measured Mr (Da)	Range of theoretical Mr (Da)	Figure
κ-CN	18,934.5082–19,974.2953	19,005–20,939	3.14.
α_{S1}-CN	22,224.7167–23,884.9178	23,487–23,739	3.15.
α_{S2}-CN	25,125.7136–26,846.1767	25,226–26,074	3.16.

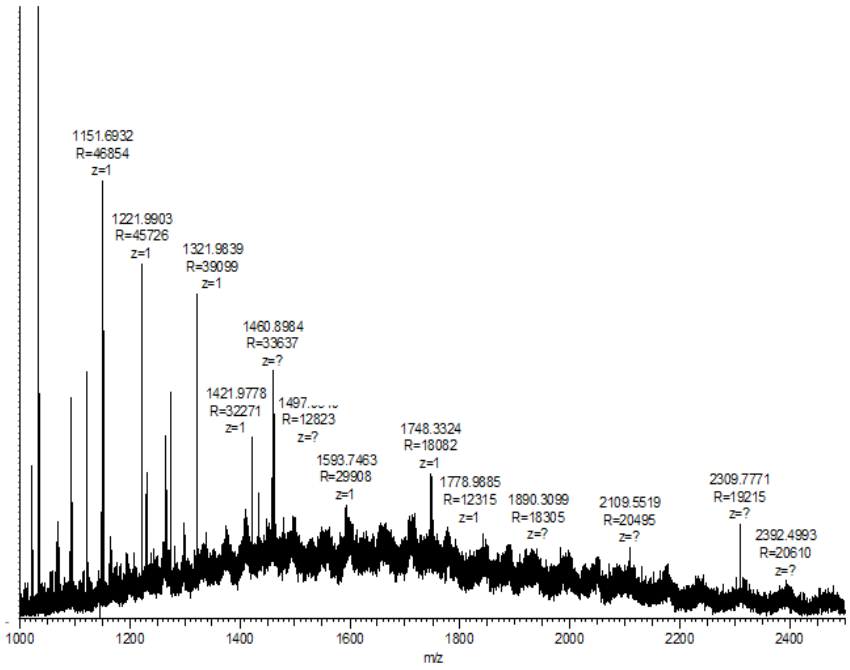


Figure 3.12. *Mass spectrum of peak 6 of TIC chromatogram in Figure 3.10. (see text). Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000-3000; maximum injection time: 100 ms for 1 microscan.*

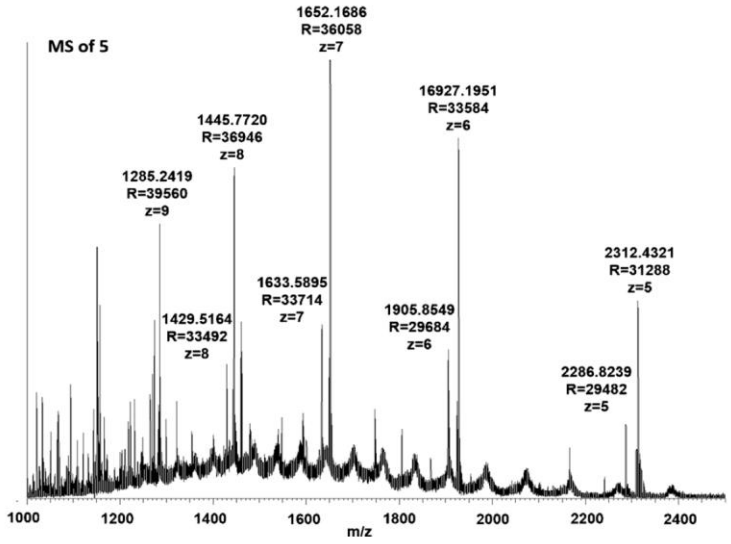


Figure 3.13. *Mass spectrum of peak 5 in region 2 in TIC chromatogram reported in Figure 3.10. Instrument: LTQ Orbitrap MS ESI (+) detector. R = 60,000, mass range: 1000–3000; maximum injection time: 100 ms for 1 microscan.*

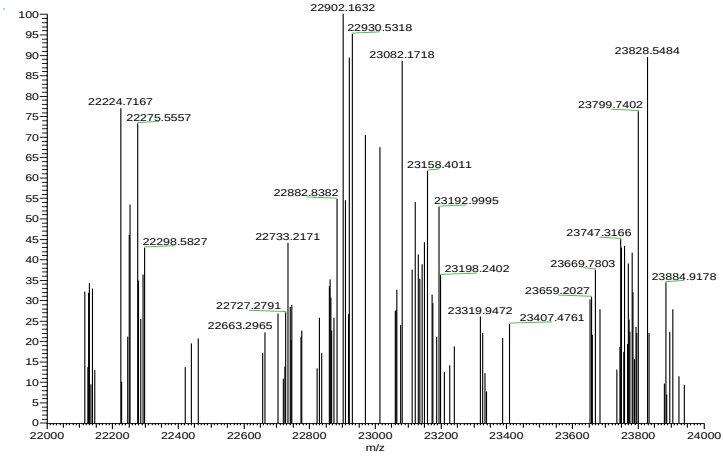


Figure 3.14. *Range of exact molecular masses of α_{S1}-CN. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.13. using Xtract software.*

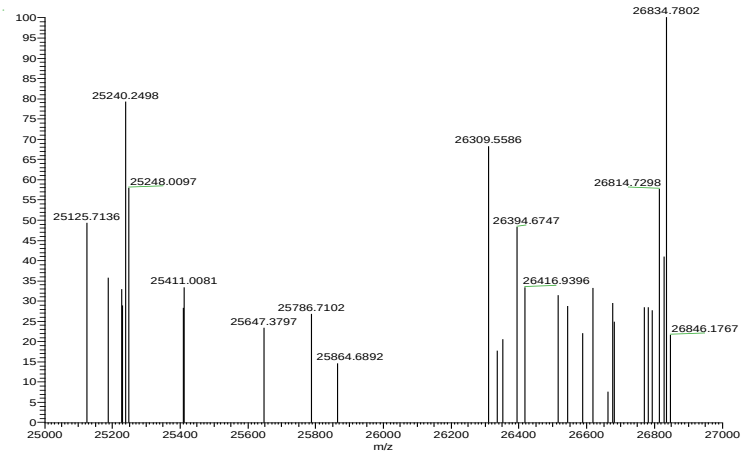


Figure 3.15. *Range of exact molecular masses of α_{S2}-CN. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.13. using Xtract software.*

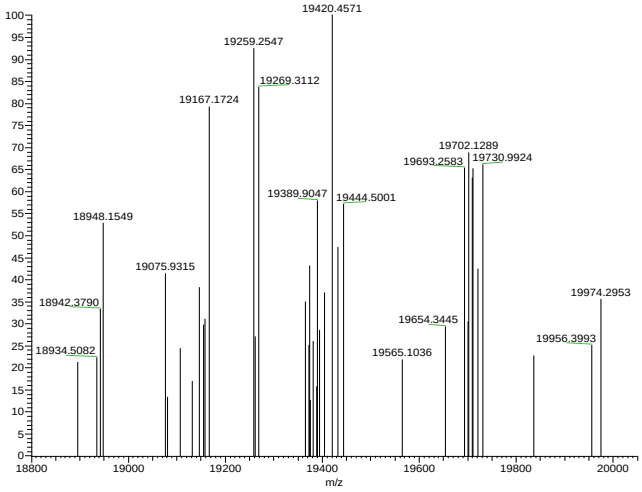


Figure 3.16. *Range of exact molecular masses of κ-CN. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum 5 in Figure 3.13. (see text) using Xtract software.*

3.4. Conclusions

In the present study, new lab-made organic monolithic capillary columns were used to analyze caseins extracted from commercially available pasteurized bovine milk samples, in an integrated capillary HPLC and high-resolution mass spectrometry approach. The lab-made monolithic capillary columns are characterized by excellent permeability and reproducibility, as well as remarkable biocompatibility, making them excellent candidates for the separation of intact proteins. Commercial standard caseins (α_S-CN, β-CN and κ-CN) were eluted through Cap-HPLC–UV on the lab-made Protein-Cap-RP-Lauryl-γ-Monolithic column (270 mm × 0.250 mm, L. × I.D.) and on two packed C4 columns (ACE 3 and Jupiter, 150 mm × 0.300 mm, L. × I.D.) to compare resolution. The results obtained on the monolithic capillary column encouraged its use in degradation studies of casein fractions extracted from bovine milk samples at 1–4 weeks after expiry date. In particular, it was observed that the degradation of casein protein was more evident in skim milk when compared to whole milk, with semi-skim milk showing an intermediate degradation pattern, suggesting a stabilizing role of the lipidic fraction towards the milk caseins. A 50-cm long monolithic column was then used to obtain higher resolution of casein fractions, both through UV detections and through HR-MS detection. Cap-LC–HRMS analysis of caseins extracted from skim milk at week IV from expiry date confirmed the degradation of β-CN and the contemporary presence of low-molecular-weight proteins with exact monoisotopic Mr between 9,444.0989 Da and 14,098.9861 Da, eluted before κ-CN.

These peptides presumably correspond to the proteose–peptones typically deriving from the proteolytic degradation of β-CN. The Cap-LC–HRMS analysis was finally sustained by direct infusion of casein standards that produced high resolution mass spectra of α_S-CN and β-CN, later used for protein identification in LC–MS.

3.5. References

[1] H.M. Farrell Jr., R. Himenez-Florez, G.T. Bleck, E.M. Brown, J.E. Butler, L.K. Creamer, C.L. Hicks, C.M. Hollar, K.F. Ng-Kwai-Hang, H.E. Swaisgood, Nomenclature of the proteins of cows' milk-sixth revision, *Journal of Dairy Science* 87 (**2004**) 1641-1674.
http://www.ncbi.nlm.nih.gov/pubmed/15453478

[2] M. Hayes, R.P. Ross, G.F. Fitzgerald, C. Stanton, Putting microbes to work: Dairy fermentation, cell factories and bioactive peptides. Part I: Overview, *Biotechnology Journal* 2 (**2007**) 426-434.
http://onlinelibrary.wiley.com/doi/10.1002/biot.200600246/abstract

[3] H. Korhonen, Milk-derived bioactive peptides: From science to applications, *Journal of Functional Foods* 1 (**2009**) 177-187.
http://www.sciencedirect.com/science/article/pii/S1756464609000085

[4] H. Korhonen, A. Pihlanto, Bioactive peptides: Production and functionality, *International Dairy Journal* 16 (**2006**) 945-960.
http://www.sciencedirect.com/science/article/pii/S0958694605002426

[5] M. Hayes, C. Stanton, G.F. Fitzgerald, R.P. Ross, Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part II: bioactive peptide functions, *Biotechnology Journal* 2 (**2007**) 435-449.
http://www.ncbi.nlm.nih.gov/pubmed/17407211

[6] M. Phelan, A. Aherne, R.J. Fitzgerald, N.M. O'Brien, Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status, *International Dairy Journal* 19 (**2009**) 643-654.
http://www.sciencedirect.com/science/article/pii/S0958694609001071

[7] L.E. Bennett, R. Crittenden, E. Khoo, S. Forsyth, Evaluation of immunomodulatory dairy peptide fractions, *Australian Journal of Dairy Technology* 60 (**2005**) 106-109.
http://www.scopus.com/record/display.url?eid=2s2.024944577031&origin=inward&txGid=8BB44815FE50D7D55647DD4E91FEBD5F.I0QkgbIjGqqLQ4Nw7dqZ4A%3a89#

[8] H. Jing, D.D. Kitts, Redox-related cytotoxic responses to different casein glycation products in Caco-2 and int-407 cells, *Journal of Agricultural and Food Chemistry* 52 (**2004**) 3577-3582.
http://www.ncbi.nlm.nih.gov/pubmed/15161233

[9] D. Migliore-Samour, F. Floc'h, P. Jollès, Biologically active casein peptides implicated in immunomodulation, *Journal of Dairy Research* 56 (**1989**) 357-362.
http://www.ncbi.nlm.nih.gov/pubmed/2668358

[10] H. Meisel, Overview on Milk Protein-derived Peptides, *International Dairy Journal* 8 (**1998**) 363-373.
http://www.sciencedirect.com/science/article/pii/S0958694698000594

[11] C. Sandre, A. Gleizes, F. Forestier, R. Gorges-Kergot, S. Chilmonczyk, J. Leonil, M.C. Moreau, C. Labarre, A peptide derived from bovine beta-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice, *Journal of Nutrition* 131 (**2001**) 2936-2942.
http://www.ncbi.nlm.nih.gov/pubmed/11694622

[12] K.F. Ng-Kwai-Hang, E.M. Kroener, Rapid separation and quantification of major caseins and whey proteins of bovine milk by polyacrylamide gel electrophoresis, *Journal of Dairy Science* 67 (**1984**) 3052-3056.
http://www.sciencedirect.com/science/article/pii/S0022030284816719

[13] H.-H.Y. Kim, R. Jimenez-Florez, Comparison of milk proteins using Preparative Isoelectric Focusing followed by Polyacrylamide Gel Electrophoresis, *Journal of Dairy Science* 77 (**1994**) 2177-2190.
http://www.sciencedirect.com/science/article/pii/S0022030294771605

[14] C.M. Hollar, A.J.R. Law, D.G. Dalgleish, R.J. Brown, Separation of major casein fractions using Cation-Exchange Fast Protein Liquid Chromatography, *Journal of Dairy Science* 74 (**1991**) 2403-2409.
http://www.sciencedirect.com/science/article/pii/S0022030291784142

[15] E. Bramanti, C. Sortino, G. Raspi, New chromatographic method for separation and determination of denatured α_{s1}-, α_{s2}-, β- and κ-caseins by hydrophobic interaction chromatography, *Journal of Chromatography A* 958 (**2002**) 157-166.
http://www.sciencedirect.com/science/article/pii/S0021967302003254

[16] S. Visser, C.J. Slangen, H.S. Rollema, Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography, *Journal of Chromatography A* 548 (**1991**) 361-370.
http://www.sciencedirect.com/science/article/pii/S0021967301886192

[17] G. Bobe, D.C. Beitz, A.E. Freeman, G.L. Lindberg, Separation and quantification of bovine milk proteins by Reversed-Phase High-Performance Liquid Chromatography, *Journal of Agricultural and Food Chemistry* 46 (**1998**) 458-463.
http://www.ncbi.nlm.nih.gov/pubmed/10554263

[18] G. Bordin, F.C. Raposo, B. de la Calle, A.R. Rodriguez, Identification and quantification of major bovine milk proteins by liquid chromatography, *Journal of Chromatography A* 928 (**2001**) 63-76.
http://www.sciencedirect.com/science/article/pii/S0021967301010974

[19] A.C.A. Veloso, N. Teixeira, I.M.P.L.V.O. Ferriera, Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea–polyacrylamide gel electrophoresis: Detection of milk adulterations, *Journal of Chromatography A* 967 (**2002**) 209-218.
http://www.sciencedirect.com/science/article/pii/S0021967302007872

