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A Complete Solution for Gelatin Species Authentication in Routine Analysis Using ProteinWorks™ Auto-eXpress Digest Kit and Xevo™ TQ-XS

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Abstract

Gelatin from different animal sources is used widely as an ingredient in many food, pharmaceutical, and cosmetic products. Species authentication of gelatin has become increasingly important due to heath and religious reasons. For example, the most widely used porcine gelatin is a non-halal ingredient which is strictly forbidden in Islam religion. In this work, we have developed a complete solution for gelatin species authentication comprising a simple and fast sample preparation with a robust LC-MS/MS method suitable for routine analysis. The workflow includes sample preparation method using ProteinWorks Auto-eXpress Digest Kit. This is a ready-to-use kit, which enables the sample digestion protocol to complete within three hours, thus allowing sample preparation, injection into the instrument and obtaining results on the same-day. The separation of markers was done on the ACQUITY™ Premier Column with an ACQUITY UPLC™ I-Class coupled to a Xevo TQ-XS tandem mass spectrometer to achieve maximum sensitivity.

Benefits

- Simple and fast three-step sample preparation protocol using ProteinWorks Auto-eXpress Digest Kit within three hours
- Multiple species detectable in gelatin-containing food samples in a single liquid chromatography-mass spectrometry (LCMS) method
- Multiple MRM transitions used for each peptide marker to increase the reliability for species identification

Introduction

Gelatin is a mixture of polypeptides obtained from the partial hydrolysis of collagens extracted from the hides, skin, and bone of animals.[1] It is used widely as an ingredient in food, pharmaceutical and cosmetic products due to its gelling property and low cost.[1,2] The primary sources of commercial gelatins are derived from bovine and porcine gelatins, although there are also other new emerging gelatin sources from fish and poultry.[3]

The source of the gelatin in products has become a major concern for various reasons such as for health and religion. For example, products containing porcine derivatives are considered non-halal and are forbidden by the Islam religion.[4] Therefore, gelatin species authentication is important to ensure the products used by the Islamic community comply with halal regulation in their respective countries.

Recently, LCMS-based methods have gained popularity in gelatin authentication due to their sensitivity and selectivity.[5] This technique commonly detects species-specific peptide markers obtained from tryptic digestion of gelatin samples. When implemented properly, it allows the detection of several species at the same time in a single analysis. However, one of the challenges faced with this technique is the long overnight tryptic digestion in the sample preparation step. When using the ProteinWorks Auto-eXpress Digest Kit, the *Rapi*Gest™ SF Surfactant improves the speed and completeness of tryptic digestion, thus the sample preparation protocol is significantly shortened to three hours. This maximizes productivity and enables the lab analyst to obtain the results within the same day of experiment.

In our previous work, a proteomics discovery workflow was demonstrated to identify species-specific peptide markers of bovine and porcine gelatin.[6] This application note is a continuation, whereby a robust LC-MS/MS method was developed for the routine analysis of bovine and porcine species-specific peptide markers found in gelatin food samples.

Experimental

Two commercially available authentic bovine and porcine gelatin reference standards (Sigma Aldrich) and eight candy samples were pre-treated with 50 mM ammonium bicarbonate (NH_4HCO_3). This is then followed by a three-step protocol using ProteinWorks Auto-eXpress Low 3 Digest Kit (p/n: 176004077 < https://www.waters.com/nextgen/global/shop/application-kits/176004077-proteinworks-auto-express-low-3-digest-kit.html>) as shown in Figure 1.

