



## APPLICATION NOTE

### LC/Mass Spectrometry

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## Milk Adulteration: Detecting Species-Specific Proteins by LC/MS

### Introduction

The adulteration of milk via the addition of low molecular weight compounds, such as melamine for economically motivated fraud,

is a well-known concern<sup>1</sup>. Methods for adulteration detection have been examined extensively using, amongst other tools, LC/MS techniques. Current methods tend to focus on small molecule adulteration; however, large molecule adulteration with other animal or plant proteins is becoming increasingly common and is not yet regulated within the dairy industry.

One of the simplest forms of milk adulteration is to dilute more expensive milks from species, such as sheep and buffalo, with less expensive cow milk. We have developed a method for measuring the addition of bovine milk to more expensive milks (goat, sheep, buffalo and camel) by using liquid chromatography coupled to electrospray time-of-flight mass spectrometry (LC-TOF MS) to detect species-specific marker proteins in the milk.

## Methods

Milk samples from different animals were spiked with varying levels of bovine milk and homogenized with a vortex mixer for 30 seconds. Proteins were then extracted from the samples for LC/MS analysis.

Whey proteins in milk were purified by liquid/liquid extraction; the proteins remain in the aqueous layer after addition by volume of 20% of dichloromethane and 10% of an aqueous solution of 5% acetic acid<sup>2</sup> to the milk to separate out the lipids.

Following extraction, the proteins were separated using a fast gradient HPLC method and detected with an AxION<sup>®</sup> 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 different proteins eluting at that time.

### 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 µL of 20x diluted sample  
Column used: Phenomenex wide pore XP-C8 2 x 100 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

### Markers for adulteration with cow milk

All mammalian milks contain lactoglobulin, lactoferrin and casein proteins at high concentrations. A liquid/liquid extraction protocol was selective for the water soluble proteins lactoferrin and lactoglobulins present in the whey fraction of milk.

Milk obtained from many mammalian species, including cow, buffalo and sheep, although not human or camel, contain the glycoprotein beta-lactoglobulin. Human allergies to cow milk may be related to the presence of this protein<sup>3</sup>.

Genetic phenotypes of beta-lactoglobulin are predominantly forms A and B, where the sequences differ by two amino acids. Cows and sheep have both A and B forms, while buffalo has only one form of beta-lactoglobulin, which is very close in sequence and mass to the cow B form. The protein sequence for beta-lactoglobulin B is highly homologous for all species that produce this protein in milk, while the sequence of beta-lactoglobulin A varies with species and has additional amino acid substitutions

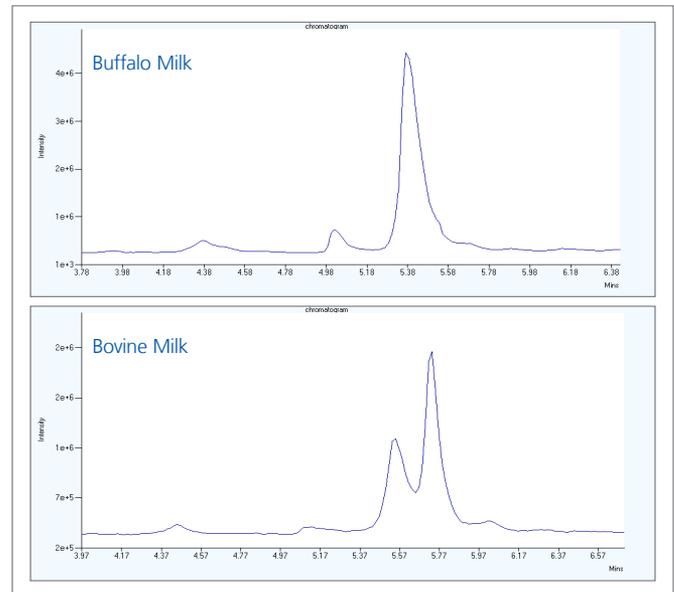


Figure 1. LC/MS analysis showing the lactoglobulin elution profiles for different milks. Buffalo milk has one form of beta-lactoglobulin, where bovine milk has two forms – beta-lactoglobulin A and B – which elute at different times in the chromatogram.

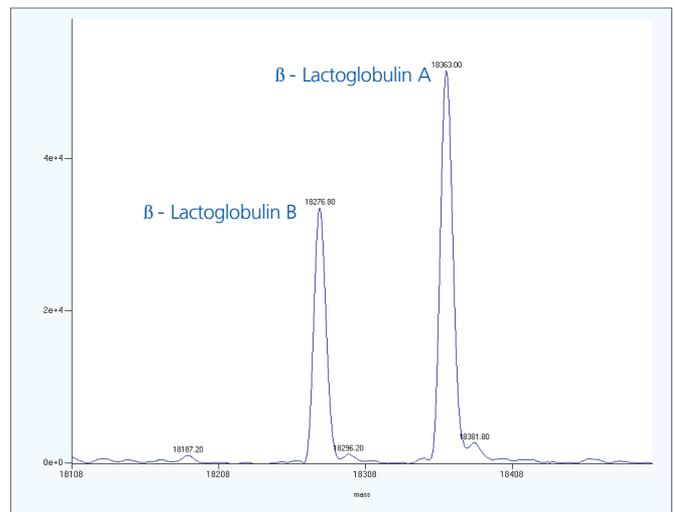


Figure 2. Deconvoluted spectrum of the beta-lactoglobulins from pure cow milk after averaging the spectra in the time range where both beta-lactoglobulin A and B elute. The B form has an average mass of 18276.8 Da and the A form of 18,363.0 Da.

due to genetic variants. The amino acid sequence differences of the A and B forms allow them to be separated by chromatography (Figure 1).