[20] I. Bonizzi, J.N. Buffoni, M. Feligini, Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context, *Journal of Chromatography A* 1216 (**2009**) 165-168.
http://www.sciencedirect.com/science/article/pii/S0021967308020268

[21] B. Miralles, V. Rothbauer, M.A. Manso, L. Amigo, I. Krause, M. Ramos, Improved method for the simultaneous determination of whey proteins, caseins and para-κ-casein in milk and dairy products by capillary electrophoresis, *Journal of Chromatography A* 915 (**2001**) 225-230.
http://www.sciencedirect.com/science/article/pii/S0021967301006173

[22] J.F. Fairise, P. Cayot, New ultrarapid method for the separation of milk proteins by Capillary Electrophoresis, *Journal of Agricultural and Food Chemistry* 46 (**1998**) 2628-2633.
http://pubs.acs.org/doi/abs/10.1021/jf970880%2B

[23] I. Recio, L. Amigo, R. López-Fandino, Assessment of the quality of dairy products by capillary electrophoresis of milk proteins, *Journal of Chromatography B: Biomedical Sciences and Applications* 697 (**1997**) 231-242.

http://www.ncbi.nlm.nih.gov/pubmed/9342674

[24] I.M.P.L.V.O. Ferreira, H. Caçote, Detection and quantification of bovine, ovine and caprine milk percentages in protected denomination of origin cheeses by reversed-phase high-performance liquid chromatography of beta-lactoglobulins, *Journal of Chromatography A* 1015 (**2003**) 111-118.
http://www.sciencedirect.com/science/article/pii/S0021967303012615

[25] B. Miralles, J. Leaver, M. Ramos, L. Amigo, Mass mapping analysis as a tool for the identification of genetic variants of bovine β-casein, *Journal of Chromatography A* 1007 (**2003**) 47-53.
http://www.sciencedirect.com/science/article/pii/S0021967303009555

[26] M. Feligini, I. Bonizzi, J.N. Buffoni, G. Cosenza, L. Ramunno, Identification and quantification of alpha-S1, alpha-S2, beta, and kappa-caseins in water buffalo milk by reverse phase-high performance liquid chromatography and mass spectrometry, *Journal of Agricultural and Food Chemistry* 57 (**2009**) 2988-2992.
http://www.ncbi.nlm.nih.gov/pubmed/19256489

[27] F. Svec, Less common applications of monoliths: preconcentration and solid-phase extraction, *Journal of Chromatography B* 841 (**2006**) 52-64.
http://www.ncbi.nlm.nih.gov/pubmed/16650811

[28] V.F. Samanidou, E.G. Karageorgou, G. Eftichia, An overview of the use of monoliths in sample preparation and analysis of milk, *Journal of Separation Science* 34 (**2011**) 2013-2025.
http://onlinelibrary.wiley.com/doi/10.1002/jssc.201100101/abstract

[29] G. Angelini, O. Ursini, F. Gasparrini, C. Villani, *PCT Int. Appl.* (**2008**), WO 2008081496 A1 20080710.

[30] E. Badaloni, M. Barbarino, W. Cabri, I. D'Acquarica, M. Forte, F. Gasparrini, F. Giorgi, M. Pierini, P. Simone, O. Ursini, C. Villani, Efficient organic monoliths prepared by γ-radiation induced polymerization in the evaluation of histone deacetylase inhibitors by capillary(nano)-high performance liquid chromatography and ion trap mass spectrometry, *Journal of Chromatography A* 1218 (**2011**) 3862-3875.
http://ac.elscdn.com/S0021967311005589/1s2.0S0021967311005589main.pdf?_tid=b1658190a56e11e4899100000aab0f27&acdnat=1422285736_73c9d8d95fdf70e7e0c1d490821872dc

[31] G. Pepe, G. Tenore, C. Conte, M. Russo, M. Manfra, P. Campiglia, Susceptibility to denaturation of caseins in milk samples for improving protein conformational study and their identification, *Natural Product Research* (**2012**) 1-5.
http://www.ncbi.nlm.nih.gov/pubmed/22978643

[32] B. Wouters, A. Vaast, A. Treumann, M. Ursem, J. Ho, M. Hornshaw, M. Raes, H. Terryn, S. Eeltink, The potential of polymeric monolithic capillary columns for the LC-MS anaylsis of intact proteins, *LC–GC Europe* 25 (**2012**) 10-19.
http://www.chromatographyonline.com/potentialpolymericmonolithiccapillary-columns-lc%E2%80%93ms-analysis-intact-proteins

[33] S. Eeltink, B. Wouters, G. Desmet, M. Ursem, D. Blinco, G.D. Kemp, A. Treumann, High-resolution separations of protein isoforms with liquid chromatography time-of-flight mass spectrometry using polymer monolithic capillary columns, *Journal of Chromatography A* 1218 (**2011**) 5504-5511.
http://www.ncbi.nlm.nih.gov/pubmed/21741050

[34] S. Zheng, C. Yoo, N. Delmotte, F.R. Miller, C.G. Huber, D.M. Lubman, Monolithic column HPLC separation of intact proteins analyzed by LC-MALDI using on-plate digestion: a new approach to integrate protein separation and identification, *Analytical Chemistry* 78 (**2006**) 5198-5204.
http://pubs.acs.org/doi/abs/10.1021/ac052284h

[35] M. Wu, R. Wu, Z. Zhang, H. Zou, Preparation and application of organic-silica hybrid monolithic capillary columns, *Electrophoresis* 32 (**2011**) 105-115.
http://www.ncbi.nlm.nih.gov/pubmed/21171117

[36] R. Bakry, C.W. Huck, G.K. Bonn, Recent applications of organic monoliths in capillary liquid chromatographic separation of biomolecules, *Journal of Chromatographic Science* 47 (**2009**) 418-431.
http://chromsci.oxfordjournals.org/content/47/6/418.full.pdf+html

[37] J. Rozenbrand, W.P. van Bennekom, Silica-based and organic monolithic capillary columns for LC: Recent trends in proteomics, *Journal of Separation Science* 34 (**2011**) 1934-1944.
http://onlinelibrary.wiley.com/doi/10.1002/jssc.201100294/pdf

[38] A. Groen, R. van der Vegt, M.S. Boeckel, O.L.A. de Rouw, H. Vos, Case study on individual animal variation in milk protein-composition as estimated by high-

pressure liquid-chromatography, *Netherlands Milk and Dairy Journal* 48 (**1994**) 201-212.
http://cat.inist.fr/?aModele=afficheN&cpsidt=3399809

[39] S. Wu, N.M. Lourette, N. Tolìc, R. Zhao, E.W. Robinson, A.V. Tolmachev, R.D. Smith, L. Păsa-Tolìc, An integrated top-down and bottom-up strategy for broadly characterizing protein isoforms and modifications, *Journal of Proteome Research* 8 (**2009**) 1347-1357.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2693490/

[40] S. Wu, F. Yang, R. Zhao, N. Tolìc, E.W. Robinson, D.G. Camp II, R.D. Smith, L. Păsa-Tolìc, An integrated workflow for characterizing intact phosphoproteins from complex mixtures, *Analytical Chemistry* 81 (**2009**) 4210-4219.
http://pubs.acs.org/doi/abs/10.1021/ac802487q

[41] W.G. Gordon, M.L. Groves, R. Greenberg, S.B. Jones, E.B. Kalan, R.F. Peterson, R.E. Townend, Probable identification of γ-, TS-, R- and S-Caseins as fragments of β-Casein, *Journal of Dairy Science* 55 (**1972**) 261-263.
http://www.journalofdairyscience.org/article/S0022-0302(72)85469-9/pdf

[42] W.G. Gordon, M.L. Groves, E.B. Kalan, S.B. Jones, Composition of bovine γ-Caseins A^1 and A^3, and further evidence for a relationship in biosynthesis of γ- and β-Caseins, *Journal of Dairy Science* 55 (**1972**) 1041-1049.
http://www.journalofdairyscience.org/article/S0022-0302(72)85621-2/pdf

[43] W.G. Gordon, M.L. Groves, Primary sequence of beta, gamma, and minor casein, *Journal of Dairy Science* 58 (**1975**) 574-582.
http://www.journalofdairyscience.org/article/S0022-0302(75)84613-3/pdf

[44] R.McL. Whitney, J.R. Brunner, K.E. Ebner, H.M. Farrell, R.V. Josephson, C.V. Morr, H.E. Swaisgood, Nomemclature of the proteins of cow's milk: fourth revision, *Journal of Dairy Science* 59 (**1976**) 795-815.
http://www.journalofdairyscience.org/article/S0022-0302(76)84280-4/pdf

[45] W.N. Eigel, Formation of γ_1-A^2, γ_2-A^2 and γ_3-A caseins by in vitro proteolysis of β-casein A^2 with bovine plasmin, *International Journal of Biochemistry* 8 (**1977**) 187-192.
http://www.sciencedirect.com/science/article/pii/0020711X7790146X

[46] A.T. Andrews, The Composition, structure and origin of proteose-peptone component 8F of bovine milk, *European Journal of Biochemistry* 90 (**1978**) 67-71.

http://onlinelibrary.wiley.com/doi/10.1111/j.1432-1033.1978.tb12575.x/pdf

[47] I. De Noni, L. Pellegrino, S. Cattaneo, P. Resmini, HPLC of proteose peptones for evaluating ageing of packaged pasteurized milk, *International Dairy Journal* 17 (**2007**) 12-19.
http://www.sciencedirect.com/science/article/pii/S0958694606000264

CHAPTER IV

Evaluation of two sub-2 µm stationary phases, core-shell and totally porous monodisperse, in the second dimension of on-line comprehensive two dimensional liquid chromatography, a case study: separation of milk peptides after expiration date

Abstract

Milk is a rich source of bioactive peptides of great interest for their healthy properties.

These peptides are usually encrypted in the sequences of proteins and are released after time dependent proteolysis as very complex hydrolysates.

In order to separate and identify the bioactive sequences, we developed an on-line comprehensive two dimensional liquid chromatography approach using the high performance combined with the ultra high performance conditions. A microbore reversed phase (C18 silica, 5 μm) column was employed in first dimension, while, in second dimension, two different UHPLC columns, packed with C18 silica, were tested: a new column based on monodisperse sub-2 μm fully porous particles with high surface area (50 mm × 3.0 mm, 1.9 μm d.p., from Supelco), and a column based on sub-2 μm core-shell particles (50 mm × 3.0 mm, 1.7 μm d.p., from Phenomenex®). Both set-ups were compared, showing high peak capacity values with respect to a high efficiency monodimensional method, maintaining the same analysis time. Satisfactory selectivity was obtained through the use of different pH between the two dimension, while a very fast continuous shifted gradient in second dimension ensured a good employment of the 2D separation space.

Keywords

Sub-2 μm particles, totally porous monodisperse particles, core-shell, RP × RP, whey protein, peptides.

Abbreviations

β-Casomorphins, β-CMs; Capillary-Liquid Chromatography-High Resolution Mass Spectrometry, Cap-LC-HRMS; Capillary Zone Electrophoresis, CZE; Centrale del Latte di Salerno, CdLdS; 2,2'-Diphenyl-1-picrylhydrazyl, DPPH; Electrospray Ionization, ESI; High Performance Liquid Chromatography, HPLC;

High Resolution Mass Spectrometry, HRMS; 1,6-Hexanediol Dimethacrylate, HDDMA; Lauryl Methacrylate, LMA; Liquid Chromatography, LC; Liquid Chromatography-Mass Spectrometry, LC-MS; Loading Pump, L.P.; Mass Spectrometry, MS; Micro Pump, μ.P.; Monodimensional, 1D; Nominal Molecular Weight Limit, NMWL; Proteose-Peptones, PP; Reversed Phase×Reversed Phase, RP × RP; Reversed Phase × Hydrophilic Liquid Chromatography, RP × HILIC; Solid Phase Extraction, SPE; Solid Phase Microextraction, SPME; Stir Bar Sorptive Extraction, SBSE; Total Ion Current, TIC; Trifluoroacetic Acid, TFA; Two Dimensional Gel Electrophoresis, 2D-GE; Two Dimensional Liquid Chromatography, 2D-LC; Ultra High Performance Liquid Chromatography, UHPLC.

4.1. Introduction

Bovine milk proteins represent a very complex biological matrix, that possesses not only an important nutritional value but has a deep connection with many physiological processes. Milk proteins, which are constituted by two major families of proteins: caseins (insoluble) and whey proteins (soluble), are involved in a wide range of biochemical mechanisms, and exert many activities including: immunomodulatory, anti-hypertensive, anti-bacterial, opioid and also anti-carcinogenic [1]. The beneficial health effects are related to the bioactive peptides, encrypted in the amino-acid sequence of milk proteins. These peptides are latent until released by enzymatic proteolysis that occurs from bacterial activity or in the gastrointestinal digestion [2]. Fermented milk is a rich source of bioactive peptides; in fact the proteolytic system of bacteria, mainly endopeptidases and exopeptidases, enhances the degradation and leads to the release of peptides having various regulatory functions [3]. The identification of bioactive peptide sequences is an important step to understanding the biological function of these compounds and their possible employment in nutraceutical formulations as well as in enriched and personalized milks. Milk protein hydrolysates represent a complex sample containing up to 103 different peptides and, their identification is a challenging task. A variety of methods are employed to analyze milk peptides and proteins: electrophoretic techniques such as 2D-GE, CZE or gel free techniques like HPLC [4,5]. Among these, HPLC allows rapid and automated analysis, high resolution, repeatability and easy hyphenation with mass spectrometry, which is the workhorse in the field of protein analysis. Despite the huge improvements in MS analyzers technology, which are more and more sensitive and accurate, front-end separation techniques are still essential in order to not overwhelm the mass spectrometer source and prevent ion suppression phenomena which lead to impaired detection of low abundant peptides [6].

Finally, in this regard monodimensional approaches usually cannot provide adequate values of resolution and more often two dimensional liquid chromatography methods are employed. 2D-LC, operated in the "comprehensive" mode (LC × LC), is the best way to obtain high values of peak capacity. In this approach the entire eluate from first dimension (1D) is continuously collected and injected on-line into the second dimension (2D) through an interface, usually represented by a multiport switching valve. Many on-line LC × LC systems have been developed for peptide analysis using a combination of separation techniques [7-9]. Among them, RP × RP represents the combination that provides the highest practical peak capacity values [10,11]. Recently, comprehensive LC × LC using RP × HILIC and HILIC × RP was described as very promising for its high degree of orthogonality [12]. Furthermore in HILIC mode, highly organic mobile phases provide high desolvation efficiency, thus better coupling with ESI-MS detection [13]. The development of sub-2 μm stationary phases and instruments capable of higher pressure limits led to the rapid expansion of ultra high performance liquid chromatography methods, characterized, with respect to conventional HPLC methods, by superior resolution and very short analysis time. Nowadays, the employment of UHPLC conditions in LC × LC has proved to be a valuable tool in terms of speed and performance for the separation of pharmaceutical samples, complex natural matrices, polyphenols and polymers [14-17].