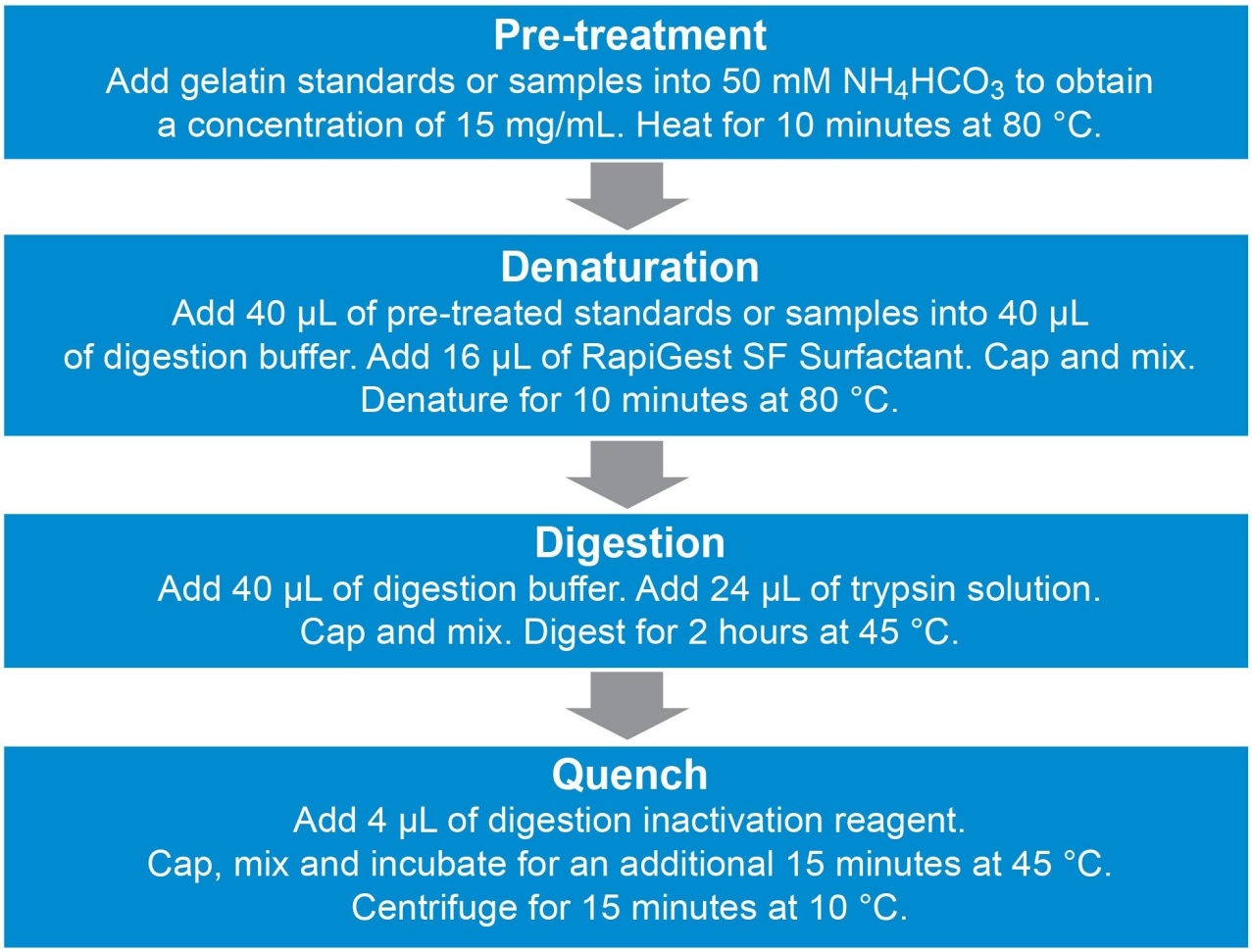

Figure 1. Three-step digestion protocol using ProteinWorks Auto-eXpress Digest Kit.

LC Conditions

LC system:	ACQUITY UPLC I-Class PLUS System
Column(s):	ACQUITY Premier UPLC HSS T3 Column, 1.8 µm, 2.1 mm x 100 mm (p/n: 186009468)
Column temp.:	40 °C
Injection volume:	2 µL
Flow rate:	0.40 mL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.4	95	5	Initial
0.5	0.4	95	5	6
12	0.4	75	25	6
12.1	0.4	25	75	6
14	0.4	25	75	6
14.1	0.4	95	5	6
16.5	0.4	95	5	6

MS Conditions

MS system:	Xevo TQ-XS

Ionization mode:	ESI (+)
Capillary voltage:	1 kV
Source temp.:	130 °C
Desolvation temp.:	600 °C
Cone gas flow:	150 L/Hr
Desolvation gas flow:	1000 L/Hr
Nebuliser gas flow:	7.0 bar

Data Management

Chromatography software:	MassLynx™ v4.2
MS software:	MassLynx v4.2
Informatics:	TargetLynx™ v4.2

MRM Transitions

The data was collected using MRM transition mode. 4–5 MRM transitions were chosen for each peptide markers as shown in Table 1 below.

