SHIMADZU
Excellence in Science

Application News

MALDI-2301

MALDI-TOF Mass Spectrometry

Optimization of Trypsin Digestion Methods for the Detection Between Mixed A1/A2 and A2-Only Cow Milk Using MALDI-TOF-MS

■ Introduction

The milk market has seen various changes over time. About 20 years ago, A2-only cow milk was introduced into the market as an alternative to traditional cow milk (A1/A2). A2-only cow milk claims better digestibility and lower risk of other ailments while keeping the same nutrition facts as mixed A1/A2 cow milk. The terms 'A1' and 'A2' refer to the variant of β-casein that is present in cow milk. A1 and A2 β-casein vary by a single amino acid at position 67; A1 β-casein H67, A2 β-casein P67 (**Figure 1**).

The current standard practice for determining if a cow will produce A2-only milk or mixed A1/A2 milk is through a genetic test to determine which alleles the cow carries. This can be a long and expensive process to have a full understanding of the genetic makeup of the herd.

The development of a technique to test milk for the presence of the A1 and A2 β-casein would allow for a more rapid detection method of the final product. MALDI-TOF-MS is a technique that allows for the rapid detection of peptides and proteins using minimal sample, making it well-suited for this application. Trypsin digestion has been commonly used as a method for peptide mass fingerprinting of large proteins, breaking them down into smaller peptides that can be easier to ionize. The single amino acid difference between the two peptides provides an avenue for detection when coupled with trypsin digestion (**Tables 1 and 2**). Trypsin selectively cleaves peptides at the cationic residues of arginine and lysine. This selective cleaving gives rise to a tryptic fragment that has a different mass due to the single amino acid difference. The use of trypsin digestion combined with MALDI-TOF-MS can be used to provide a fast method of determining which β-casein is present in the final product.

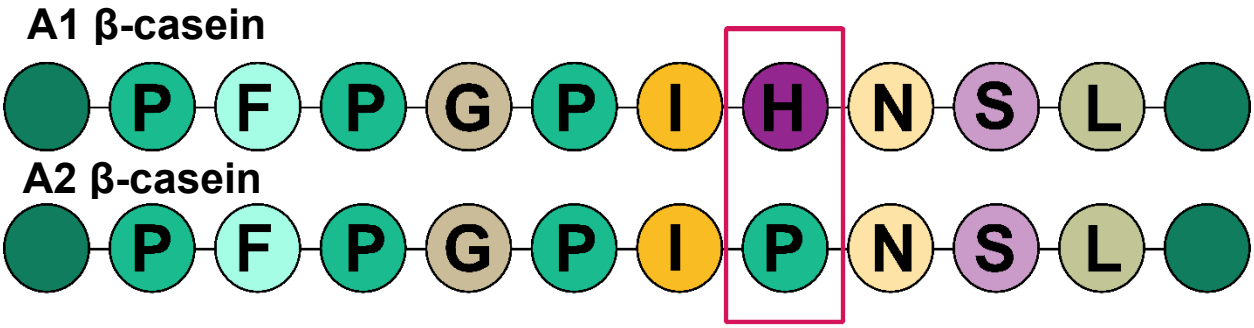

Figure 1: Amino acid sequence of A1 and A2 β-casein from position 61-70, highlighting the difference in position 67.

Table 1: Tryptic fragment results generated from PeptideMass for A1 β-casein. Proteolytic fragment (49-97) masses and the 67th amino acid were highlighted.

Mass	Position	Peptide Sequences for A1 β-Casein
6363.3300	114-169	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK
5360.2816	49-97	IHPFAQTQSLVTPFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK
2647.8494	2-25	ELEELNVPGEIVESLSSSEESITR
2187.6045	187-202	DMIPQAFLLYQEPVLGPVR
1983.0088	33-48	FQSEEQQQTEDELQDK
830.8617	171-183	AVPYPQR
780.9855	170-176	VLPVPQK
748.9149	108-113	EMPFPK
742.9360	203-209	GPFPIIV
646.7792	100-105	EAMPK

Table 2: Tryptic fragment results generated from PeptideMass for A2 β-casein. Proteolytic fragment (49-97) masses and the 67th amino acid were highlighted.

Mass	Position	Peptide Sequences for A2 β-Casein
6363.3300	114-169	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK
5320.2572	49-97	IHPFAQTQSLVTPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSK
2647.8494	2-25	ELEELNVPGEIVESLSSSEESITR
2187.6045	187-202	DMIPQAFLLYQEPVLGPVR
1983.0088	33-48	FQSEEQQQTEDELQDK
830.8617	171-183	AVPYPQR
780.9855	170-176	VLPVPQK
748.9149	108-113	EMPFPK
742.9360	203-209	GPFPIIV
646.7792	100-105	EAMPK

■ Milk Sample Preparation and Trypsin Digestion

Milk samples were collected from local grocery stores. To determine the difference between mixed A1/A2 and A2-only cow milk, milk proteins were extracted. The protein of interest, β-casein, can be easily isolated through decreasing the pH of the solution to cause the proteins to become insoluble and crash out of solution. A standard protein extraction method was used to isolate β-casein and other related proteins.

