



## APPLICATION NOTE

### LC/Mass Spectrometry

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## LC/MS Study of Casein Proteins in Milk

### Introduction

We have developed a method for extracting and measuring the masses of proteins in milk by using liquid chromatography coupled to electrospray time-of-flight mass spectrometry (LC-TOF MS). This method was used to look at the patterns and masses of the casein proteins in cow milk.

### Methods

Proteins were extracted from milk by the addition of a two-fold volume of an aqueous denaturing solution containing 8 M urea, 165 mM TRIS, 44 mM sodium citrate and 0.3% (v/v) mercaptoethanol, followed by filtration<sup>1</sup>. This method extracted fat soluble casein proteins in addition to whey proteins.

Following extraction, the proteins were separated using a fast gradient HPLC method and detected with an AxION® 2 TOF mass spectrometer, with ion optics tuned to optimize transmission of the mass range  $m/z$  1000-2000.

Spectra in the acquired datasets were averaged for selected time ranges and deconvoluted using a proprietary algorithm to create zero charge spectra which show both the accurate mass and intensity ratios of the proteins eluting at that time. The high resolution and high mass accuracy of TOF MS allows for measurement of a protein average mass to within 1 Da of the calculated mass.

#### LC conditions:

Pump: PerkinElmer Flexar™ FX-15 pump  
 Flow: 0.4 mL/min  
 Mobile phase A: Water + 0.1% formic acid + 0.01% trifluoroacetic acid  
 Mobile phase B: Acetonitrile + 0.1% formic acid + 0.01% trifluoroacetic acid  
 Gradient conditions: 25-43% B over 6 min  
 Injection volume: 2  $\mu$ L of 20x diluted sample  
 Column used: Phenomenex wide pore XP-C8 2 x100 mm, heated to 50 °C

#### MS conditions:

Mass spectrometer: PerkinElmer AxION 2 TOF MS  
 Ionization source: PerkinElmer Ultraspray™ 2 (Dual ESI source)  
 Ionization mode: Positive  
 Spectral acquisition rate: 3 spectra/sec

## Results

Milk contains lactoglobulin, lactoferrin and casein proteins at high concentrations. Caseins are relatively hydrophobic phosphoproteins, which comprise 80% of the cow milk proteins; there are several related caseins, which are phosphorylated to different degrees. The alpha and beta caseins are insoluble in water and are bound together with the more soluble kappa-casein to form micelles.

The caseins are extracted from milk with a denaturing 8 M urea protocol, which breaks apart the micelles and solubilizes the proteins. Chromatography of the proteins in the urea extract separates the various phosphorylated variants of alpha, beta and kappa forms of casein (Figure 1). Other non-casein proteins from the milk are also observed. Peak identification is based on the protein masses in the deconvoluted spectra.

#### Alpha S1-Casein

Alpha S1-casein is a heavily phosphorylated protein which causes the most common milk allergic reaction<sup>2</sup>. Detection of this protein is of interest in food allergy studies.

A deconvoluted spectrum from the averaged alpha S1-casein elution time-range spectra clearly shows the presence of two major different forms of the protein (Figure 2), with 8 and 9 phosphorylated serine residues.

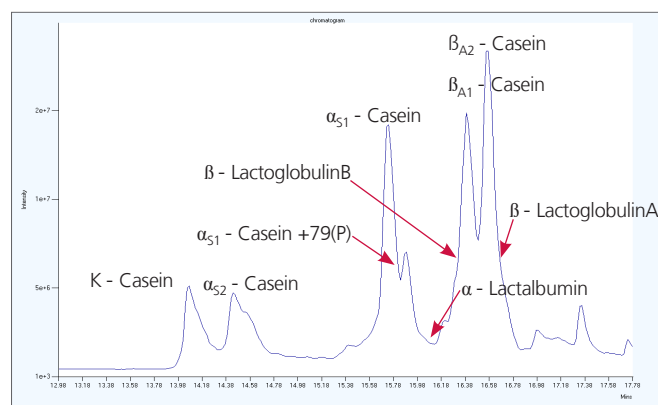


Figure 1. Total ion current trace showing the profile of the proteins in the separation of urea-extracted US supermarket cow's milk.

