Milk Allergen Identification and Quantitation in Wine with LC-MS/MS Approaches

T. Zhang, Y. Zhang, D. Horn, Z. Hao and G. Jiang, **Thermo Fisher Scientific, San Jose, California, USA**

Overview

Purpose: LC-MS/MS methods were developed for milk allergen identification and quantitation in wine using high resolution/accurate mass and linear ion trap mass spectrometers.

Methods: LC-MS/MS analyses were performed on an Thermo Scientific UltiMate 3000 x 2 Dual RSLC system coupled to a Thermo Scientific Velos Pro or Q Exactive mass spectrometer.

Results: Both top down (intact protein) and bottom up (peptides) approaches were investigated for milk allergen protein analysis. Allergen proteins and the peptides could be quantified and confirmed by one LC-MS run with the method. Three orders of magnitude linear range were realized for bottom up method. The low ppb level of limit of detection (LOD) for milk allergen in wine was achieved.

Introduction

Food allergy is a major public health concern. Cow's milk is one of the most common causes of allergic reactions in the early life [1]. Milk contains approximately 3% protein, about 80% of the milk proteins are caseins. The casein fraction is composed of alpha S1-, alpha S2-, beta-, and k-casein, of which alpha S1-casein seems to be a major allergen [2]. More recent studies have shown that even low concentration milk proteins are potential allergens [3].

The analytical methods used for food allergen testing are enzyme-linked immunosorbent assay (ELISA), and real-time Polymerase Chain Reaction (PCR) detection. Both methods had their disadvantages. ELISA, depend on the quality of antigen used and quality of the antibodies against antigen, may lead to false-negative results. PCR method detects DNAs, which is an indirect measurement of proteins and could result in false positive and false negative detection. With the recent new method development in food allergen test, LC-MS/MS analyses have shown the advantages over ELISA and PCR. The high selectivity with monitoring for specific masses of interest and the capability for compounds identification/confirmation with their MS/MS spectra of the LCMSMS methodology attracted more attentions and became a very hot area for food allergen testing.

Methods

Sample Preparation

Casein was purchased from Sigma Aldrich. Nonfat milk powder and white wine matrix was purchased from Safeway. The casein and nonfat milk stock solution were prepared in 50mM ammonium bicarbonate buffer. The nonfat milk solution protein concentration was determined by measure absorbance of the stock solution at 280 nm.

$$\text{Protein concentration} = A_{280}$$

The intact protein solution was directly injected for LC-MS analysis with the top-down method. The protein solution was enzyme digested and peptides were injected for LC-MS analysis with the bottom up approach. Figure 1 illustrates the workflow for the general bottom up approach.

Liquid Chromatography

Columns:	Thermo Scientific Hypersil C8 column (1.0 x 100 mm, 5.0 µm) Thermo Scientific Acclaim 120 C18 (3.0 x 10 mm, 5.0 µm particle) Thermo Scientific Acclaim PepMap100 C18 (1.0 x 150 mm, 3 µm)
Mobile Phase:	[A] Water containing 0.1% formic acid [B] Acetonitrile containing 0.1% formic acid
Column temperature:	30 °C for bottom up, 50 °C for top-down
Injection volume:	2 µl
Needle wash:	80:20 (v/v) acetontrile:water
Gradient:	Table 1 for top-down Table 2 for bottom up

Mass Spectrometry

Velos Pro

S-lens:	59	Capillary Temp:300 degree
MSMS Microscan:	5 microscans	Voltage:4.5kv

FIGURE 1. Bottom up approach workflow diagram

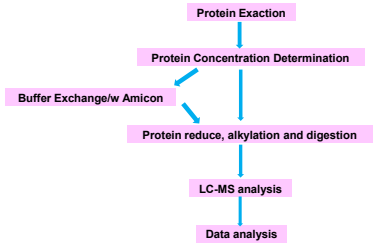


Table 1

Time	A %	B %	µL/min
0.0	90	10	150
0.5	90	10	150
0.6	85	15	150
2.0	70	30	150
13.00	50	50	150
14.0	5	95	150
14.9	5	95	150
16.0	90	10	150

Table 2

Time	A %	B %	Flow µL/min
0.0	98	2	150
3.0	98	2	150
3.6	90	10	150
9.0	70	30	150
13.0	40	60	150
14.0	2	98	150
14.9	2	98	150
15.0	98	2	150

Mass Spectrometry

Intact Protein - Q Exactive™ MS

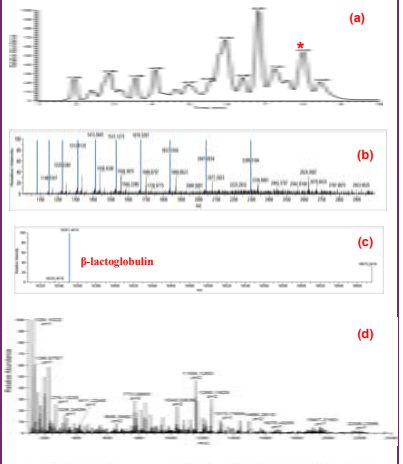
Full MS Scan		MS/MS	
Mass Range	1000 to 3000 m/z	Collision Energy	28%+30% Step
Resolution	70,000	FT AGC	2e5
Injection Time	500 ms	FT Injection	500 ms
AGC Target	5e5	Microscans	2

Peptides

tSIM Scan		dd-MS/MS	
Microscan 1		Collision Energy	26%
Resolution	70,000	FT AGC	5e4
Injection Time	500 ms	FT Injection	500 ms
AGC Target	2e5	Microscans	1
		Resolution	17,500

FIGURE 2. Intact protein analyses with LCMSMS for Nonfat milk.