This is why, in the present work, the described technique has been used to study the degradation pattern of low-medium molecular weight peptides derived from soluble fraction of semi-skimmed milk after four weeks. For this purpose, after an ultrafiltration through centrifugal filters with different cutoff and subsequent SPE, the peptide fraction with molecular weight < 3,000 Da was collected. A comprehensive RP × RP platform, consisting of a microbore (5 μm, 1.0 mm I.D.) C18 column in 1D, and in 2D, was developed while two different sub-2 μm (3.0 mm I.D.) stationary phases were investigated, based on 1.9 μm totally porous

monodisperse particles, and 1.7 μm core-shell column, respectively. The two dimensions were coupled through an ultra high pressure 10 port-two position switching valve and two different modulation times (60 s and 45 s) were used together with a 2D shifted gradient in order to maximize the peak capacity. Different pH conditions of mobile phase were used in the two dimensions (basic in 1D and acidic in 2D) allowing significant selectivity, despite the employment of the same separation mode (RP). Results were compared with those obtained with a high efficient monodimensional separation with the same analysis time, and corrected taking into account both the under-sampling effect, and the orthogonality of the system developed. Results obtained highlighted the effectiveness of the LC × UHPLC analysis system developed, presenting itself as a valuable tool, for the study of time dependent degradation of proteins belonging to the soluble fraction milk.

4.1.1. Aim of work

In the last year of my PhD, the degradation pattern of the soluble fraction of milk samples stored at room temperature, after expiration date, was analyzed in order to identify a time window for the recovery of bioactive molecules.

With the aim to extend the time-dependent degradation study also at the soluble fraction of milk, a LC × UHPLC platform was developed. The new 2D chromatographic system allows high values of resolution and peak capacity for the separation of the peptides derived from milk soluble fraction after four weeks from the expiration date. These peptides, mainly in the low-medium molecular weight range, represent a rich source of bioactive peptides and may be used in the formulation of functional foods and cosmetics.

4.2. Materials and methods

4.2.1. Chemicals

Ultra pure water (H_2O) was obtained by a Direct-8 Milli-Q system (Millipore, Milan, Italy), LC-MS grade acetonitrile (ACN) and additives formic acid (HCOOH), ammonium acetate (CH_3COONH_4), TFA, reagent grade glacial acetic acid (CH_3COOH) and 1 M sodium-acetate (CH_3COONa) solution, the standard peptide mixture and the small molecule test mixture were all purchased from Sigma Aldrich (St. Louis, Mo, USA). Centrifugal Filter Devices Amicon®Ultra-4 3 K and 10 K and Strata C18-SPE cartridge were purchased by Millipore© and Phenomenex® (Castel Maggiore, Bologna, Italy), respectively, while filter paper Whatman® 540 from Sigma Aldrich.

4.2.2. Sampling and sample preparation

Commercial samples of semi-skim milk from Centrale del latte di Salerno (Salerno, Campania, Italy) were analyzed; samples were stored at room temperature and the corresponding peptide fractions, generated by enzymatic hydrolysis in the products during fermentation, were extracted at four weeks after expiration date. Samples were prepared by separating the residual fat fraction from expired semiskim milks: 50 mL of milk were subjected to centrifugation at 4000 × *g* for 30 min at 4 °C (Mikro 220R, Hettich, Germany). To harden the cream, the tubes were kept at −20 °C for 10 min and finally the cream was removed. Skim milk sample was diluted with distilled water (1:10, *v/v*) and the solution thus obtained was shake vigorously. The caseins were removed from skim milks by precipitation at pH 4.3 (20 °C) with addition of glacial acetic acid (10% *v/v*) and 1 M sodium-acetate solution. The acidified milk was centrifuged for 15 min at 3000 × *g* (20 °C) and subsequently the precipitated caseins were eliminated by double filtration using, firstly by filter

paper (particle retention 22 μm) for retention of coarse and gelatinous precipitates and subsequently again by filter paper (8 μm), to a final volume of 40 mL.

The soluble fraction thus obtained was analyzed by Capillary HPLC-UV and Capillary-LC-HRMS and subsequently the whey was processed to collect the low-medium molecular weight peptides (see afterwards).

4.2.3. Peptides isolation

The supernatant of skimmed milk samples were ultra-filtered by Millipore's Amicon®Ultra-4 centrifugal filter devices at different cutoffs. A preliminary filtration was carried out for all the samples using filters with 10,000 NMWL and subsequently 4 mL of permeate were loaded on devices with 3000 NMWL. The devices were centrifuged for 40 min at 6000 rpm at 25 °C, using a centrifuge with fixed angle rotor (35 °). In order to recover the peptides, devices were washed with 4 mL of acidified water at pH 3.0 by formic acid. A peptide fraction of about 3,5 mL, with molecular weight <3000 Da was collected, filtered through a 0.45 μm pore cellulose membrane (Millipore©) and lyophilized for 24 h (LyoQuest-55, Telstar Technologies, Spain). This fraction was solubilized in 0.1% (*v/v*) aqueous TFA, and purified from salts and sugars with a Strata C18-E cartridge (Phenomenex®), previously equilibrated in 0.1% *v/v* TFA, then eluted with 70/30/0.1 ACN/water/TFA (*v/v/v*) and finally re-lyophilized and stored at −20 °C. Twenty milligram of the lyophilized were reconstitued in 1 mL of water and analyzed.

4.2.4. Columns

4.2.4.1. Organic monolithic column

The degradation of whey proteins fractions at different times (week I-IV) after expiring date was investigated by injecting the soluble fractions on a Protein-Cap-

RP-Lauryl-γ-Monolithic lab made column (250 × 0.250 mm, L. × I.D.) prepared following the protocol reported in section 3.2.3.

4.2.4.2. HPLC columns

For high efficiency monodimensional comparative analysis two Titan^{TM} 100 mm × 2.1 mm 1.9 μm (80 Å) (Supelco, Bellefonte, PA, USA), and two Kinetex^{TM} 100 + 50 × 2.1 mm, 1.7 μm (Phenomenex®) were employed; the columns were placed in series by a 6 cm × 0.100 mm I.D. viper capillary (Thermo Fisher Scientific, Milan, Italy).

In LC × UHPLC analysis a Discovery®C18 Bio Widepore 150 mm × 1.0 mm, 5 μm (300 Å) from Supelco was used in 1D of the LC × UHPLC system. In 2D two different columns were tested: the Kinetex^{TM} C18 50 mm × 3.0 mm, 1.7 μm (100 Å) from Phenomenex® and a Titan^{TM} C18 50 mm × 3.0 mm, 1.9 μm (80 Å) from Supelco. Moreover a Kinetex^{TM} C18 50 mm × 3.0 mm, 2.6 (100 Å) from Phenomenex® was used for comparison purpose in kinetic performance evaluation.

4.2.5. Evaluation of column perfomances

4.2.5.1. Instrumentation

The monolithic column was characterized in terms of efficiency and permeability by HPLC system (Model UltiMate LPG-3000, Dionex, Sunnyvale, CA, USA), equipped with two low-pressure gradient micro-pumps (Model DGP-3600M), a vacuum degasser (Model DG-1210), a micro-autosampler (Model WPS-3000TPL) equipped with a 5 μL loop, and a UV-detector (Model VWD-3100, cell 20 nL).

For the evaluation of kinetic performances of the sub-2μm columns a Waters Acquity UPLC (Waters, Millford, MA, USA) was employed, consisting of a binary solvent manager, a column heater (from 4 to 65 °C) and a Waters 2996 PDA detector with a 500 nL flow cell. To reduce the extra-column volume nano viper capillaries of 250 mm × 0.075 mm L. × I.D. (internal volume: 1.10 μL) were used

(Thermo Fisher Scientific, Milan, Italy). The total extra-column volume was determined injecting uracile by using a zero dead volume union and was estimated in 4.11 μL. The instrument variance, calculated at peak width half height, ranges from 0.58 to 0.87 μL^2 (from 0.1 to 2.0 mL/min).

4.2.5.2. Chromatographic conditions: kinetic evaluation of monolithic column

The organic monolithic column was characterized in terms of efficiency and permeability using a set of small molecule probes [1) uracile, 2) phenol, 3) benzaldehyde, 4) nitro-benzene, 5) benzene, 6) toluene, 7) ethyl-benzene, 8) *n*-propyl-benzene 9) *n*-butyl-benzene, 10) *n*-pentyl-benzene] eluted with CH_3CN/H_2O, 60/40 (*v/v*) as mobile phase at different flow rates and a fixed temperature of 25 °C.

4.2.5.3. Efficiency evaluation of sub-2 μm columns

The van Deemter analysis and Poppe plot were used to compare the efficiency of the three tested columns and data fitting of the curves was performed using Origin 6.0 software. Mobile phase used was composed by ACN/H_2O; 60/40, *v/v*; (viscosity given as (η): 0.59×10^{-3} Pa s at 35 °C) and column oven was set at 35 °C. The UV wavelength detector was set at 214 nm, while detector parameters were as follows:

- Sampling rate: 80 points/s;
- Resolution: 4.8 nm;
- No filters.

A volume of 0.5 μL was injected for each analysis. Plots were produced using a mixture of test compounds solubilized in mobile phase. The number of theoretical plates was calculated according to the European Pharmacopeia, using peak width at half height as implemented in the software. 2D columns were also tested with peptides < 3,000 Da fraction in fast gradient elution using (A) H_2O/ACN 95/5 (*v/v*)

+ 0.1% (*v/v*) TFA; (B) ACN/H_2O 95/5 (*v/v*) + 0.1% (*v/v*) TFA as eluents and a flow rate of 1.5 mL/min. The column oven was set 40 °C and the gradient for both column was: 0-5 min, 0-15% B, 5-10 min, 15-40% B, hold for 2 min at 40% B.

4.2.6. Analysis of soluble fractions

4.2.6.1. Analysis of whey proteins by Cap-HPLC-UV

Whey proteins extracted at different weeks (I-IV) after expiring date was investigated by injecting the soluble fraction previously described on a 25-cm-long lab-made monolithic column and a trapping column (Protein-Cap-RP-Lauryl-γ-Monolithic column, 50 mm × 0.200 mm, L. × I.D.). The chromatographic system used was the UltiMate LPG-3000 instrument.

The mobile phases A and B consisted of 95/5 water/acetonitrile (*v/v*) and 5/95 water/acetonitrile (*v/v*), respectively. Both mobile phases were acidified with 0.1% *v/v* TFA.

Load-trapping injection mode was performed using a 10-port switching valve, as described earlier. The sample was dissolved in mobile phase A and was pumped into the loading column (valve in position 10_1) using the L.P., at a flow rate of 10 µL/min, during 5 min. It was then transferred to the separation column (valve in position 1_2) using the µ.P. with a linear gradient of mobile phase B at a flow rate of 10 µL/min. Finally, suitable column regeneration and equilibration was performed before starting a second analysis. A 65-min gradient elution from 10% to 35% of eluting mobile phase (B) was used. The column temperature was set at 60 °C. Injection volume of samples was 0.5 µL. The UV detector was set at a time constant of 0.10s and a data collection rate of 40Hz. UV detection was performed at 214 nm. Chromatographic data were collected and processed with Chromeleon 6.8 (Dionex, Sunnyvale, CA, USA).

4.2.6.2. Analysis of whey proteins by Cap-LC-MS

Mass spectrometry detection was performed on an Exactive Orbitrap Mass spectrometer (Thermo Fisher Scientific, San José, CA, USA), using a mass range of 500-4000 m/z. A resolution of 10,000 in positive ESI mode was used with a sheath gas flow of 15.0 units, an auxiliary gas of 5 units and a spare gas of 4.0 units. Spray voltage was set at 2.3 kV, capillary voltage at 30 V, skimmer voltage at 25 V, capillary temperature at 275 °C and tube lens voltage at 240 V. Mass spectra were collected and processed using Xcalibur software and deconvoluted mass spectra were obtained by using Xtract.

4.2.7. 1D and LC × UHPLC analysis of peptides

4.2.7.1. Instrumentation

1D and LC × UHPLC analyses were performed on a Shimadzu Nexera LC-30A (Shimadzu, Milan, Italy), consisting of a CBM-20A controller, four LC-30AD dual-plunger parallel-flow pumps, a DGU-20 A_{R5} degasser, an SPD-M20A PDA detector (equipped with 2.5 μL detector flow cell volume), a CTO-20AC column oven, a SIL-30AC autosampler. The two dimensions were connected by a ultra high pressure 10 port-two position switching valve with micro-electric actuator (model FCV-12 AH, 1.034 bar; Shimadzu, Kyoto, Japan), placed inside the column oven and equipped with two identical 0.254 mm I.D. stainless steel sample loops (22 μL each) to alternatively collect and deliver fractions from 1D to 2D. Two mixers were housed inside the column oven too and were 180 μL and 40 μL for 1D and 2D, respectively. A 55 cm × 0.100 mm I.D. stainless steel tubing was used to connect the autosampler to 1D column, while a 10 cm × 0.100 mm I.D. viper capillary was used to connect the 10 port switching valve with 2D column (Thermo Fisher Scientific, Milan, Italy). All other connections were 0.100 mm I.D. and kept of the shortest length possible. A total extra-column volume of 28.6 μL was determined injecting toluene by using a zero dead volume union in place of the

column. Both dimensions and the switching valve were controlled by the LCMS solution® software (Version 5.54, Shimadzu). The instrument was coupled online with a LCMS-IT-TOF (Shimadzu) equipped with an electrospray source operated in positive mode. The LC × LC data were visualized and elaborated into two and three dimensions using Chromsquare® ver. 1.5.01 software (Chromaleont, Messina, Italy).

4.2.7.2. Chromatographic conditions: comparative high efficiency monodimensional (1D) analysis

1D analysis were carried out with the following parameters: the mobile phases (A) and (B) consisted of water and acetonitrile respectively, and were buffered with 0.1% (*v/v*) of TFA. The selected gradient: 0-70 min, 0-30% B, 70-85 min, 35-45% B, 85-90 min, 45-90% B. A flow rate of 0.6 mL/min was employed and the column oven was set at 60 °C. The PDA detector parameters were: sampling rate 12.5 Hz, time constant 0.1 s, wavelength 220-214 nm. A volume of 3 μL was injected.

4.2.7.3. Chromatographic conditions: LC × UHPLC (set-up 1 and 2)

1D mobile phase was (A): 10 mM CH_3COONH_4 in water (adjusted at pH 8.9 with NH_4OH), (B): ACN. The employed gradient was: 0-5 min, 0% B, 5-20 min, 0-35% B, 20-85 min, 35-50% B, 85-90 min, 50-95% B. Flow rate was set at 20 μL/min. Column oven was set at: 25 °C. 2 μL were injected. 2D mobile phase was (A) 0.1% TFA in water pH^w 2 *v/v*, (B) ACN (plus 0.1% TFA) *v/v* pH^a 2. Gradient was operated in continuous shifting mode as depicted in Figure 4.1. Flow 2.5 mL/min. Column oven was set at 60 °C. PDA detection parameters were: sampling rate 100 Hz, time constant 0.025 s, wavelength 220-214 nm. Two modulation times were tested: 60 s (condition a), 45 s (condition b) corresponding to an injection volume of 20 and 15 μl, respectively.