Species	Sequence	MRM
Bovine	GATGPAGVR	393.2>402.2
		393.2>499.3
		393.2>556.3
		393.2>657.4
	GETGPAGPAGPIGPVGAR	780.91>570.3
		780.91>823.48
		780.91>766.5
		780.91>991.6
	IGQPGAVGPAGIR	596.8>513.3
		596.8>894.5
		596.8>669.4
		596.8>740.4
		596.8>797.5
	QGPSGASGER	473.22>576.3
		473.22>519.3
		473.22>498.2
		473.22>585.3
		473.22>760.4
	SGDRGETGPAGPAGPIGPVGAR	659.34>766.5
		659.34>823.5
		659.34>985.4
		659.34>991.6
Porcine	GETGPAGPAGPVGPVGAR	773.9>499.3
		773.9>809.5
		773.9>752.4
		773.9>991.5
	GlpGEFGLpGPAGPR	727.4>642.3
		727.4>837.5
		727.4>780.4
		727.4>984.5
		727.4>1283.6
	SGDRGETGPAGPAGPVGPVGAR	654.7>809.5
		654.7>752.4
		654.7>880.5
		654.7>977.6
		654.7>985.4
	TGQPGAVGPAGIR	590.82>570.3
		590.82>513.3
		590.82>669.4
		590.82>894.5
		590.82>797.5

Table 1. MRM transitions of nine bovine and porcine species-specific

peptide markers.

Results and Discussion

The main advantage of this sample preparation method is that this entire process can be completed within three hours from sample pretreatment to the quenching step. The use of *Rapi*Gest SF, an anionic surfactant which is MS-friendly in the denaturation step, helps to accelerate the speed of tryptic digestion. Unlike other protocols which require an overnight digestion, this allows a same-day sample preparation, injection, and data processing which is ideal for fast and routine analysis.

From the previous discovery work,[6] further study has been done to establish a method for an LCMS analysis. A total of nine peptide markers; five bovine, and four4 porcine markers were chosen and for every peptide marker, 4 to 5 MRM transitions were selected as confirmation ions to increase the robustness and reliability of the detection method.

At least three of the MRM transitions with a minimum of signal-to-noise ratio of three must be present for a peptide marker to be detected and at least two peptide markers must be present for species assignment.[7]

Eight commercial gelatin-containing candy samples were analyzed using the established method and the results were tabulated in Table 2.

Sample	Halal declaration	No. of peptide markers detected	
		Bovine	Porcine
Candy 1	Yes	5	N.D
Candy 2	Yes	5	N.D
Candy 3	Yes	5	N.D
Candy 4	Yes	5	N.D
Candy 5	No	N.D	4
Candy 6	No	5	4
Candy 7	No	5	4
Candy 8	No	4	4

Table 2. Results of the screening of Eight commercial candy samples.

One of the samples, Candy 1 is declared as halal-certified on its product labelling. The chromatograms obtained from the LCMSMS analysis matches with the product label as there were no porcine markers detected in the sample as shown in Figure 2. Another sample, Candy 5 is a non-halal product where the gelatin used is from porcine source. The chromatograms of Candy 5 (Figure 3) match with the product label with the detection of four porcine markers.

