Allergen Detection in Wine by Micro Flow Liquid Chromatography Tandem Mass Spectrometry microLC-MS/MS

Stephen Lock
AB SCIEX Warrington (UK)

Overview

A rapid, robust, sensitive and specific LC-MS/MS assay has been developed for the simultaneous detection of milk and egg proteins in white wine. The method utilizes a simplified sample preparation protocol, the Eksigent ekspert™ microLC 200, and the AB SCIEX QTRAP® 5500 system with the *Scheduled* MRM™ algorithm to detect below 0.1 ppm casein in wine.

Introduction

In wine production fining a wine eliminates any appearance of cloudiness by removing sediment. In this process fining agents, such as casein, are stirred into barrels of wine where they act as magnets by picking up the sediment in the wine and depositing it at the bottom of the barrel. Once the wine has been clarified, racking of the wine is done to separate the wine from the sediment.

In 2011 EFSA concluded that wines fined with casein, caseinate and milk products may trigger adverse reactions in susceptible individuals following a survey of wine where the detection of casein was reported in trace amounts (<2 mg/L [2 parts-per-million]) in two (out of 32) experimental wines without bentonite treatment and in three (out of 61) commercial wines with unknown treatment.[1, 2] This fact together with a new European Union legislation (that states that wine after 30 June 2012 must disclose on the label if fining reagents such as casein and egg ovalbumin have been used in processing)[3] has driven the need for methods which are capable of detecting casein products in wine at low levels.

Here we present new data using micro flow LC in combination with an LC-MS/MS method developed on an Eksigent ekspert™ microLC 200 and AB SCIEX QTRAP® 5500 system utilizing the *Scheduled* MRM™ algorithm which detects casein in wine at sub ppm levels. The method utilizes a simple protein digestion of the wine followed by dilution and injection and has been designed to limit extensive sample preparation and perform all protein modification in the same Eppendorf tube. In this paper we will discuss the benefits of micro flow LC over higher flow rate separations.

Experimental

Standards

For this work the target proteins were commercially available as well as reagents used for alkylating, reducing and digesting the samples and all were purchased from Sigma Aldrich. Wine for spiking experiments was obtained from a local supermarket.

Sample Preparation

The wine samples (0.5 mL) were reduced by adding TCEP (tris(2-carboxyethyl)phosphine, 0.2 M, 50 µL) and agitating using a thermal mixer for 60 minutes at 60°C. The samples were cooled to room temperature and alkylated by adding a solution of MMTS (S-methyl methanethiosulfonate, 0.2 M, 100 µL in iso-propanol) and storing protected from light for 30 minutes at ambient temperature. This process cleaves the disulfide bridges of the allergenic proteins and then alkylates the free cysteine residues preventing reformation of the bridges and aids trypsin digestion. The extracts containing the modified proteins were diluted 1 in 4 with a ammonium bicarbonate buffer and rapidly digested over a one hour period using trypsin and thermal mixing (60 minutes at 40°C). After 1 hour digestion the samples were further diluted 1 in 2 with 0.1% formic acid to deactivate the trypsin and stop the digestion and prepare the sample for LC-MS/MS analysis.

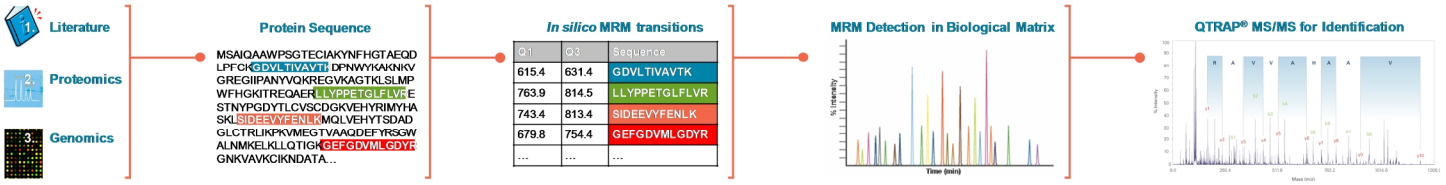

Figure 1. The MIDAS™ workflow (MRM-initiated detection and sequencing)

LC

The initial high flow LC analysis used a Shimadzu UFLC$_{XR}$ system and the conditions shown in Table 1 where A = water and B = acetonitrile both containing 0.1 % formic acid. A volume of 10 µL of sample was injected onto a Phenomenex Kinetex 2.6 um XB-C18 100A (2.1 x 50 mm) column held at 40°C.