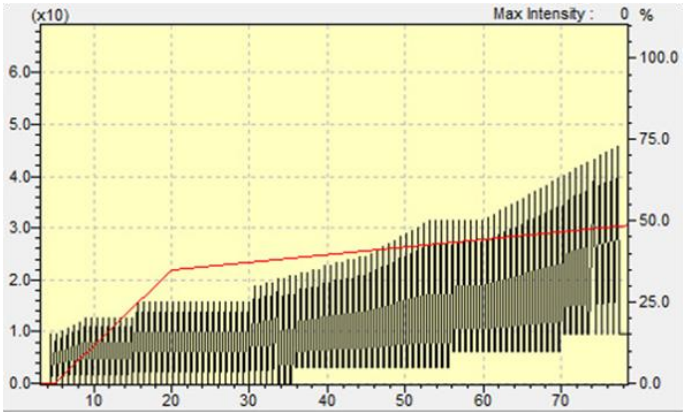


Figure 4.1. *Expansion of software window showing the ^{2}D continuous shifted gradient.*

4.2.8. Identification of peptides

The identity of peptides extracted was assessed by 1D-LC-ESI-IT-TOF, with the following parameters:

ESI^{+}, curve desolvation line (CDL) 200 °C, Block Heater 200 °C, Nebulizing gas 1.5 L/min, Interface Voltage + 4.5 kV, Drying gas 100 kPa. MS: Scan 200-2000 m/z, Ion trap repeat: 3, ASC 70%. MS/MS: data dependent mode, precursor ion triggering at BPC: 150,000, scan 150-2000. Exclusionion: by list. CID 50%, Energy 50%.

The Instrument was tuned with a Sodium trifluoroacetate solution, in automatic mode. Sample: Semi-skimmed milk T4. The data were elaborated with the Protein analysis software (Shimadzu).

4.3. Results and discussion

4.3.1. Analysis of whey proteins present in bovine milk

4.3.1.1. Kinetic and thermodynamic evaluation of monolithic column

The Protein-Cap-RP-Lauryl-γ-Monolithic column (250 × 0.250 mm L. × I.D.) was characterized in terms of efficiency and permeability. Flow resistance properties of the monolithic columns were observed by monitoring the pressure drop across 250 mm length columns at different flow-rates using a water/acetonitrile 40/60 (*v/v*) mobile phase. Strictly linear plots were obtained for a linear velocity range extending from 1.00 to 3.00 μL/min, indicating complete absence of compression phenomena of the monolith in response to pressure stress (Figure 4.2.).

The column permeability K_0 was calculated according to equation (1), where μ_0 represents the linear flow velocity, η is the viscosity of the solvent, L is the column length, Φ is the flow rate and ΔPc is the pressure drop across the column.

$$K_0 = \frac{\mu_0 \eta L}{\Delta Pc} \tag{1}$$

Assuming viscosity value as $\eta = 0.72 \times 10^{-3}$ Pa s at 25 °C, the equation 1 gave $K_0 = 7.01 \times 10^{-14}$ m^2, with a total porosity $\varepsilon_T = 0.72$, as calculated by equations 2 and 3, where K_F represents the specific permeability and r is the radius of column.

$$\varepsilon_T = \frac{K_0}{K_F} \tag{2}$$

$$K_F = \frac{\eta L \Phi}{r^2 \pi \Delta Pc} \tag{3}$$

The good permeability obtained allowed the use of high eluent flow-rates under "high speed" conditions, and could, in principle, be used for the preparation of longer columns affording increased resolution and peak capacity.

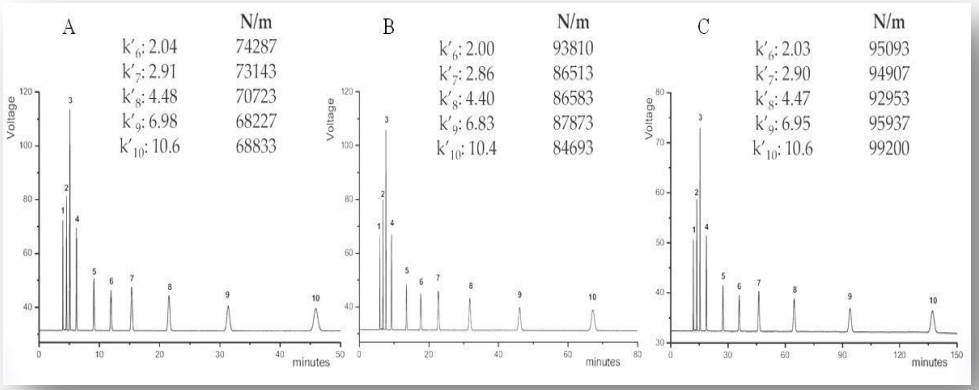


Figure 4.2. *Chromatographic characterization and efficiency test of Protein-Cap-RP-Lauryl-γ-Monolithic column (250 × 0.250 mm L. × I.D.). (A): Φ: 3.00 μL/min, ΔP_c: 817 psi; (B) Φ: 2.00 μL/min, ΔP_c: 523 psi; Φ: 1.00 μL/min, ΔP_c: 287 psi.*

The lowest achievable plate height H_{min} and the corresponding optimal linear velocities (Figure 4.3.) were calculated from the van Deemter equation (4), where A represents the eddy diffusion (6.06 μm^2), B/μ is longitudinal diffusion (1.11 μm^2 /ms) and $C \cdot \mu$ is the mass transfer (4.84 ms):

$$H = A + \frac{B}{\mu} + C \cdot \mu \qquad (4)$$

A value of H_{min}= 10.6 μm (N/m = 99,200), corresponding to optimum linear velocity μ_{opt} = 0.47 mm/s, and optimum superficial linear velocity $\mu_{SF,opt}$ = 0.34 mm/s (referred to *n*-pentyl-benzene).

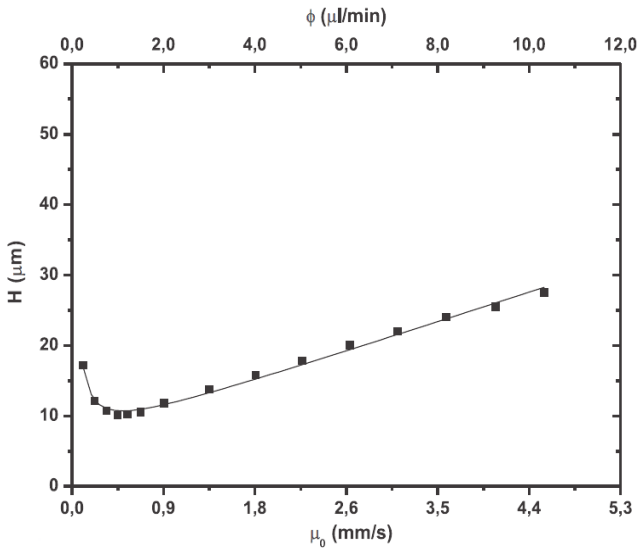


Figure 4.3. *Experimental van Deemter for n-pentyl-benzene, column: Protein-Cap-RP-Lauryl-γ-Monolithic column (250 × 0.250 mm, L. × I.D.). Mobile phase: ACN/H_2O; 60/40, v/v; η: 0.72 × 10^{-3} Pa s at 25 °C. Column oven: 25 °C; Detection: UV at 214 nm. Injection volume: 50 ηL.*

4.3.1.2. Analysis of whey proteins in Cap-LC-UV and Cap-LC-HRMS

For the chromatographic characterization of the whey proteins a monolithic support previously developed was used as stationary phase, allowing a good separation and efficient identification of the main proteins both using data gathered from literature and analitycal parameters of commercially available standards (Figure 4.4.).

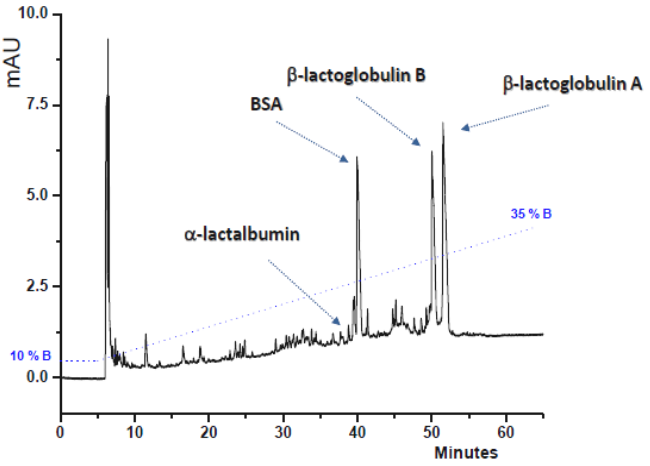


Figure 4.4. *Chromatographic profile of whey fraction. Column: Protein-Cap-RP-Lauryl-γ-Monolithic (250 × 0.250 mm, L. × I.D.); Pre-column: Protein-Cap-RP-Lauryl-γ-Monolithic (50 × 0.200 mm, L. × I.D.). Elution: from 10% to 35% of mobile phase B with a 65-min gradient time; flow rate: 10 µL/min. Tcol = 60 °C. UV detection at 214 nm. See Section 4.2.6.1. for full experimental details.*

The identity of whey proteins was confirmed by Cap-LC-MS studies. Figure 4.5. shows the total ion current (TIC) chromatograms obtained through Cap-LC-HRMS during the analysis of whey proteins deriving from semi-skimmed milk.

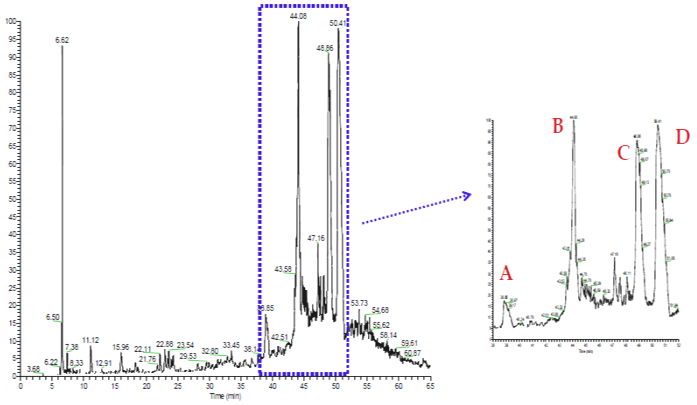


Figure 4.5. *Total ion current of whey fraction using a 250 cm long protein-Cap-RP-Lauryl-γ-Monolithic column (0.250 mm I.D.). Elution: 70-min gradient from 15% to 45% of mobile phase (B); flow rate: 10 μL/min. Tcol = 60 °C. Exactive Orbitrap MS-ESI (+) detector. R = 10,000, mass range: 500-4000 amu. Full experimental details in Section 4.2.6.2.*

Monoisotopic and averaged molecular masses values reported in Figure 4.6. and 4.7. are normally reported in literature for α-Lactalabumin and Bovine Serum Albumin, respectively.

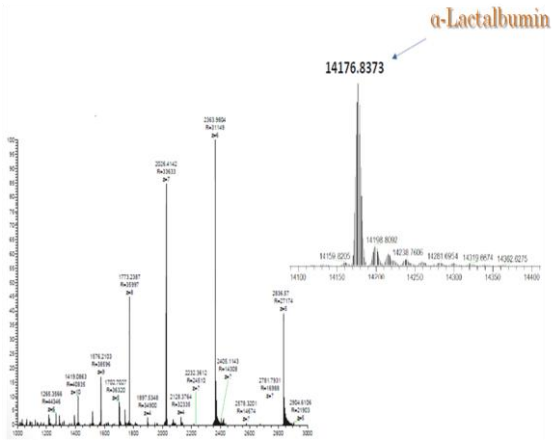


Figure 4.6. *Mass spectrum of peaks A in TIC chromatogram of whey protein. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum in Figure 4.5. using Xtract software. Averaged molecular masses are reported, determined by matching the measured isotopic pattern to a calculated isotope pattern obtained from the elemental composition of an average hypothetical amino acid.*

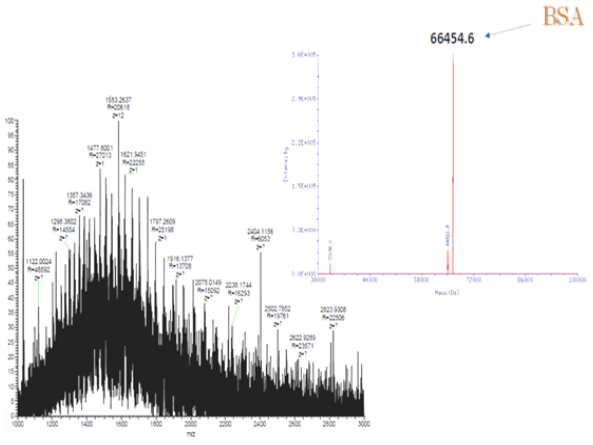


Figure 4.7. *Mass spectrum of peaks B in TIC chromatogram of whey fraction. Deconvoluted spectra of BSA obtained using the Promass software.*

The deconvolution of mass spectra reported in Figure 4.8. and 4.9., corresponding to two different β-Lactoglobulin variants, identified as isoform B and A, respectively.

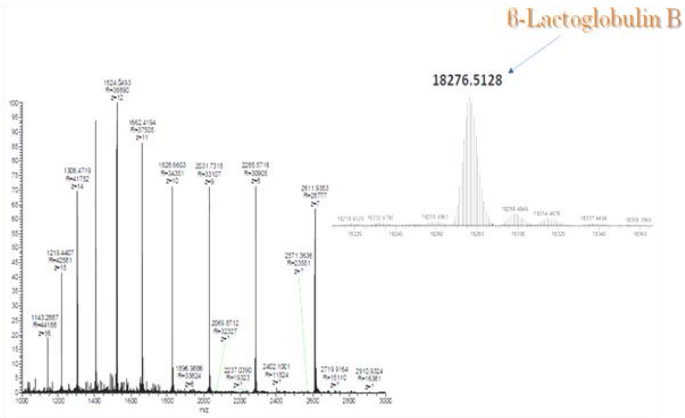


Figure 4.8. *Mass spectrum of β-Lactoglobulin B. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum in Figure 4.5. using Xtract software.*

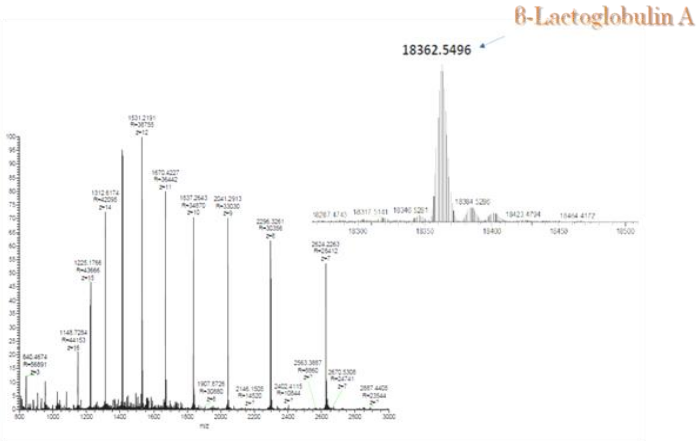


Figure 4.9. *Range of exact molecular masses of β-Lactoglobulin A. Monoisotopic exact molecular masses were obtained by deconvolution of high resolution mass spectrum in Figure 4.5. using Xtract software.*

4.3.1.3. Cap-LC-UV analysis of whey proteins extracted from semi-skim milk after expiration date.

Commercial samples of milk from CdLdS were preserved at room temperature and the corresponding soluble fractions, were extracted and analyzed at different weeks (weeks I–IV) after expiration date. In Figure 4.10. the chromatographic profile of the whey protein are shown.