A deconvoluted spectrum for the averaged spectra of the two forms shows the intensity ratio and masses of bovine beta-lactoglobulin A and B forms (Figure 2). The high resolution and high mass accuracy of TOF MS enables measurement of a protein average mass to within 1 Da of the calculated mass. The theoretical average masses of bovine lactoglobulin A and B forms are calculated as 18,363.3 Da and 18,277.2 Da respectively for the forms in which four of the cysteine residues are in the oxidized form and one in the reduced form<sup>4</sup>. The experimentally determined lactoglobulin masses from bovine milk were within 0.4 Da of the theoretical masses.

Measurement of the proteins in the buffalo milks spiked with cow milk provides unambiguous confirmation of the presence of bovine lactoglobulin A (Figure 3) down to low levels resulting from 5% adulteration.

Thus beta-lactoglobulin A is a marker for the presence of bovine milk, even in the presence of lactoglobulin B, and the specific mass of this marker protein is linked to the breed of cow<sup>5</sup>. Similar results were obtained for other milks, such as sheep (Figure 4) and camel (Figure 5).

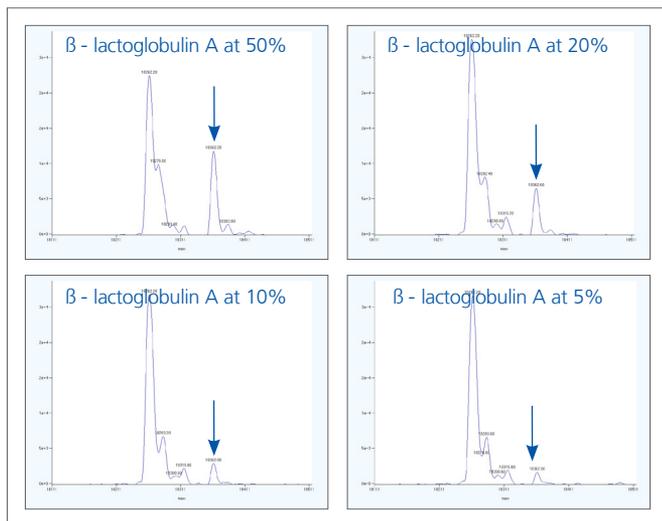


Figure 3. Adulteration levels down to 5% of cow milk in buffalo milk are detected in the deconvoluted spectrum of the averaged lactoglobulin elution region using  $\beta$ -lactoglobulin A as the marker protein.

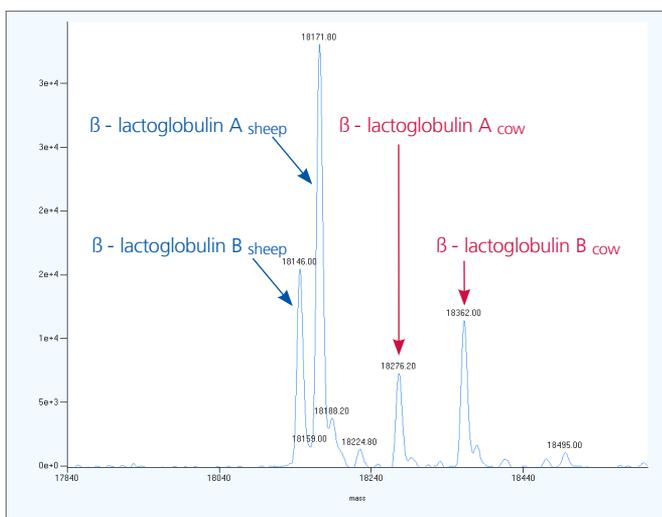


Figure 4. Adulteration of sheep milk by cow milk is detected by the different masses of both the A and B forms of lactoglobulin for each species.

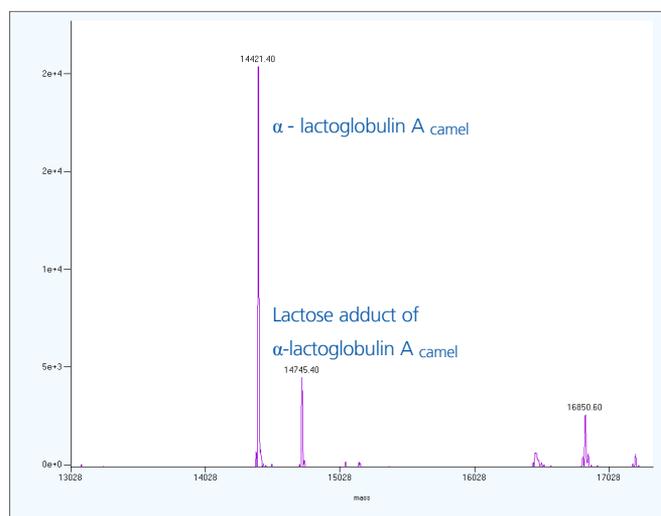


Figure 5. Camel milk contains no lactoglobulin B; only lactoglobulin A and the lactosylated form of the protein are detected.

## Conclusions

A fast and simple sample preparation, combined with a rapid reversed-phase LC/MS analysis, provided information on the masses and levels of a number of different proteins in the milk of different species and breeds.

A protein of a specific mass only found in bovine milk was used as a marker protein for the detection of adulteration of expensive milks by less expensive bovine milk. Low levels of 5% adulteration were detected. This detection method was successfully applied to buffalo, goat, camel and sheep milk.

## Acknowledgements

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## References

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