Table 1. Gradient conditions used for 'high flow' LC separation at a flow rate of 300 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	2	98	2
2	8	60	40
3	8.2	2	98
4	9.0	2	98
5	9.1	98	2
6	10	98	2

Table 2. Gradient conditions used for micro flow LC separation at a flow rate of 25 µL/min

Step	Time	A (%)	B (%)
0	0	98	2
1	0.3	98	2
2	4	60	40
3	4.1	5	95
4	4.3	5	95
5	4.4	98	2
6	5.5	98	2

All micro flow LC method development and analysis was done using an Eksigent ekspert™ microLC 200 system. Final extracted samples (10 µL) were separated over a 5.5 minute gradient (Table 2) of A = water and B = acetonitrile both containing 0.1 % formic acid. Peptides were separated on a reversed-phase YMC Triart C18 2.7 µm (50 x 0.5 mm) column held at 40ºC.

MS/MS

All analyses were performed on an AB SCIEX QTRAP® 5500 LC/MS/MS system using a TurboV™ source, with a standard electrospray ionization (ESI) probe used with the high flow LC system and for micro flow LC analysis the ESI electrode was changed to a micro LC hybrid electrode (50 µm ID).[4]

The initial method development was carried out using the MIDAS™ workflow (MRM-initiated detection and sequencing). MIDAS uses a set of predicted MRM transitions from the known protein sequence as a survey scan to trigger the acquisition of QTRAP® full scan MS/MS spectra (Figure 1). This data was then submitted to a database search engine for confirmation of peptide identification and of the feasibility of the MRM transition for casein, milk, and egg product detection in wine. With this workflow MRM transitions were designed without the need for synthetic peptides.

In the final micro flow LC method the following Turbo V™ source conditions were used: Gas 1, Gas 2, and the CUR set at 30 psi, the ion source temperature (TEM) at 350°C and IS voltage of 5500 V. The peptides were detected in Multiple Reaction Monitoring (MRM) mode for best selectivity and sensitivity using the *Scheduled* MRM™ algorithm with an MRM detection window of 40 sec and a target scan time of 0.30 sec. Q1 resolution was set to low and Q3 resolution was set to unit. A total of 44 MRM transitions (Tables 3 and 4) were evaluated for over 16 target peptides from milk and egg. This meant that there is plenty of scope to add further markers in the future.

Source conditions of the high flow method were optimized for 300 µL/min, but all other setting were identical.

Table 3. MRM transitions and retention times (RT) of peptides for the detection of egg and milk protein in wine

Peptides for the detection of egg protein					
Identity	**RT (min)**	**Q1 (amu)**	**Q3 (amu)**	**DP (V)**	**CE (V)**
egg protein 1 1_1	3.2	563.3	631.3	100	29
egg protein 1 1_2	3.2	563.3	732.4	100	29
egg protein 1 2_1	2.9	791.4	951.4	76	39
egg protein 1 2_2	2.9	791.4	1052.5	96	43
egg protein 1 3_1	3.2	845.0	860.4	161	47
egg protein 1 3_2	3.2	845.0	1007.5	136	47
egg protein 1 4_1	3.6	930.0	1116.6	186	49
egg protein 1 4_2	3.6	930.0	888.5	166	49
egg protein 1 4_3	3.6	930.0	1017.3	216	49
egg protein 1 5_1	1.9	390.7	667.3	90	20.9
egg protein 1 5_2	1.9	390.7	504.2	90	20.9
egg protein 1 5_3	1.9	390.7	433.2	90	20.9
egg protein 2 1_1	1.9	437.7	452.2	90	31
egg protein 2 1_2	1.9	437.7	680.3	90	27
egg protein 2 1_3	1.9	437.7	737.4	90	27
egg protein 2 2_1	2.4	714.8	1152.5	139	37
egg protein 2 2_2	2.4	714.8	951.5	139	38
egg protein 2 2_3	2.4	714.8	804.4	139	39
Peptides for the detection of milk protein					
milk protein 1 1_1	3.2	587.3	758.4	91	27
milk protein 1 1_2	3.2	587.3	871.5	76	27
milk protein 1 1_3	3.2	587.3	790.4	81	29
milk protein 1 2_1	3.9	634.4	771.5	80	37
milk protein 1 2_2	3.9	634.4	934.5	80	37
milk protein 1 2_3	3.9	634.4	991.6	80	37
milk protein 1 3_1	2.8	598.3	911.5	81	25
milk protein 1 3_2	2.8	598.3	456.3	71	27
milk protein 1 3_3	2.8	598.3	266.2	76	49
milk protein 1 4_1	4.0	692.8	920.5	91	29
milk protein 1 4_2	4.0	692.8	991.5	106	31
milk protein 1 4_3	4.0	692.8	1090.6	106	29
milk protein 1 5_1	3.2	880.5	436.2	211	49
milk protein 1 5_2	3.2	880.5	663.0	206	51
milk protein 1 5_3	3.2	880.5	408.2	236	55