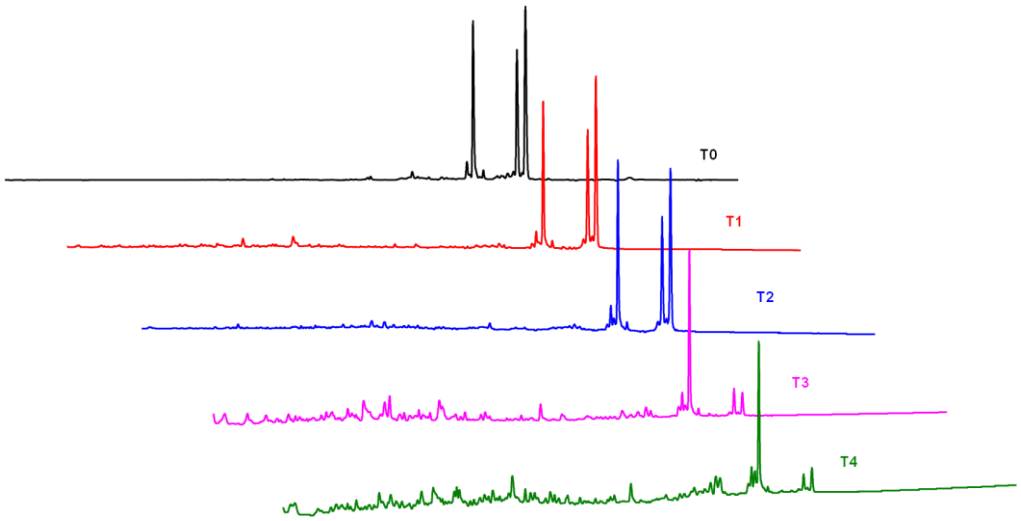


Figure 4.10. *Chromatographic profile of whey fraction extracted from semi-skimmed milk at weeks I-IV from expiry date. Column: Protein-Cap-RP-Lauryl-γ-Monolithic (25 cm × 0.250 mm, L. × I.D.). Elution: from 10% to 35% of mobile phase B with a 70-min gradient time; flow rate: 10 μL/min. Tcol = 60 °C. UV detection at 214 nm. See Section 4.2.6.1. for full experimental details.*

The chromatographic profile of T0 milk (black line) corresponds to the whey fraction extracted from milk before its expiring date, when the degradation of proteins due to milk acidity and bacteria is almost totally lacking. Comparing T0

milk with the soluble fractions at week I (red line) and week II (blue line), a very similar profile was obtained.

In weeks III and IV (pink and green lines, respectively), the chromatographic profiles of the soluble fraction significantly change. In particular, there is an increment of peaks with lower retention times and a contemporary loss of peaks in the area of the chromatogram between 50 and 55 min, where the different isoforms A and B of β-Lactoglobulin are eluted, witnessing a huge increase in major proteins degradation.

The comparison of the UV chromatographic profiles of semi-skimmed milk show that α-Lactalabumin and Bovine Serum Albumin are stable at room temperature after long period of times (4 weeks after expiring date) while it is evident the degradation of β-Lactoglobulin.

4.3.2. Analysis of peptides

4.3.2.1. Kinetic and thermodynamic evaluation of 2D columns

In online comprehensive LC × LC the sampling period is a crucial factor. Ideally, at least 3-4 fractions of each peak should be collected in the first-dimension and transferred in the second-dimension separation, to not incur in the "*undersampling*" effect [19]. In this regard 2D separation should be as fast as possible, and thus, short very efficient column are required [20]. In this work we compared the performance of two sub-2 μm commercial columns: a C-18 Kinetex™ core shell and a C18-Titan™ fully porous monodisperse column. The RP Kinetex™ column based on 1.7 μm particles represents the second generation of stationary phases developed on core shell silica supports where the fused core technology meets the particles of sub-2 μm diameter [21]. As a consequence of this, the 1.7 μm Kinetex™ delivers efficiency gain compared to the 2.6 μm parent (black data for 1.7 μm column and green data for 2.6 μm column in Figure 4.11.A.) especially at higher linear velocities, realizing very fast separations with a limited loss of efficiency. Similar

trend of H vs μ plot was recorded for the fully porous monodisperse Titan™ C18 column, recently introduced in the market and deeper characterized by Gritti et al. [22]. In particular, the lowest achievable plate height H_{min} and the corresponding optimal linear velocities (μ_{opt}) were calculated from the van Deemter equation (4). A value of H_{min} = 3.46 μm, corresponding to μ_{0opt} = 4.73 mm/s, was found for the Titan™ column, while the Kinetex™ column slightly better performs at higher linear velocities having the coordinates of minimum (H_{min}= 3.27 μm, μ_{0opt} = 5.65 mm/s) shifted in the right zone of the curve. In both cases, the sub-2 μm columns maintain high efficiency at linear velocities over 6.00 mm/s (≈ 1.3 mL/min).

Kinetic performance data have been confirmed by Poppe plot (Figure 4.11.B.) [23]. Again, in the short length/high velocities zone (bottom left corner in Figure 4.11.B.) the two sub-2 μm columns showed very similar performances much better than 2.6 μm Kinetex. The comparable kinetic performances make these columns ideal supports for the second dimension of LC × UHPLC platform, where very fast separations are needed [6]. Instead, a different behavior in terms of retention (thermodynamic parameter) was observed between the two columns. As shown in Figure 4.12. by eluting a mixture of small molecules, the retention factors of Titan™ column were higher respect to the core shell counterpart. The effect, clearly evident from the fourth eluted peak, correlates with the higher surface area of fully porous particles. Being the speed of second dimension cycle time crucial, we eluted the peptides derived from the soluble fraction on both columns with a fast gradient (tg= 10 min) and high flow rates (1.5 mL/min) obtaining a complete analysis in less than 10 min (Figure 4.13.). Finally, to improve the analysis time of 2D, a flow rate of 2.5 mL/min was selected, which was the best compromise in terms of resolution, re-equilibration time, and system backpressure. Taking into account the differences of kinetic (1.7 μm Kinetex™ better performs) and thermodynamic (1.9 μm Titan™ is more retentive) properties, the employ of

Titan^{TM} column is preferred whit slightly larger injection volumes (20 μL respect to the 15 μL).

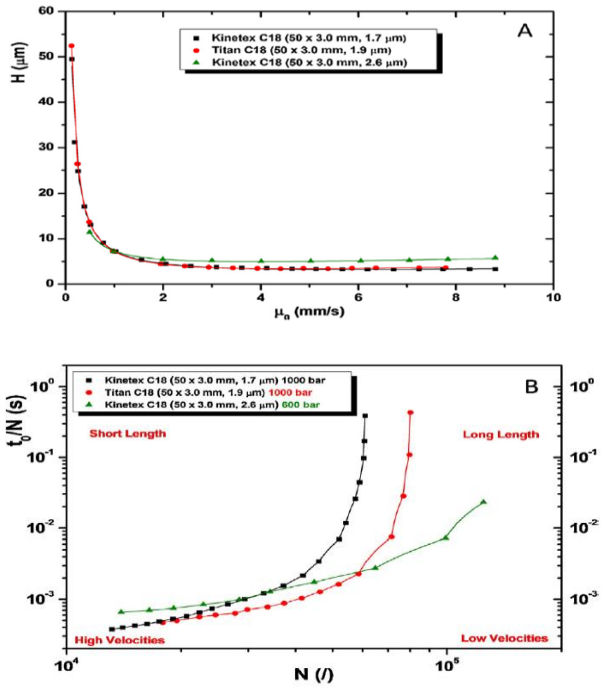


Figure 4.11. *Experimental van Deemter (A) and Popple plot (B) for ethylbenzene, columns: (■) Kinetex^{TM} C18 50 × 3.0 mm, 1.7 μm, (●) Titan^{TM} C18 50 × 3.0 mm, 1.9 μm, (▲) Kinetex^{TM} 50 × 3.0 mm, 2.6 μm. Mobile phase: ACN/H_2O; 60/40, v/v; (viscosity given as (η): 0.59 × 10^{-3} Pa s at 35 °C). Column oven: 35 °C; Detection: UV at 214 nm, sampling rate: 80 points/s; resolution: 4.8 nm; no filter. Injection volume: 0.5 μL.*

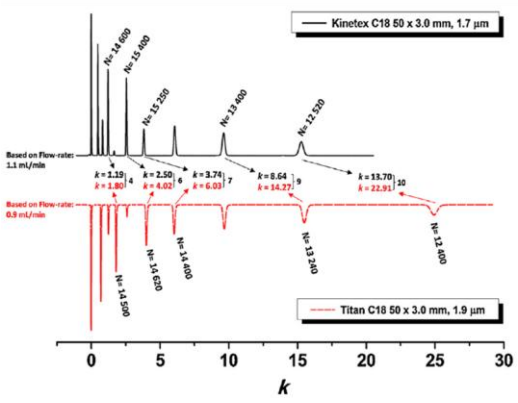


Figure 4.12. *Comparison of isocratic elution on the Kinetex™ C18 50 × 3.0 mm, 1.7 µm (black line), Titan™ C18 50 × 3.0 mm, 1.9 µm (red dotted line). Column oven: 35 °C; Detection: UV at 214 nm, Sampling rate: 80 points/s; resolution: 4.8 nm; no filter. Injection volume: 0.5 µL. Sample: small molecule mixture. Results are expressed as retention factors (k) and theoretical plates per column (N).*

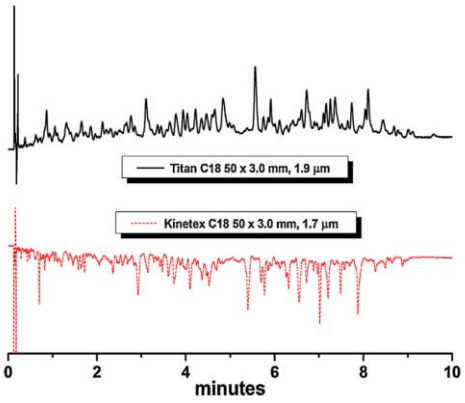


Figure 4.13. *Comparison of a fast gradient elution on the Kinetex™ C18 50 × 3.0 mm. 1.7 µm (red dotted line), Titan™ C18 50 × 3.0 mm, 1.9 µm (black line). Instrument: Waters UPLC; Detector: PDA; Cell: 500 nL; UV at 214 nm; Tcol: 40 °C; Vinj: 6.0 µL; Mobile phase: (A) H_2O/ACN 95/5 v/v + 0.1% v/v TFA; (B) ACN/H_2O 95/5 v/v + 0.1% v/v TFA; Flow rate: 1.5 mL/min. Sample: peptides < 3,000 Da fraction.*

4.3.2.2. Preliminary search of chromatographic condition in both dimensions

After the isolation of the peptide fraction of molecular weight < 3,000 Da through centrifugal filters [24], and subsequent enrichment and cleaning from contaminants, mainly oligosaccharides, by SPE, we firstly optimized the separation in the two dimension independently before tuning together. Since an RP × RP strategy was adopted, particular attention has been paid to several factors such as the orthogonality of the system, on-column peak focusing and fast 2D separation [11]. In 1D, the choice of operating under basic pH was aimed to obtain selectivity with respect to 2D, carried out using acidic mobile phase, due to the changes in charge on amines and carboxylic acids [10]. A microbore (1.0 mm I.D.) column was employed as 1D in order to inject small volumes in the second dimension and suppress band broadening phenomena [25-27] that can derive from the injection of large volumes in the second dimension, furthermore no flow splitting was necessary, thus avoiding possible loss of sensitivity and additional dispersion in the flow split. A flow rate of 20 μL/min gave the best results, in terms of peak overlap and separation. The separation in 2D was carried out a temperature of 60 °C for both columns to further speed the separation, shorten the re-equilibration times, and improve peak shapes. As expected, trifluoroacetic acid gave better peak shape, in comparison with formic acid [28].

4.3.2.3. Application of the LC × UHPLC approach to the separation of peptides of soluble fraction of milk

In peptide mapping, when the amount of sample is limited, miniaturized separation techniques such as micro-nano HPLC, are necessary. However when sample is sufficient, UHPLC systems can be employed without loss of sensitivity [29,30], furthermore peak capacity in miniaturized system is often affected from delay volumes and extra-column volume contributions. In our approach we coupled a 150 mm × 1.0 mm I.D. column with a 50 mm × 3.0 mm I.D. The small volumes

transferred from 1D to 2D ensured adequate peak focusing at the head of the 2D column. The choice of a 3.0 mm I.D. column was the best compromise between the sensitivity of a 2.1 mm I.D. column and the versatility of a 4.6 mm I.D. column, being less affected from extra-column band broadening effects, with respect to 2.1 mm I.D. columns [31]. Two different modulation times were tested, 60 and 45 s, corresponding to an injection volume of 20 and 15 μL of 1D eluent were transferred to 2D. We used 22 μL sampling loops in this method due to the fact that sampling loop must be slightly larger than the volume of the fraction being transferred, as previously reported in literature [32]. Despite the employment of the same stationary phase in both dimensions good selectivity was ensured through the use of different pH, furthermore 2D was carried out with a shifted gradient approach, in which the mobile phase composition varies continuously according to the retention, this approach is particularly useful with LC × LC separations that possess partly correlated retention, such as RP × RP [33-35]. Doing so, a better separation space coverage was obtained, with peaks not concentrated around the main diagonal line of the separation area, as can be observed from 2D maps, as well as good band compression (see afterwards).

4.3.2.4. Performance evaluation and comparison of set-ups #1 and #2

System parameters and comparison of performance, in peak capacity values, of different set-ups are summarized in Table 4.1.