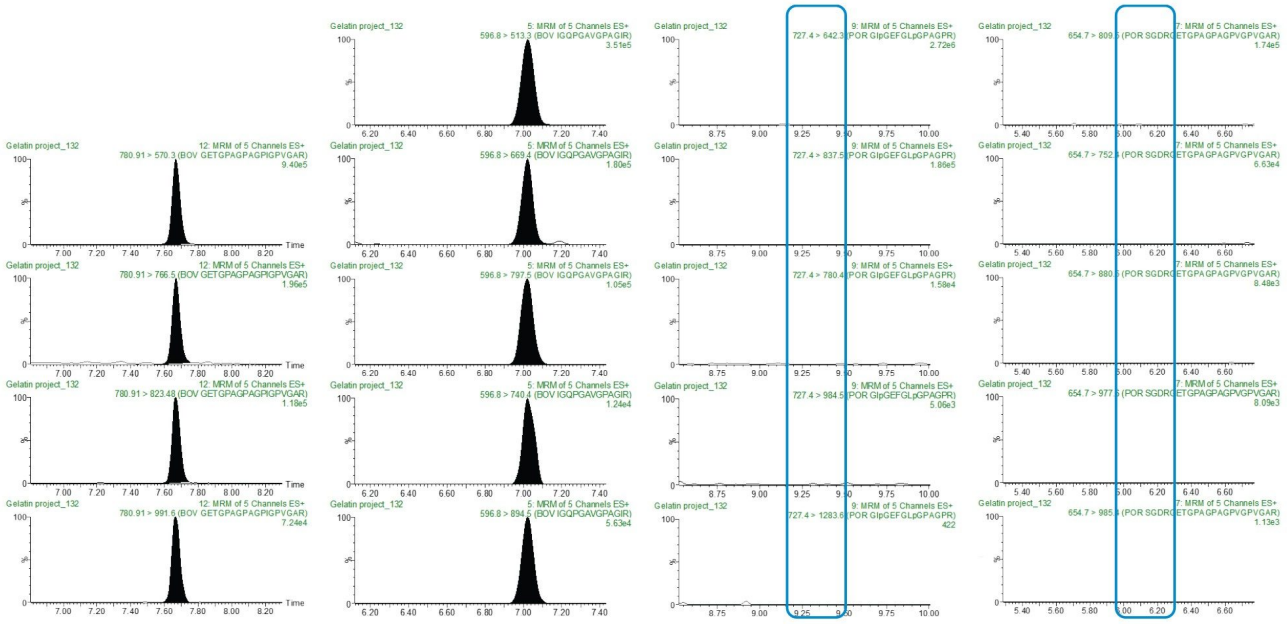

Figure 2. Chromatograms of sample Candy 1 (halal labeled). The MRM transitions shown are of
(a) Two bovine markers (GETGPAGPAGPIGPVGAR and IGQPGAVGPAGIR) and
(b) Two porcine markers (GlpGEFGLpGPAGPR and SGDRGETGPAGPAGPVGPVGAR). The expected retention times of the Two porcine markers (not presence) are highlighted with the light blue boxes.

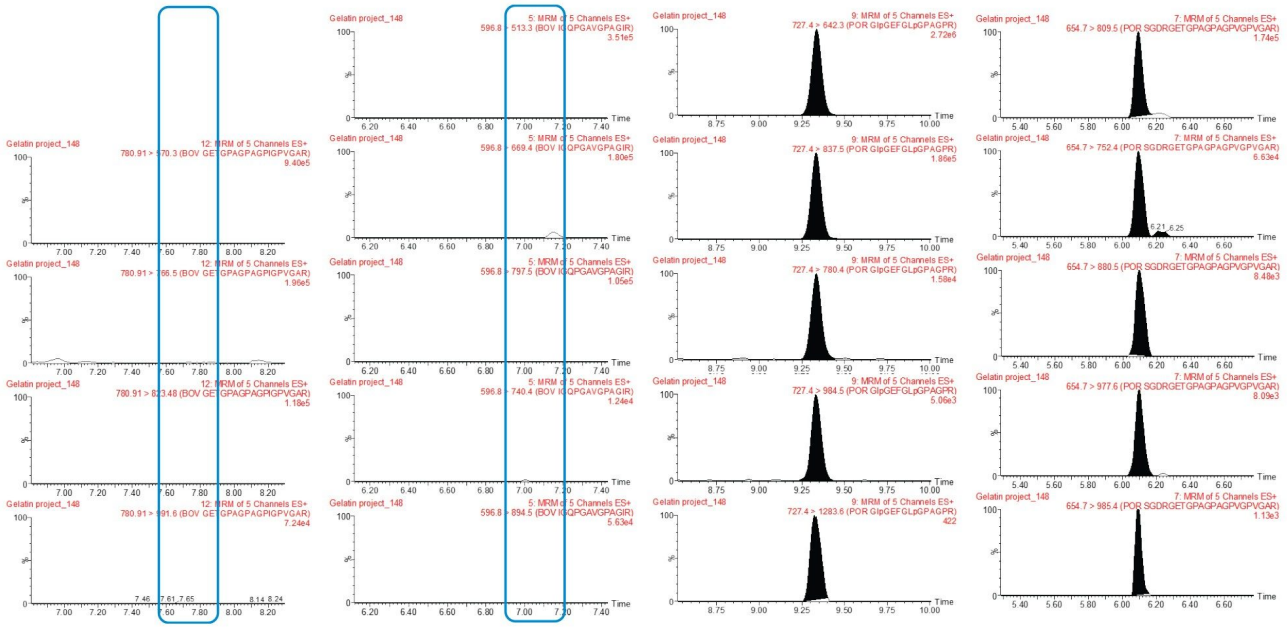

Figure 3. Chromatograms of sample Candy 5 (non-halal labeled). The MRM transitions shown are of
(a) Two bovine markers (GETGPAGPAGPIGPVGAR and IGQPGAVGPAGIR) and
(b) Two porcine markers (GlpGEFGLpGPAGPR and SGDRGETGPAGPAGPVGPVGAR). The expected retention times of the Two bovine markers (not presence) are highlighted with the light blue boxes.

Porcine gelatin standard was spiked into bovine gelatin standard to simulate porcine gelatin adulteration. Figure 4 shows the chromatograms of 1% porcine gelatin spiked into bovine gelatin and an unspiked bovine gelatin. It clearly shows that the porcine markers could be easily detected at this level with the established method on the Xevo TQ-XS.