Table 3. continued

Identity	RT (min)	Q1 (amu)	Q3 (amu)	DP (V)	CE (V)
milk protein 2 2_1	2.6	467.3	707.4	101	21
milk protein 2 2_2	2.6	467.3	608.3	101	25
milk protein 2 2_3	2.6	467.3	379.2	101	33
milk protein 3 1_1	2.7	348.7	421.2	80	22
milk protein 3 1_2	2.7	348.7	550.2	80	22
milk protein 4 1_1	2.2	415.7	563.3	80	26
milk protein 4 1_2	2.2	415.7	660.4	80	26
milk protein 4 1_3	2.2	415.7	759.4	80	26
milk protein 4 2_1	2.4	390.8	471.3	80	25
milk protein 4 2_2	2.4	390.8	568.4	80	25
milk protein 4 2_3	2.4	390.8	681.4	80	25

Results and Discussion

Before analyzing a batch of wine samples the micro flow LC method was first compared to a high flow method that had previously been developed for allergen detection in baked goods.[5]

A spiked sample at a concentration of 1 ppm in white wine was analyzed using a Phenomenex Kinetex 2.6 µm column at a flow rate of 300 µL/min and then compared to the result obtained using a YMC Triart C18 2.7 µm column with micro flow LC at 25 µL/min. The gradient conditions were kept the same as was the injection volume and column temperature for both separations, and the results are shown in Figure 2.

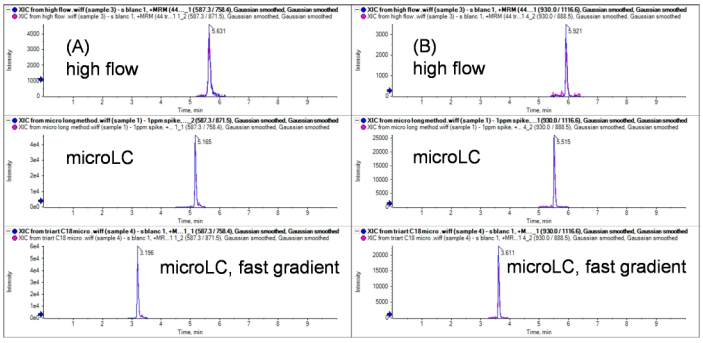

Figure 2. Comparison of high flow vs. microLC using a 1 ppm protein spike in white wine. A milk peptide is shown on the left (A) and an egg peptide is shown on the right (B).

Figure 2 and Table 4 show that moving to micro flow LC increases sensitivity by typically a factor of 4 to 13 fold in signal-to-noise (S/N), compared to the high flow LC method. Further to this the runtime could be halved without any detrimental effect on S/N.

Table 4. Signal-to-noise (S/N) improvements when using microLC and microLC with a faster gradient over the traditional high flow LC method

	Milk peptide	Egg peptide
S/N high flow LC	41.5	65.0
S/N microLC	539.5	260.6
S/N gain	**13x**	**4.2x**
S/N microLC with fast gradient	381.5	354.4
S/N gain	**9.2x**	**5.7x**

These results demonstrated the low gradient delay volume of the microLC system which enables rapid gradients even at flow rates ranging from 10 to 40 µL/min. The sensitivity increase was not only due to improved peak shape (peak width of 6 sec using micro flow LC and 8 sec for high flow LC) but was mainly down to the improved ionization efficiency which is possible at these lower flow rates, a fact that nanoLC has taken advantage of historically in proteomics applications.