Table 4.1. *Peak capacity values for comparative monodimensional analysis and for comprehensive LC × UHPLC set-ups #1 and #2, values are corrected for both orthogonality and undersampling effect.*

Parameters	**Monodimensional 1D**		**First dimension, LC × UHPLC ^{1}D set-ups # 1-2**
Stationary phase	Titan C18	Kinetex C18	Discovery BWP
Column geometry	2 × 100 × 2.1 mm, 1.9 μm	100 + 50 × 2.1 mm, 1.7 μm	150 × 1.0 mm, 5 μm
Gradient	90 min, stepwise	90 min, stepwise	90 min, stepwise
Flow rate	0.6 mL/min	0.6 mL/min	20 μL/min
Temperature	60 °C	60 °C	25 °C
n_c	454	490	43.5

	Second dimension, LC × UHPLC ^{2}D			
	Set-up #1		**Set-up #2**	
Stationary phase	Titan C18		Kinetex C18	
Column geometry	50 × 3.0 mm, 1.9 μm		50 × 3.0 mm, 1.7 μm	
Gradient	Continuosly shifted		Continuosly shifted	
Flow rate	2.5 mL/min		2.5 mL/min	
Temperature	60 °C		60 °C	
Modulation time	[a] 60 sec	[b] 45 sec	[a] 60 sec	[b] 45 sec
$4\sigma_{average}$	0.60 sec	0.57 sec	0.74 sec	0.52 sec
n_c	71	69	62	76
Practical $2Dn_c$	1652	1872	1545	2056

The first-dimension peak capacity in gradient conditions was calculated according to equation (5):

$$ {}^{1}n_c = \frac{\left(t_{r,l} - t_{r,f}\right)}{{}^{1}w_{\text{avg}}} \qquad (5) $$

where $t_{r,l}$ and $t_{r,f}$ are the retention times of the last and first peaks eluted in the 2D map, and w_{avg} is the average 4σ peak width of five selected peaks in the 2D map, covering the entire separation window in the 2D space. The value of 43 was obtained. The second dimension peak capacity was calculated according to (6):

$$^{2}n_c = \frac{^{2}t_g}{^{2}w_{avg}} \quad (6)$$

In which $^{2}t_g$ is the second-dimension gradient time, without the re-equilibration time (which is 6 and 9 s for the 45 and 60 s modulation times, respectively), and w_{avg} is the average 4σ peak width of the second dimension separation of the same five selected peaks used for previous calculation. The peak capacity of the two dimensional system can be calculated by multiplying the individual peak capacities obtained for the two dimensions, according to equation (7):

$$n_c2D = n_{c1} \times n_{c2} \quad (7)$$

As reported in previous studies [36], this equation largely over-estimates the practical peak capacity and should be corrected taking into account both the undersampling effect and the orthogonality of the system. For the estimation of the undersampling effect, the equation (7) can be corrected with (8):

$$n'_c 2D = \frac{(^{1}n_c \times {}^{2}n_c)}{\langle\beta\rangle} \quad (8)$$

where β is the correction factor described in (9):

$$\beta = \sqrt{1 + 3.35\left(\frac{^{2}t_c}{1_{\bar{w}}}\right)^{2}} \quad (9)$$

In which ${}^{2}t_c$ is second dimension cycle time (which is equal to the 2D gradient time, plus the 2D re-equilibration time), and *w* average first dimension peak width. 2D separations rarely are fully orthogonal, due to the correlations between the solute retention in the two dimension, and hence this effect reduces the available 2D space. As observed by Liu et al. [37], for a given spreading angle $\langle\beta\rangle$, the effective available 2D area, or the practical peak capacity, can be described as (10):

$$N_p = N_t - (A + C) \tag{10}$$

In which A and C are the unavailable 2D space described by the correlation angles α and γ. In this way the practical peak capacity can be finally calculated by (11) [15]:

$$\text{Practical 2D}n_c = n_{c1} \times n_{c2} - \frac{1}{2}\left[n_{c2}^2 \tan(\gamma) + n_{c1}^1 \tan(\alpha)\right] \tag{11}$$

In which the theoretical "product rule" $n_{c1} \times n_{c2}$ has been already corrected for the undersampling. The values reported in Table 4.1. are already corrected considering both undersampling and orthogonality. Firstly, as can be appreciated from 2D plots, decreasing the modulation time from 60 s (Figures 4.14. and 4.15.) to 45 s (Figures 4.16. and 4.17.) resulted in an improved separation, and hence higher peak capacity values. This is due to a major sampling and thus a better fractionation of the first dimension, being transferred 105 fractions (45 s modulation time) with respect to 70 (60 s modulation time) [38]. With modulation time of 60 s, the fully porous monodisperse column performed slightly better (nc: 71) than the core-shell column (nc: 62). Decreasing the sampling time to 45 s, the reverse situation occurs; in fact very thin peaks ($4\sigma_{avg}$ 0.52 s vs 0.74 s) were obtained with the Kinetex™ and an increase (+24%) of peak capacity (from 1545 to 2056) was observed in practical 2Dn_c.

One of the possible explanation is the lower loading capacity of the core-shell stationary phase, that is more suitable for very fast (less than 60 s) analysis, and thus small injected volumes, in order to avoid overloading effects and severe peak broadening, this observation is in good accordance with a recent study [12]. From the y axis of the 2D maps (Figures 4.16. and 4.17.), representing the 2D retention time, the different behavior in retention of the two columns can be appreciated, especially for first eluting peptides, in the upper left part of the 2D map, with the Titan™ column being more retentive for the smaller and more polar peptides (Figure 4.18.A.) with respect to the Kinetex™ (Figure 4.18.), as mentioned above. The choice of a different pH in the two dimension as well as a continuous shifting gradient in 2D ensured a good employment of the 2D separation space, and a satisfactory selectivity. Results for the two set-ups are summarized in Table 4.1., as can be appreciated, both the Titan™ and the Kinetex™ columns allow to obtain high peak capacity values, which is interesting and show how the Titan™ column possesses excellent kinetic and thermodynamic properties. The Kinetex™ column, with a modulation time of 45 s provided the highest value of peak capacity (2Dn_c: 2056). This value is significantly higher with respect to a high efficiency monodimensional separation (n_c: 454-490) carried out with both fully porous monodisperse and core shell packed columns coupled in series (Figure 4.19.), with the same analysis time (90 min). Analyses of the peptides on the LC × UHPLC platform were run in triplicate, to assess the repeatability of the system, CV % values of 0.3 and 0.4% for retention time and peak area, respectively were obtained by using as control five selected peaks regularly distributed in gradient window.

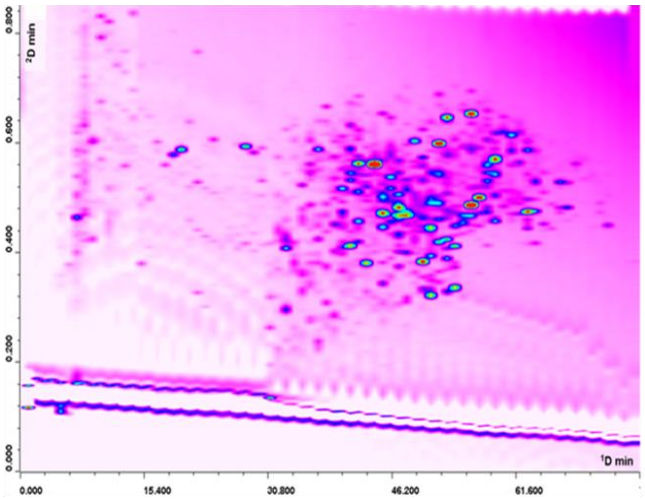


Figure 4.14. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. [1]D column Discovery® 150 × 1.0 mm, 5 µm, flow rate 20 µL/min; column oven: 25 °C, injection volume 2 µL. [2]D column Titan™ C18 50 × 3.0 mm, 1.9 µm.; flow rate 2.5 mL/min. Modulation time 60 sec; column oven: 60 °C.*

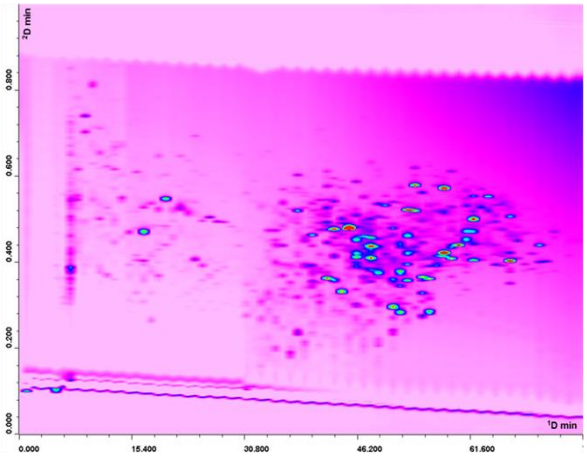


Figure 4.15. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. [1]D column Discovery® 150 × 1.0 mm, 5 µm; flow rate 20 µL/min; column oven: 25 °C; injection volume 2 µL. [2]D column Kinetex™ C18 50 × 3.0 mm, 1.7 µm; flow rate 2.5 mL/min. Modulation time 60 sec; column oven: 60 °C.*

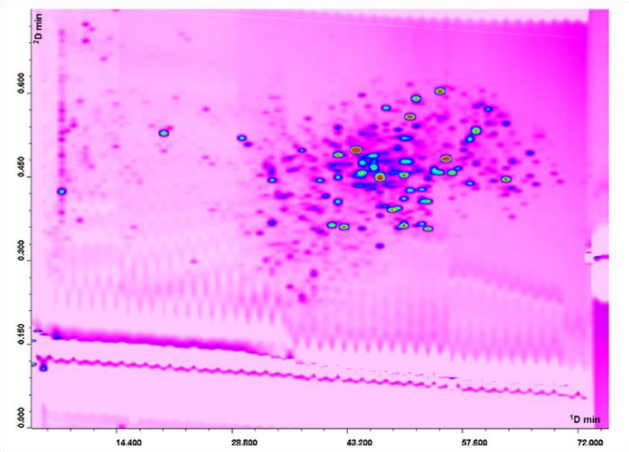


Figure 4.16. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. 1D column Discovery® 150 × 1.0 mm, 5 μm, flow rate 20 μL/min; column oven: 25 °C, injection volume 2 μL. 2D column Titan™ C18 50 × 3.0 mm, 1.9 μm; flow rate 2.5 mL/min. 2D Modulation time 45 s; column oven: 60 °C.*

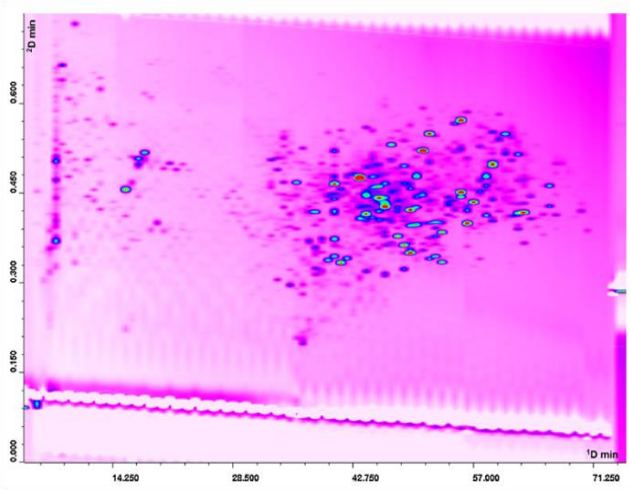


Figure 4.17. *2D LC × UHPLC plot of peptides < 3,000 Da fraction after four week from expiration date. 1D column Discovery® 150 × 1.0 mm, μm; flow rate 20 μL/min; column oven: 25 °C; injection volume 2 μL. 2D column Kinetex™ C18 50 × 3.0 mm, 1.7 μm; flow rate 2.5 mL/min. Modulation time 45 s; column oven: 60 °C.*

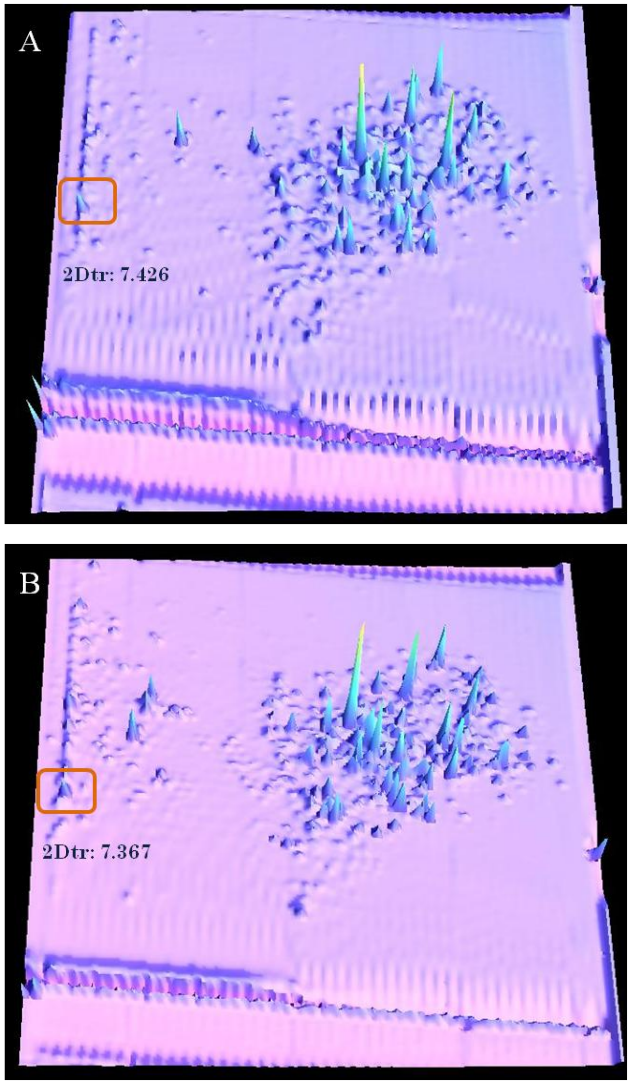


Figure 4.18. *Three dimensional projection of 2D LC × UHPLC plot of peptides < 3,000 Da fraction obtained using in second dimension a (A) Titan™ C18 and (B) Kinetex™ C18 columns, respectively, with a modulation time of 45 s. The LC × LC data were visualized and elaborated in three dimensions using Chromsquare® ver. 1.5.01 software.*

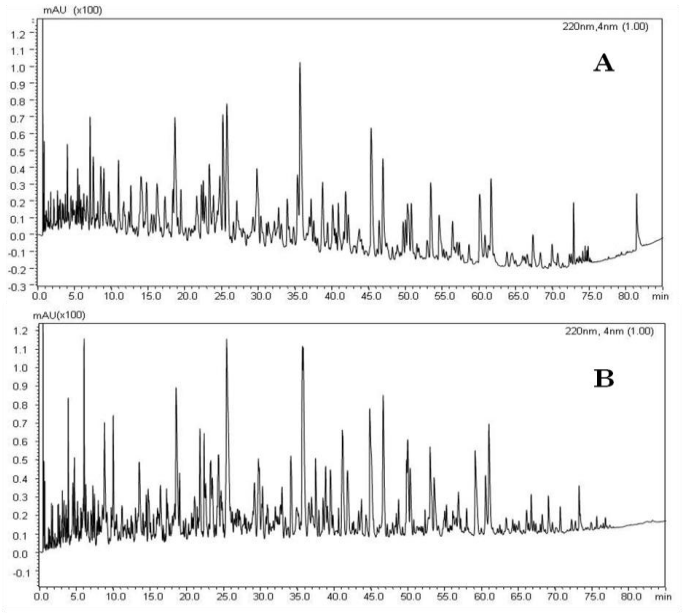


Figure 4.19. *Chromatograms of high efficiency monodimensional comparative analysis of the peptides belonging to < 3,000 Da fraction. Titan™ C18 2 × 100 × 2.1 mm, 1.9 µm, n_c: 454 (A); Kinetex™ C18 100 + 50 × 2.1 mm, 1.7 µm, n_c: 490 (B).*

Those results are very promising, since a high peak capacity is essential to ensure protein sequence coverage and peptide identification [39]. Clearly, in peptide mapping tandem MS (MS/MS) is mandatory, a next step of this study is to optimize an MS/MS method capable of obtain good spectra also for the very thin peaks (less than 1 s) of the LC × UHPLC system employed, this is currently under evaluation with two different mass analyzers, an IT-TOF and a high speed triple quadrupole, and will be presented in a subsequent work.