(a) Chromatogram of protein separations (b) The MS spectrum of protein * labeled in (a). (c) Deconvoluted MS spectrum (β-lactoglobulin). (d) HCD MSMS spectrum of m/z 1670.3214. (e) Identified protein sequence, β-lactoglobulin, by database search.



Results

1. Intact protein analysis (Top down)

The nonfat milk protein solution was directly injected and separated on a C8 reverse phase column (Figure 2). A full MS scan followed by dd-MS2 experiment was executed. The protein molecular weight was obtained by deconvoluting the MS full scan spectrum. And the proteins were identified by database search using its dd-HCD MSMS spectrum. Figure 2 shows the flow of the analysis, from protein separations to protein identifications. Multiple proteins were separated and identified in the nonfat milk solution, including β–lactoglobulin, α-casein, β-casein, k-casein.

2. Protein analyses with proteolytic digestion (Bottom up)

The bottom up experiments were performed on an Ultimate™ 3000 RSRP HPLC system coupled with either a linear ion trap MS (Velos™ Pro) or an Orbitrap™ MS (Q Exactive).

a. Linear ion trap mass spectrometer (Velos Pro)

The enzyme digested casein, spiked in white wine, was injected on an Acclaim C18 column. A full scan MS with targeted MSMS method was performed. The peptides were separated by HPLC and identified by their full MSMS spectra (Figure 3). Their MSMS fragments were used for peptide quantitation. Figure 3 illustrates the extracted fragments ion chromatogram for two peptides and their MSMS spectra. Figure 4 shows the calibration curve for peptide FALPQYLK. Excellent linearity of detector response was achieved, with correlation coefficient 0.9921. Three order of magnitude linear range, from 25ppb to 5ppm range was obtained. A 25ppb limit of detection and 50ppb limit of quantitation were achieved with this method

Figure 3. Extracted fragments ion chromatogram of two peptides at m/z of 490.3 and 692.8 (Top panel) and their MSMS spectra (Bottom panel).

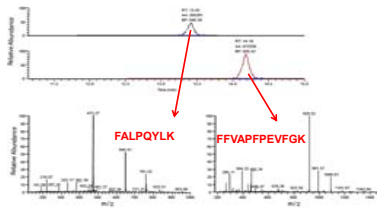
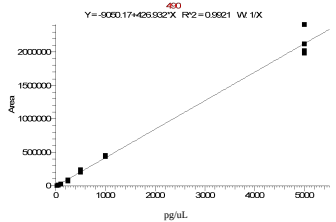


Figure 4. Calibration curve of peptide FALPQYLK in wine matrix.



b. Orbitrap mass spectrometer (QExactive)

The enzyme digested casein, spiked in white wine, was injected on an Acclaim™ C18 column. A targeted selected ion monitor (tSIM) followed by data dependent MSMS (dd-MS2) method was employed. Extracted ion of the target with 3ppm mass tolerance were used for peptides quantiation (Figure 5). Their dd-MS^2 spetra were used for peptides identification and conformation. A calibration curve range from 25ppb to 5ppm was plotted as shown in figure 6. The 25ppb level could be accurately quantified in wine matrix.

FIGURE 5. The extracted ion chromatogram of two target peptides (Top panel) and their isotope-resolved spectra of precursor 490.2835 and 692.8686 (Bottom panel).

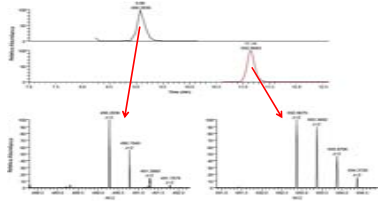
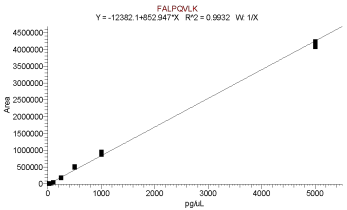


FIGURE 6. Calibration curve of peptide FALPQVLK in wine matrix with Q Exactive MS



Conclusion

- Allergen proteins could be separated and identified by a RPLC/MS method.
- The targeted full-scan MS/MS method using Velos Pro linear ion trap mass spectrometer provided excellent selectivity and sensitivity for the identification and quantitation of the peptides in wine matrix with low ppb level detection limits.
- The targeted selected ion monitor (tSIM) method followed by data dependent MSMS (dd-MS^2) using Q Exactive mass spectrometer achieved identification and quantitation of peptides in one LC-MS run, capable of quantifying as low as 25ppb peptides in wine matrix with accurate mass tSIM scans.

References

1.Host, A., & Halken, S (1990) Allergy 45, 587-596
2.Spuergin, P., et al., Allergy (1996) 51, 306-312
3. Wal, J., M. Bovine milk allergenicity, Ann. Allergy Asthma Immunol. (2004), 93 (5 suppl. 3), S2-S11