The ionization efficiency gains of microLC are not as great as those seen in nanoLC, which runs at sub µL/min, but microLC has the advantage over nanoLC that runtimes can be a lot

shorter (< 6 minutes, Table 2) compared to a traditional nanoLC run which can take from 40 minutes to over 1 hour.[6] Also as microLC uses the TurboV™ source this technique has been shown to be very robust.[7]

To assess the sensitivity of this approach egg and milk proteins were spiked into white wine from 0.05 to 2 ppm concentrations. Figures 3 and 4 demonstrate that both egg and milk could be detected in wine at 50 ppb or below and that the response was linear over the 2 orders tested. This linearity of response is typical for LC-MS/MS which can easily exceed 3 orders of linearity which is far greater than commercial ELISA techniques.

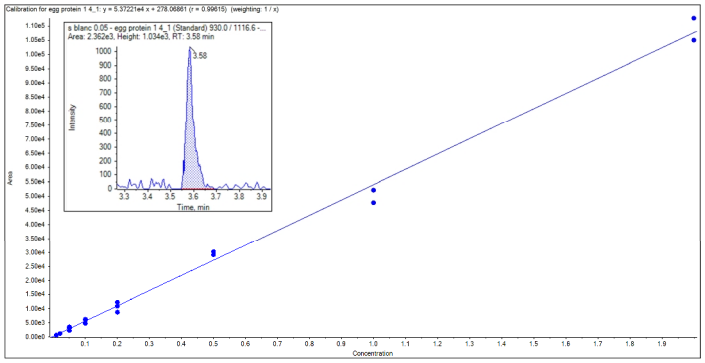

Figure 3. Calibration line from a peptide from egg which had been spiked into a sauvignon blanc wine (0.01 to 2 ppm) and chromatogram of the 50 ppb spike sample. The linearity is provided without the use of any internal standards.

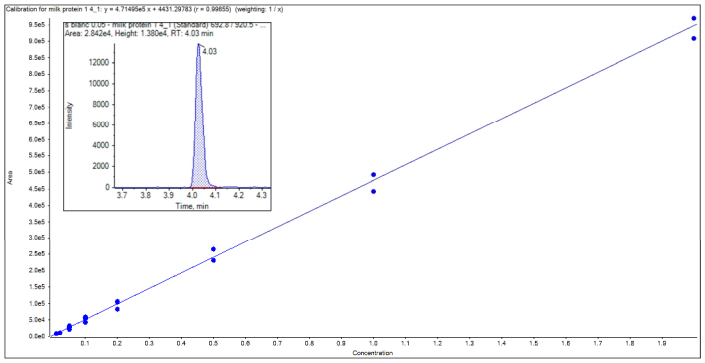

Figure 4. Calibration line from a peptide from milk which had been spiked into a sauvignon blanc wine (0.01 to 2 ppm) and chromatogram of the 50 ppb spike sample. The linearity is provided without the use of any internal standards.

One of the big advantages that LC-MS/MS has over other techniques used for allergen detection, such as ELISA and PCR, is its ability to acquire multiple points of identification. This is clearly shown in Figure 5 where MRM transitions are used to trigger the acquisition of full scan data. In this figure the AB SCIEX QTRAP® 5500 system was used to analyze a wine sample which had been spiked at 0.5 ppm. At this level multiple peptides for egg and milk were detected which were used to trigger full scan MS/MS spectra given unambiguous identification of these proteins in samples.

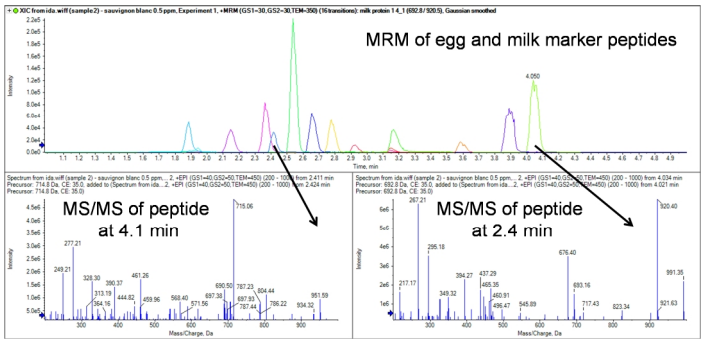

Figure 5. Micro flow LC-MS/MS analysis of 0.5 ppm spike of egg and milk proteins into a sauvignon blanc sample analyzed using the MIDAS™ workflow. The top pane shows the extracted ion chromatogram for the peptides of milk and egg and the bottom two panes show examples of MS/MS spectra for target peptides

Finally the effect of the white wine variety was tested by spiking 0.5 ppm of the proteins into different white wine samples. Figure 6 shows that the white wine variety did not have a major effect on response of the peptides or the peptide profile. However, for accurate quantitation the addition of internal standard of the proteins into wine would be recommended or the use of standard addition (as done previously in baked goods[4]).