4.3.3. Identification of peptides by 1D high efficiency monodimensional-MS analysis

Total ion chromatograms of < 3,000 Da peptides extracted from semi-skimmed milk after four week from expiration date are reported in Figure 4.20.

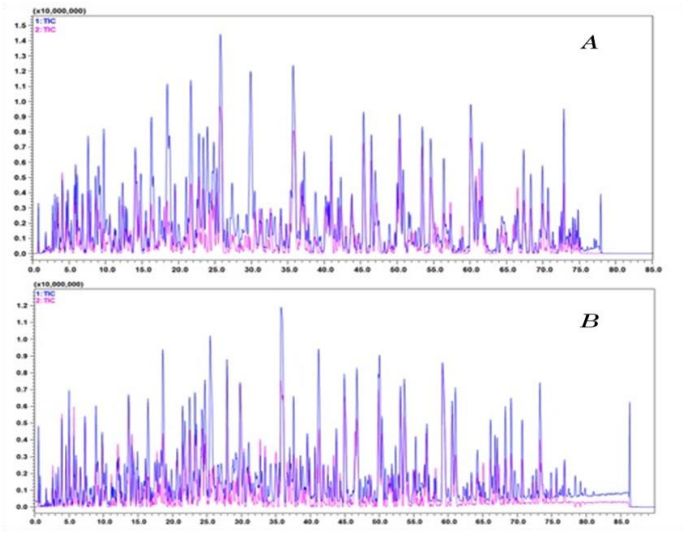


Figure 4.20. *Total ion chromatograms of high efficiency monodimensional comparative analysis of the peptides < 3,000 Da fraction. Columns top: Titan™ C18 2 × 100 × 2.1 mm, 1.9 µm; bottom: Kinetex™ C18 100 + 50 × 2.1 mm, 1.7 µm.*

The criteria used for peptide identification were mass accuracy of precursors versus the score of confidence calculated by the software, accurate MS/MS fragments (b and y) obtained with collision induced dissociation (CID) were used for peptide sequence determination. Peptide sequences are reported in Table 4.2.

Table 4.2. *Sequence and masses of identified milk peptides after four week from expiration date by 1D-LC-ESI-IT-TOF.*

Peptide	-10lgP	Mass	Error (ppm)	m/z	z	Rt	Protein
L.YQEPVLGPVRGPFPIIV	32.69	1780.9875	-1.9	891.4994	2	74.26	P02666|CASβ_BOVIN
L.YQEPVLGPVRGPFPII.V	31.79	1590.9246	3.7	796.4725	2	51.31	P02666|CASβ_BOVIN
K.VLPVPQKAVPYPQR.D	29.86	1434.8235	-1.6	718.4178	2	53.66	P02666|CASβ_BOVIN
K.VLPVPQKAVPYPQ.R	29.07	1450.7893	-2.3	726.4003	2	74.42	P02666|CASβ_BOVIN
T.PVVVPPFLQPEVM.G	27.70	1667.9034	-0.8	834.9583	2	72.32	P02666|CASβ_BOVIN
L.YQEPVLGPVRGPFPI.I	27.40	1299.6863	-0.7	650.8500	2	69.90	P02666|CASβ_BOVIN
L.VYPFPGPIPNSL.P	26.03	1507.8108	1.2	754.9136	2	74.06	P02666|CASβ_BOVIN
T.PVVVPPFLQPEVMG.V	23.00	1471.8398	-1.6	736.9260	2	73.35	P02666|CASβ_BOVIN
D.VENLHLPLPLLQS.W	22.33	1129.6859	-1.3	565.8495	2	73.11	P02666|CASβ_BOVIN
N.LHLPLPLLQS.W	21.56	1412.7704	-1.3	707.3915	2	73.30	P02666|CASβ_BOVIN
S.LVYPFPGPIPNSL.P	21.32	1446.7242	-2.7	724.3674	2	61.29	P02666|CASβ_BOVIN
Y.PVEPFTESQSLTL.T	20.63	1186.6022	-12.1	594.3012	2	58.31	P02666|CASβ_BOVIN
L.VYPFPGPIPNS.L	18.75	1091.6378	1.1	546.8268	2	71.32	P02666|CASβ_BOVIN
T.PVVVPPFLQP.E	18.55	1484.7445	4.1	743.3826	2	50.76	P02666|CASβ_BOVIN
K.AVPYPQRDMPIQA.F	18.36	1027.5087	0.9	514.7621	2	35.17	P02666|CASβ_BOVIN
K.IHPFAQTQS.L	17.88	1472.8239	-2.8	737.4172	2	75.04	P02666|CASβ_BOVIN
L.TDVENLHLPLPLL.Q	17.75	1140.5928	5.4	571.3068	2	48.03	P02666|CASβ_BOVIN
K.IHPFAQTQSL.V	17.67	1299.6863	-9.9	650.8440	2	65.11	P02666|CASβ_BOVIN
S.LVYPFPGPIPNS.L	17.22	1687.9144	0.9	844.9652	2	73.61	P02666|CASβ_BOVIN
L.TDVENLHLPLPLLQS.W	16.93	1382.7921	-55.0	692.3653	2	58.92	P02666|CASβ_BOVIN
F.LLYQEPVLGPVR.G	16.73	1156.6240	-0.8	579.3188	2	46.51	P02666|CASβ_BOVIN
L.YQEPVLGPVR.G	16.70	1269.7081	3.6	635.8636	2	51.53	P02666|CASβ_BOVIN
L.LYQEPVLGPVR.G	15.83	1378.7721	-4.3	690.3904	2	39.12	P02666|CASβ_BOVIN
L.PVPQKAVPYPQR.D	15.51	753.4425	4.0	754.4528	1	60.92	P02666|CASβ_BOVIN
T.PVVVPPF.L	15.03	664.4523	-6.9	665.4550	1	72.57	P02666|CASβ_BOVIN
H.LPLPLL.Q	14.96	1200.6179	8.2	1201.6350	1	66.94	P02666|CASβ_BOVIN
V.YPFPGPIPNSL.P	14.89	645.3156	-4.3	646.3201	1	19.34	P02666|CASβ_BOVIN
K.EAMAPK.H	14.82	878.4360	4.8	440.2274	2	52.03	P02666|CASβ_BOVIN
E.MPFPKYP.V	14.72	619.3363	-0.5	620.3433	1	25.18	P02666|CASβ_BOVIN
E.VMGVSK.V	13.82	734.4003	2.9	735.4097	1	59.94	P02666|CASβ_BOVIN
S.LVYPFP.G	13.13	529.2900	-4.2	530.2950	1	51.54	P02666|CASβ_BOVIN
R.GPFPI.I	12.30	688.4272	-2.2	689.4329	1	61.10	P02666|CASβ_BOVIN
N.LHLPLP.L	12.25	1705.9514	-1.4	853.9818	2	51.14	P02666|CASβ_BOVIN
R.FFVAPFPEVFGKEK.V	22.24	1640.8602	2.2	821.4392	2	72.71	P02662|CASα1_BOVIN
D.VPSERYLGY.L	19.05	1082.5397	10.1	542.2826	2	43.87	P02662|CASα1_BOVIN
F.FVAPFPEVFGKEK.V	18.04	1493.7917	-3.0	747.9009	2	65.96	P02662|CASα1_BOVIN
F.SDIPNPIGSENSEK.T	16.84	1485.6947	-1.5	743.8535	2	42.08	P02662|CASα1_BOVIN
A.RPKHPIK.H	16.04	874.5500	-1.6	438.2816	2	20.92	P02662|CASα1_BOVIN
S.FSDIPNPIGSENSEK.T	14.36	1632.7631	-4.8	817.3849	2	49.72	P02662|CASα1_BOVIN
F.FVAPFPEV.F	13.08	904.4694	5.0	905.4812	1	64.57	P02662|CASα1_BOVIN

K.HQGLPQE.V	12.32	807.3875	1.3	808.3959	1	27.06	P02662|CASα1_BOVIN
F.FVAPFPEVFGK.E	12.24	1236.6542	0.6	619.3347	2	69.46	P02662|CASα1_BOVIN
L.SRYPSYGLNY.Y	24.82	1218.5669	0.9	610.2913	2	46.30	P02668|CASk_BOVIN
A.VRSPAQILQWQVL.S	16.72	1536.8776	-1.1	769.4452	2	72.42	P02668|CASk_BOVIN
F.LPYPYYAK.P	16.03	1013.5222	-2.7	507.7670	2	45.01	P02668|CASk_BOVIN
E.SPPEIN.T	13.10	655.3177	-0.6	656.3246	1	25.75	P02668|CASk_BOVIN
Y.VLSRYPSYGLNY.Y	12.41	1430.7194	5.4	716.3708	2	52.65	P02668|CASk_BOVIN
F.LPYPYY.A	12.26	814.3901	1.6	815.3987	1	49.40	P02668|CASk_BOVIN

Peptide sequences corresponding to the β-Casomorphins have been identified by 1D-LC-ESI-IT-TOF analysis. β-CMs are a group of exogenous opioid-like peptides derived from the hydrolysis of β-casein and were initially isolated from an enzymatic casein digest [40]. These results confirm that β-casein is the main protein undergoing to proteolytic degradation in samples of semi-skimmed milk stored at room temperature four weeks after expiration date.

β-Casomorphins may be also produced by the enzymatic action of different proteases released from tumor cells [41-43]. Indeed, Hatzoglou et al. [44] have shown that five different casomorphins, α-casein fragments 90–95 and 90–96 [45], β-Casomorphins 7 fragment 60–66, β-Casomorphins 5 fragment 60–64, and the morphiceptin, the amide of β-Casomorphins 4, have an antiproliferative action on T47D cells, blocking cells in G0/G1 phase. Hence, two important considerations can be derived: firts of all, this inhibitory effect should be reasonably due to the interaction between the casomorphins and opioid receptors binding sites, decreasing cell proliferation in a dose-dependent manner; secondly, casomorphins possess the minimum structural requirements to interact with opioid receptors, represented by the hydrophobic character of proteins, their high content of proline and the presence of tyrosine residues at the N-terminus [46]. Also, it appears that, with the exception of morphiceptin whose action is mediated by type II somatostatin receptors, all peptides interact with to δ- and κ-opioid binding sites of T47D cells with different selectivity. It is interesting to note that all casomorphins

show a significant interaction with somatostatin receptors in T47D cells, as these peptides may have a major physiological role in breast cancer. Furthermore, the interaction of casomorphins with opioid and somatostatin receptors leads to the inhibition of intracellular levels of cAMP. These peptides could reduce colon cells proliferation by interaction with specific opioid, and somatostatin receptors, present in the intestinal tract of mammals. These biological properties may play an important role in the development of medical foods for the treatment of cancer associated diseases.

4.4. Conclusions

In this contribution, an online comprehensive LC × UHPLC platform was developed for the analysis of peptides of the soluble fraction of milk after four week from the expiration date for the first time. Two set-ups were described, employing two different sub-2 μm columns in 2D, and their performance and behavior were evaluated. Despite the use of the same separation method (RP) in both dimension, high values of peak capacity, satisfactory selectivity as well as good employment of the 2D separation space were obtained, through the use of a different pH between the two dimensions, and, for the first time for peptide separation, with a continuous shifted gradient in 2D. Results are encouraging and the further coupling with mass spectrometry for peptide deconvolution and identification, could be a powerful tool for the identification of bioactive sequences.

4.5. References

[1] R.J. Fitzgerald, B.A. Murray, Bioactive peptides and lactic fermentations, *International Journal of Dairy Technology* 2 (**2006**) 118-125. http://onlinelibrary.wiley.com/doi/10.1111/j.1471-0307.2006.00250.x/abstract

[2] H. Korhonen, A. Pihlanto, Bioactive peptides: production and functionality, *International Dairy Journal* 16 (**2006**) 945-960. http://www.sciencedirect.com/science/article/pii/S0958694605002426

[3] H. Meisel, W. Bockelmann, Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties, *Antonie VanLeeuwenhoek* 76 (**1999**) 207-215. http://www.ncbi.nlm.nih.gov/pubmed/10532380

[4] J.R. Yates, C.I. Ruse, A. Nakorchevsky, Proteomics by mass spectrometry: approaches, advances, and applications, *Annual Review of Biomedical Engineering* 11 (**2009**) 49-79. http://www.ncbi.nlm.nih.gov/pubmed/19400705

[5] B. Hernández-Ledesma, L. Amigo, M. Ramos, I. Recio, Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion, *Journal of Chromatography A* 17 (**2004**) 107-114. http://www.ncbi.nlm.nih.gov/pubmed/15499922

[6] P. Donato, F. Cacciola, L. Mondello, P. Dugo, Comprehensive chromatographic separations in proteomics, *Journal of Chromatography A* 1218 (**2011**) 8777-8790. http://www.ncbi.nlm.nih.gov/pubmed/21689821

[7] G.J. Opiteck, K.C. Lewis, J.W. Jorgenson, R.J. Anderegg, Comprehensive on-line LC/LC/MS of proteins, *Analytical Chemistry* 69 (**1997**) 1518-1524. http://pubs.acs.org/doi/abs/10.1021/ac961155l

[8] T. Kajdan, H. Cortes, K. Kuppannan, S.A. Young, Development of a comprehensive multidimensional liquid chromatography system with tandem mass spectrometry detection for detailed characterization of recombinant proteins, *Journal of Chromatography A* 1189 (**2008**) 183-195. http://www.ncbi.nlm.nih.gov/pubmed/18078946

[9] F. Bedani, W.Th. Kok, H.G. Janssen, A theoretical basis for parameter selection and instrument design in comprehensive size-exclusion chromatography × liquid chromatography, *Journal of Chromatography A* 1133 (**2006**) 126-134.
http://www.ncbi.nlm.nih.gov/pubmed/16959256

[10] I. Francois, D. Cabooter, K. Sandra, F. Lynen, G. Desmet, P. Sandra, Tryptic digest analysis by comprehensive reversed phase × two reversed phase liquid chromatography (RP-LC × 2RP-LC) at different pH's, *Journal of Separation Science* 32 (**2009**) 1137-1144.
http://www.ncbi.nlm.nih.gov/pubmed/19360782