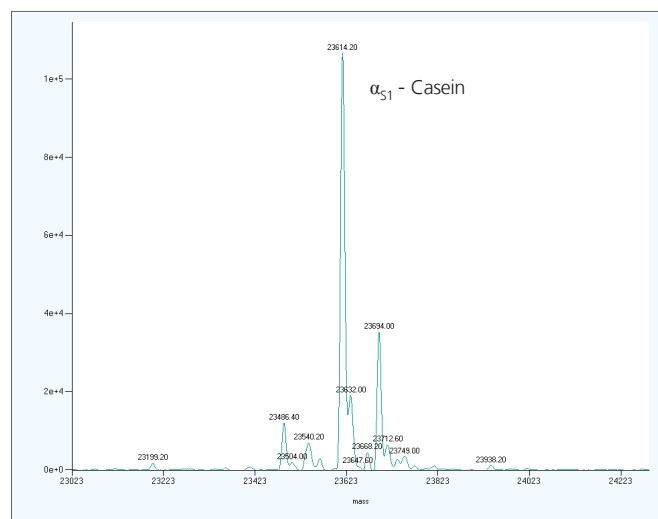


Figure 2. Deconvoluted spectrum of the averaged alpha-S1-casein elution region showing two major highly phosphorylated forms of the protein (23,614 Da) and (23,694 Da), differing by one phosphorylation site (80 Da).

#### A1 and A2 Beta-Casein

Cows, depending on the breed, have genetic variants resulting in the production of different sequences of beta-casein. Older breeds of African and Indian cows and water buffalo have the A2 beta-casein genetic allele and produce only this protein. Some cows, such as Holsteins and Friesians, have a genetic mutation which causes them to produce a variant protein, A1 beta-casein. These breeds carry A1 and A2 alleles in equal distribution, while Jersey and Guernsey cows have higher ratios of the A2 allele. Consequently, milk from most European herds contains a mix of A1 and A2 beta-caseins.

The presence of A1 beta-casein in bovine milk has been linked to health concerns. The A1 variant of beta-casein differs from the A2 variant by one amino acid at position 67, having Ile-His67 in A1 and Ile-Pro67 in A2. This difference of 40 Da is easily detected. The A1 form cleaves at the Ile-His peptide bond during human digestion, to produce the bioactive peptide beta-casomorphin-7. This peptide has been suggested as a potential disease risk factor<sup>3</sup>. Thus measurement of A1 and A2 beta-casein levels in the milk supply is of interest.

In the U.S. milk supply, the breeds of cows, predominantly Holstein, provide milk with A1 and A2 beta-casein proteins in approximately equal ratios. Peaks for the two forms of A1 and A2 beta-casein are shown in the elution profile of supermarket milk (Figure 1). The deconvoluted spectra from the two peaks in this milk sample show the A1 form at a slightly higher level than the A2 form (Figure 3).

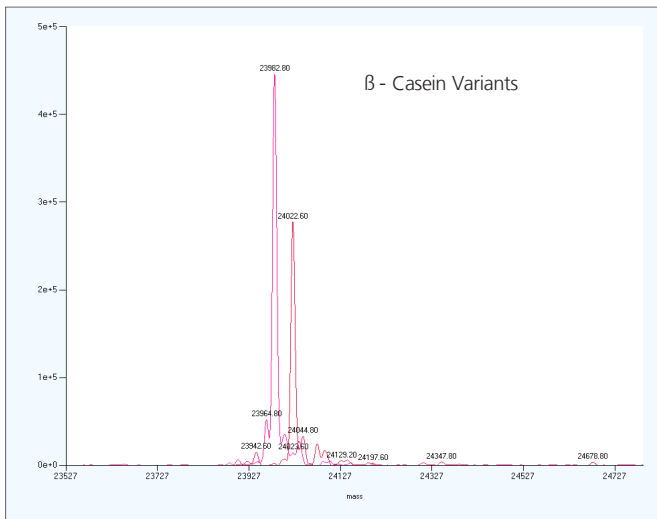


Figure 3. Overlay of the deconvoluted spectra of the two beta-casein peaks showing that this milk sample contains both of A1 (23,982 Da) and A2 (24,022 Da) variants. The variants are due to a His/Pro substitution differing by 40 Da. Both beta-caseins are multiply phosphorylated.

## Conclusions

A fast and simple sample preparation, combined with a rapid reversed-phase LC/MS analysis, was used to investigate the presence of different caseins and phosphorylation levels of caseins in different milk samples.

## References

1. Ivan Bonizzi, Joanna Natalia Buffoni, Maria Feligini, Quantification of bovine casein fractions by direct chromatographic analysis of milk, *Journal of Chromatography A*, 1216 (2009) 165–168
2. Høst A, Frequency of cow's milk allergy in childhood, *Ann. Allergy Asthma Immunology (December 2002)*, 89: 33–37
3. A.S. Truswell, *Eur J Clin Nutr*. The A2 milk case: a critical review, (2005) May 59(5):623-31.