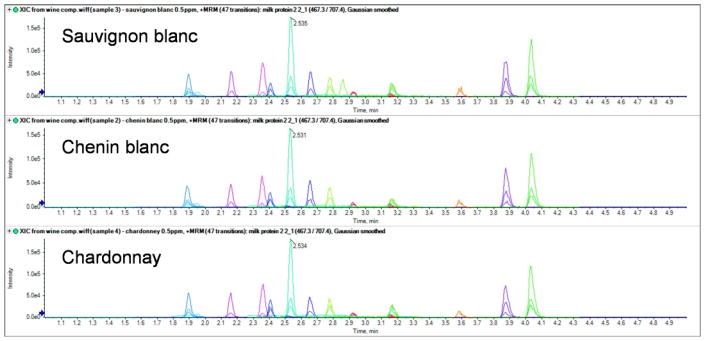

Figure 6. microLC-MS/MS analysis of 0.5 ppm spiked samples of egg and milk proteins into 3 different white wines

Summary

MicroLC-MS/MS using the Eksigent ekspert™ microLC 200 system coupled to an AB SCIEX QTRAP® 5500 system has been shown to offer a rapid, robust, sensitive and specific assay for the simultaneous detection of a series of milk and egg markers in white wine. A simple sample preparation was used with the complete extraction procedure in the same Eppendorf tube. The method is capable of providing detection levels below 100 ppb.

Sensitivities achieved were equivalent to sensitivities of some currently available methods based on ELISA and real-time PCR methods. The microLC-MS/MS approach has the additional advantage of being a potential multi-allergen screen unlike ELISA where different allergens, like egg and milk, are detected by separate kits. Using the MIDAS™ workflow full scan QTRAP® MS/MS spectra were obtained at the same time as quantitative information, confirming multiple peptide target identification and reducing the occurrence of false positives associated with other techniques.

Micro flow LC has been able to show that analysis times can be halved and sensitivities increased by upwards of a factor of 10 with also the additional reduction in solvent consumption which leads to the added benefit of a cost saving for the allergen analysis.

References

1 EFSA: 'Scientific opinion related to a notification from the International Organisation of Vine and Wine (OIV) on casein/caseinate/milk products to be used in the manufacture of wine as clarification processing aids pursuant to Article 6, paragraph 11 of Directive 2000/13/EC – for permanent exemption from labelling' EFSA Journal 10 (2011) 2384-2369

2 DIRECTIVE 2000/13/EC: 'on the approximation of the laws of the Member States relating to the labelling, presentation and advertising of foodstuffs' March 2000

3 COMMISSION REGULATION (EU) No 1266/2010 'regards labelling requirements for wines' December 2010

4. K. Mriziq et al.: 'Higher Sensitivity and Improved Resolution Microflow UHPLC with Small Diameter Turbo V™ Source Electrodes and Hardware for use with the Eksigent expressHT™ Ultra System' Technical Note Eksigent (2011) # 4590211-01

5. Stephen Lock et al.: 'The Detection of Allergens in Bread and Pasta by Liquid Chromatography Tandem Mass Spectrometry' Application Note AB SCIEX (2010) # 1830610-01

6 K. Sandra et al.: 'The Q-Trap mass spectrometer, a novel tool in the study of protein glycosylation' J Am Soc Mass Spectrom 15 (2004) 413-423

7. Stephen Lock: 'The Use of Micro Flow UHPLC in Pesticide Screening of Food Samples by LC-MS/MS' Application Note AB SCIEX (2012) # 6330212-01

For Research Use Only. Not for use in diagnostic procedures.

Publication number: 8880213-01

Headquarters
500 Old Connecticut Path, Framingham, MA 01701 USA
Phone 508-383-7700
www.sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
www.absciex.com/offices