[11] P. Donato, F. Cacciola, E. Sommella, C. Fanali, L. Dugo, M. Dachà, P. Campiglia, E. Novellino, P. Dugo, L. Mondello, Online comprehensive RPLC × RPLC with mass spectrometry detection for the analysis of proteome samples, *Analytical Chemistry* 83 (**2011**) 2485-2491.
http://www.ncbi.nlm.nih.gov/pubmed/21384902

[12] A. D'Attoma, S. Heinisch, On-line comprehensive two dimensional separations of charged compounds using reversed-phase high performance liquid chromatography and hydrophilic interaction chromatography. Part II: Application to the separation of peptides, *Journal of Chromatography A* 1306 (**2013**) 27-36.
http://www.sciencedirect.com/science/article/pii/S0021967313010923

[13] A. Periat, J. Boccard, J.L. Veuthey, S. Rudaz, D. Guillarme, Systematic comparison of sensitivity between hydrophilic interaction liquid chromatography and reversed phase liquid chromatography coupled with mass spectrometry, *Journal of Chromatography A* 1312 (**2013**) 49-57.
http://www.ncbi.nlm.nih.gov/pubmed/24034137

[14] A.L. Huidobro, P. Pruim, P. Schoenmakers, C. Barbas, Ultra rapid liquid chromatography as second dimension in a comprehensive two-dimensional method for the screening of pharmaceutical samples in stability and stress studies, *Journal of Chromatography A* 1190 (**2008**) 182-190.
http://www.ncbi.nlm.nih.gov/pubmed/18400227

[15] F. Cacciola, P. Donato, D. Giuffrida, G. Torre, P. Dugo, L. Mondello, Ultra high pressure in the second dimension of a comprehensive two-dimensional liquid chromatographic system for carotenoid separation in red chili peppers, *Journal of Chromatography A* 1255 (**2012**) 244-251.
http://www.ncbi.nlm.nih.gov/pubmed/22796028

[16] M. Kivilompolo, T. Hyötyläinen, Comparison of separation power of ultra performance liquid chromatography and comprehensive two-dimensional liquid chromatography in the separation of phenolic compounds in beverages, *Journal of Separation Science* 31 (**2008)** 3466-3472.
http://www.ncbi.nlm.nih.gov/pubmed/18792012

[17] E. Uliyanchenko, P.J. Cools, S. van der Wal, P.J. Schoenmakers, Comprehensive two-dimensional ultra high-pressure liquid chromatography for separations of polymers, *Analytical Chemistry* 84 (**2012**) 7802-7809.
http://www.ncbi.nlm.nih.gov/pubmed/22928586

[18] G. Pierri, D. Kotoni, P. Simone, C. Villani, G. Pepe, P. Campiglia, P. Dugo, F. Gasparrini, Analysis of bovine milk caseins on organic monolithic columns: an integrated capillary liquid chromatography-high resolution mass spectrometry approach for the study of time-dependent casein degradation, *Journal of Chromatography A* 1313 (**2013**) 259-269.
http://www.ncbi.nlm.nih.gov/pubmed/24011725

[19] R.E. Murphy, M.R. Schure, J.P. Foley, Effect of sampling rate on resolution in comprehensive two-dimensional liquid chromatography, *Analytical Chemistry* 70 (**1998**) 1585-1594.
http://pubs.acs.org/doi/abs/10.1021/ac971184b

[20] G. Guiochon, N. Marchetti, K. Mriziq, R.A. Shalliker, Implementations of two-dimensional liquid chromatography, *Journal of Chromatography A* 1189 (**2008**) 109-168.
http://www.sciencedirect.com/science/article/pii/S0021967308001271

[21] S. Fekete, K. Ganzler, J. Fekete, Efficiency of the new sub-2 µm core-shell (Kinetex^{TM}) column in practice, applied for small and large molecule separation, *Journal of Pharmaceutical and Biomedical Analysis* 54 (**2011**) 482-490.
http://www.ncbi.nlm.nih.gov/pubmed/20940092

[22] F. Gritti, D.S. Bell, G. Guiochon, Particle size distribution and column efficiency. An ongoing debate revived with 1.9 µm Titan-C18 particles, *Journal of Chromatography A* 1355 (**2014**) 179-192.
http://www.ncbi.nlm.nih.gov/pubmed/25001333

[23] H. Poppe, Some reflections on speed and efficiency of modern chromatographic methods, *Journal of Chromatography A* 778 (**1997**) 3-21.
http://www.sciencedirect.com/science/article/pii/S0021967397003762

[24] R.S. Tirumalai, K.C. Chan, D.A. Prieto, H.J. Issaq, T.P. Conrads, T.D. Veenstra, Characterization of the low molecular weight human serum proteome, *Molecular & Cellular Proteomics* 2 (**2003**) 1096-1103.
http://www.ncbi.nlm.nih.gov/pubmed/12917320

[25] P. Dugo, F. Cacciola, T. Kumm, G. Dugo, L. Mondello, Comprehensive multidimensional liquid chromatography: theory and applications, *Journal of Chromatography A* 1184 (**2008)** 353-368.
http://www.sciencedirect.com/science/article/pii/S002196730701151X

[26] I. Francois, K. Sandra, P. Sandra, Comprehensive liquid chromatography: fundamental aspects and practical considerations: a review, *Analytica Chimica Acta* 641 (**2009**) 14-31.
http://www.ncbi.nlm.nih.gov/pubmed/19393362

[27] K.M. Kalili, A. de Villiers, Systematic optimisation and evaluation of on-line, off-line and stop-flow comprehensive hydrophilic interaction chromatography × reversed phase liquid chromatographic analysis of procyanidins. Part II: Application to cocoa procyanidins, *Journal of Chromatography A* 1289 (**2013**) 69-79.
http://www.ncbi.nlm.nih.gov/pubmed/23570856

[28] D.V. McCalley, Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography, *Journal of Chromatography A* 1038 (**2004**) 77-84.
http://www.sciencedirect.com/science/article/pii/S0021967304004893

[29] M. Rodriguez-Aller, R. Gurny, J.L. Veuthey, D. Guillarme, Coupling ultra high-pressure liquid chromatography with mass spectrometry: constraints and possible applications, *Journal of Chromatography A* 1292 (**2013**) 2-18.
http://www.ncbi.nlm.nih.gov/pubmed/23062879

[30] G. Desmet, S. Eeltink, Fundamentals for LC miniaturization, *Analytical Chemistry* 85 (**2013**) 543-556.
http://pubs.acs.org/doi/abs/10.1021/ac303317c

[31] P.J. Eugster, D. Biass, D. Guillarme, P. Favreau, R. Stöcklin, J.L. Wolfender, Peak capacity optimisation for high resolution peptide profiling in complex mixtures by liquid chromatography coupled to time-of-flight mass spectrometry: application to the Conus consors cone snail venom, *Journal of Chromatography A* 1259 (**2012**) 187-199.

http://www.ncbi.nlm.nih.gov/pubmed/22658136

[32] A. Van der Horst, P.J. Schoenmakers, Comprehensive two-dimensional liquid chromatography of polymers, *Journal of Chromatography A* 1000 (**2003**) 693-709.
http://pubs.acs.org/doi/abs/10.1021/ac3011582

[33] D. Li, O.J. Schmitz, Use of shift gradient in the second dimension to improve the separation space in comprehensive two-dimensional liquid chromatography, *Analytical and Bioanalytical Chemistry* 405 (**2013**) 6511-6517.
http://www.ncbi.nlm.nih.gov/pubmed/23760137

[34] P. Jandera, T. Hajek, P. Česla, Effects of the gradient profile, sample volume and solvent on the separation in very fast gradients, with special attention to the second-dimension gradient in comprehensive two-dimensional liquid chromatography, *Journal of Chromatography A* 1218 (**2011**) 1995-2006.
http://www.ncbi.nlm.nih.gov/pubmed/21081232

[35] G. Mazzi Leme, F. Cacciola, P. Donato, A.J. Cavalheiro, P. Dugo, L. Mondello, Continuous vs. segmented second-dimension system gradients for comprehensive two-dimensional liquid chromatography of sugarcane (Saccharum spp.), *Analytical and Bioanalytical Chemistry* 406 (**2014**) 4315-4324.
http://www.ncbi.nlm.nih.gov/pubmed/24951131

[36] M.R. Filgueira, Y. Huang, K. Witt, C. Castells, P.W. Carr, Improving peak capacity in fast on-line comprehensive two-dimensional liquid chromatography with post first dimension flow-splitting, *Analytical Chemistry* 83 (**2011**) 9531-9539.
http://www.ncbi.nlm.nih.gov/pubmed/22017622

[37] Z. Liu, D.G. Patterson Jr., M.L. Lee, Geometric approach to factor analysis for the estimation of orthogonality and practical peak capacity in comprehensive two-dimensional separations, *Analytical Chemistry* 67 (**1995**) 3840-3845.
http://pubs.acs.org/doi/abs/10.1021/ac00117a004

[38] J.M. Davis, D.R. Stoll, P.W. Carr, Dependence of effective peak capacity in comprehensive two-dimensional separations on the distribution of peak capacity between the two dimensions, *Analytical Chemistry* 80 (**2008**) 8122-8134.
http://pubs.acs.org/doi/abs/10.1021/ac800933z

[39] J.N. Fairchild, M.J. Walworth, K. Horvátha, G. Guiochon, Correlation between peak capacity and protein sequence coverage in proteomics analysis by liquid

chromatography-mass spectrometry/mass spectrometry, *Journal of Chromatography A* 1217 (**2010**) 4779-4783.
http://www.ncbi.nlm.nih.gov/pubmed/20554284

[40] A. Henschen, F. Lottspeich, V. Brantl, H. Teschemacher, Novel opioid peptides derived from casein (β-casomorphins). II. Structure of active components from bovine casein peptone, *Hoppe-Seyler's Zeitschrift fur Physiologische Chemie* 360 (**1979**) 1217-1224.
http://www.ncbi.nlm.nih.gov/pubmed/511111

[41] Z. Sun, Z. Zhang, X. Wang, R. Cade, Z. Elmir, M. Fregly, Relation of β-casomorphin to apnea in sudden infant death syndrome, *Peptides* 24 (**2003**) 937-943.
http://www.ncbi.nlm.nih.gov/pubmed/12948848

[42] F. Jänicke, M. Schmitt, L. Pache, K. Ulm, N. Harbeck, H. Höfler, H. Graeff, Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer, *Breast Cancer Research and Treatment* 24 (**1993**) 195-208.
http://www.ncbi.nlm.nih.gov/pubmed/8435475

[43] A.K. Tandon, G.M. Clark, G.C. Chamness, J.M. Chirgwin, W.L. McGuire, Cathepsin D and prognosis in breast cancer, *New England Journal of Medicine* 322 (**1990**) 297-302.
http://www.nejm.org/doi/full/10.1056/NEJM199002013220504

[44] A. Hatzoglou, E. Bakogeorgou, C. Hatzoglou, P.M. Martin, E. Castanas, Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line, *European Journal of Pharmacology* 310 (**1996**) 217-223.
http://www.ncbi.nlm.nih.gov/pubmed/8884220

[45] S. Loukas, D. Varoucha, C. Zioudrou, R.A. Streaty, W.A. Klee, Opioid activities and structures of α-casein-derived exorphins, *Biochemistry* 22 (**1983**) 4567-4573.
http://www.ncbi.nlm.nih.gov/pubmed/6313043

[46] H. Teschemacher, Opioid receptor ligands derived from food proteins, *Current Pharmaceutical Design* 9 (**2003**) 1331-1344.
http://www.ncbi.nlm.nih.gov/pubmed/12769741

CHAPTER V

Conclusions

The main objective of my PhD has been focused to the analysis and characterization of milk and dairy products derived from "Centrale del latte di Salerno" in order to assess their potential use as dietary supplements and functional foods.

To fulfill these objectives, in these three years my research group developed innovative analytical methods for the qualitative characterization of the protein fraction (casein and whey proteins), contained in commercial bovine milk products and preserved at room temperature after expiration date.

The chromatographic characterization of caseins is a challenging task due to the tendency of such molecules to form, in aqueous solution, micellar structures, highly stabilized by non-covalent interactions. For this reason, in order to optimize chromatographic resolution, we studied the effects of various solvents and denaturant solutions capable of altering the three-dimensional structure, for a better identification of the individual components.

The best chromatographic separation was obtained by incubation of casein powder, for one hour at room temperature, with a denaturing solution containing 8 M urea, 165 mM Tris-HCl, 44 mM sodium citrate and 0.3% β-mercaptoethanol. This method has shown excellent accuracy in terms of reproducibility and repeatability. The caseins were, thereafter, characterized by Cap-LC-HRMS experiments, carried out by employing a lab-made monolithic capillary column (Protein-Cap-RP-Lauryl-γ-Monolithic). In comparison with the commercially available columns, the monolithic capillary column showed improved selectivity, efficiency and chromatographic resolution. The newly developed method allowed to study the degradation pattern of the protein fraction in milk samples. Results obtained highlighted the stability of α_S- and κ-casein to proteolysis as well the formation of polypeptides (proteose-peptones) deriving from cleavage of the N-terminal and of the C-terminal β-CN by bovine plasmin.

Moreover, a different protein degradation pattern was observed in different milk samples (whole, semi-skimmed and skimmed milk). We hypothesized that a possible stabilizing role is exerted by the lipid fraction, able in inhibiting casein degradation in whole milk. This effect was, in fact, not evidenced in skimmed milk and only partially observed in semi-skimmed milk.

Similarly, the whey protein stability in semi-skimmed milk samples has been studied, underlining a time-dependent degradation of β-lactoglobulin A and B, associated to the formation of a large number of peptides.

Analysis of soluble peptides fraction four week after the expiry date, was performed by an on-line comprehensive two dimensional liquid chromatography, using the high performance combined with the ultra high performance conditions. Through the use of a differential pHs between the two dimensions and a continuous shifted gradient in 2D, high values of peak capacity, satisfactory selectivity as well as good employment of the 2D separation space were obtained. At the best of our knowledge this is the first time that these chromatographic conditions are applied for peptide separation and analysis.

Finally, peptide sequences corresponding to the β-Casomorphins have been identified by 1D-LC-ESI-IT-TOF analysis. These results confirmed that β-casein is the protein mainly subjected to proteolytic degradation in semi-skimmed milk samples, stored at room temperature four weeks after expiration date.

Peptides derived from milk proteins exerts beneficial effects on human health, as profusely described in literature. These biological properties may play an important role in the development of medical foods to treat or mitigate several diseases. Moreover their qualitative separation and identification could be also important for the development of new potent drugs having well-defined pharmacological effects. With the rise of consumer concerns about the deleterious effects of chemical preservatives and the increasing preference for natural components, the importance of milk-derived bioactive substances is further increased. Application of

enrichment protocols such as membrane processing and chromatographic isolation is also an area of future interest in the extraction of potent biofunctional peptides from milk and dairy products and their following utilization as functional food ingredients. Molecular studies are, anyway, required to clarify the pharmacological mechanisms by which the bioactive peptides exert their activities.