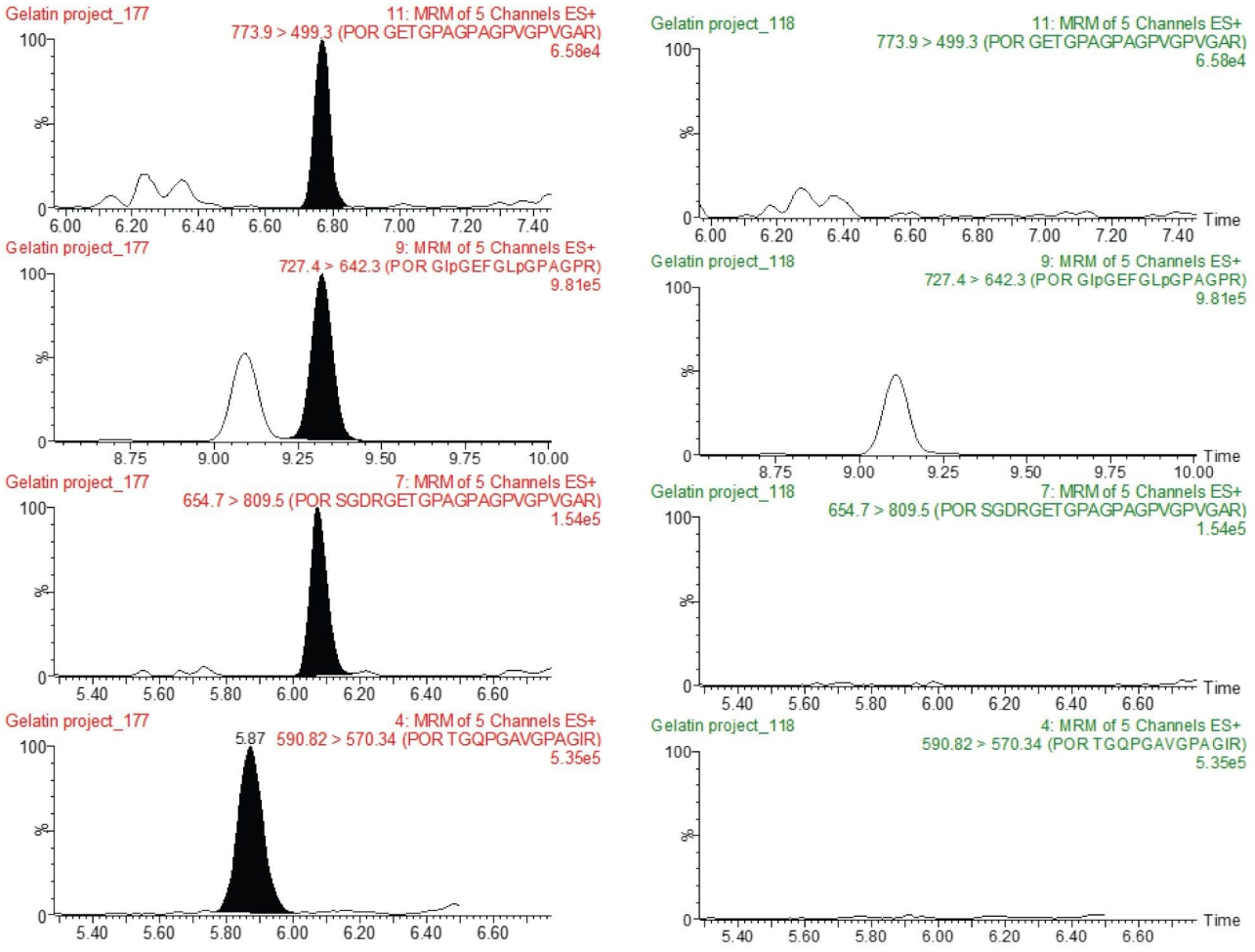

Figure 4. Chromatograms of four porcine markers from

(a) bovine gelatin adulterated with porcine gelatin at 1% and

(b) unadulterated bovine gelatin.

Conclusion

This work demonstrates the complete solution of gelatin species authentication from sample preparation to peptide markers separation using LC-MS/MS suitable for routine analysis of gelatin-containing food products such as candies. The established method enables detection of gelatin origin species (bovine or porcine) at 1%

adulteration. The ProteinWorks Auto-eXpress Digest Kit allows a simple and fast digestion protocol which could be completed within 3 hours. This kit-based approach also ensures the easiness of method transfer in a routine testing setting.

References

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