Extracted protein was used for an in-solution trypsin digest and made into a 5 mg/mL solution in 50 mM Tris-HCl (pH 8.0). Raw milk samples were diluted in 50 mM Tris-HCl (pH 8.0) (1:50, *v:v*). Sinapinic acid was used as the MALDI matrix, prepared as a 20 mg/mL solution in 50% Acetonitrile water with 0.1% TFA.

In-solution trypsin digestion was conducted using 50 mg of extracted protein; trypsin was added in a 25:1 protein-to-protease ratio. The resulting solution sat overnight at 37°C before being ended with the addition of TFA. Solution was centrifuged and the supernatant was analyzed via MALDI-TOF-MS.

■ Analysis of Digestion Methods

Trypsin digestion protocols require an overnight digestion step to prevent autolysis of trypsin through using a lower concentration of enzyme.

In-solution trypsin digestion was used to first ensure detection of the tryptic fragments of interest. This method allowed for visualization of the two key tryptic peptide fragments from A1 and A2 β-casein (**Figure 2**). Confirming the differences between A1 and A2 β-casein are detectable via MALDI-TOF-MS. Although successful at differentiating A1/A2 from A2 milk, a lengthy overnight digestion procedure is not amenable to high throughput and for large-scale analysis of milk samples.

To improve sample work-up and turnaround time, an on-plate digestion method was developed. In this method, trypsin was dried onto the target before addition of the extracted milk protein. The plate was incubated in a humid environment for 15 minutes before digestion was quenched and analyzed with MALDI. The characteristic A1 and A2 β-casein peaks were easily identifiable with this method (**Figure 3**). This procedure brought detection from *a day down to just over an hour*, including milk protein extraction. To further simplify and speed up the detection process, the same procedure was tested with diluted raw milk samples, shortening the procedure down to *under 20 minutes*. This minimal sample prep method produced spectra that allowed for the detection of mixed A1/A2 from A2-only cow's milk (**Figure 4**). An overview of the different sample preparation techniques is visualized in **Figure 5**.

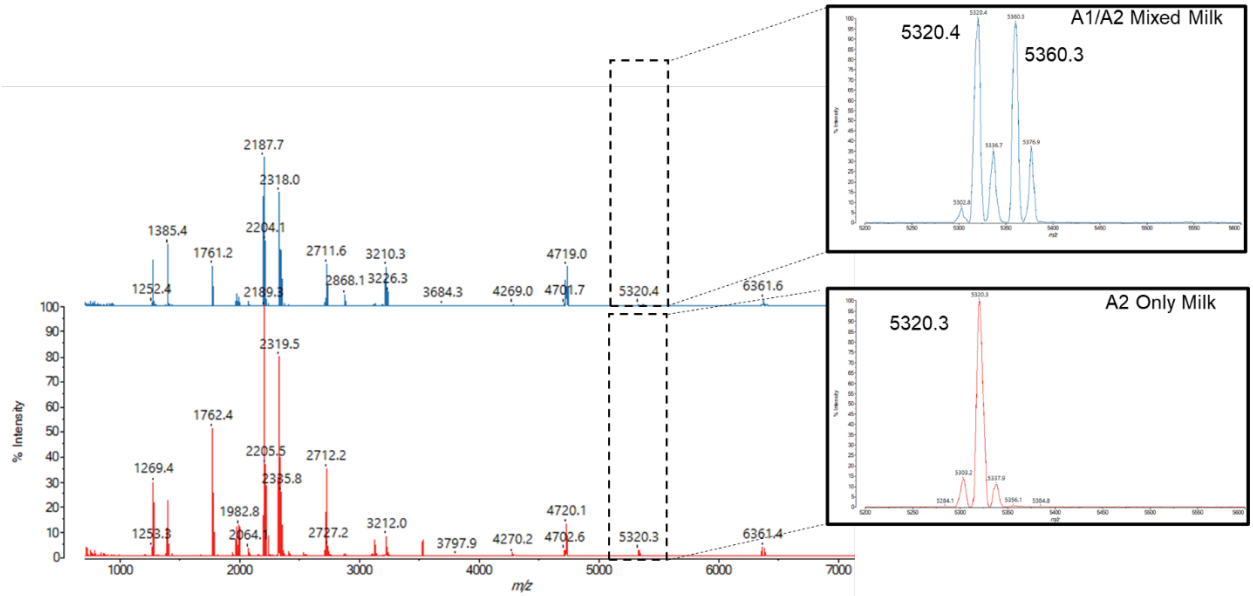

Figure 2: MALDI spectrum of in solution trypsin digestion of mixed A1/A2 milk and A2-only milk.

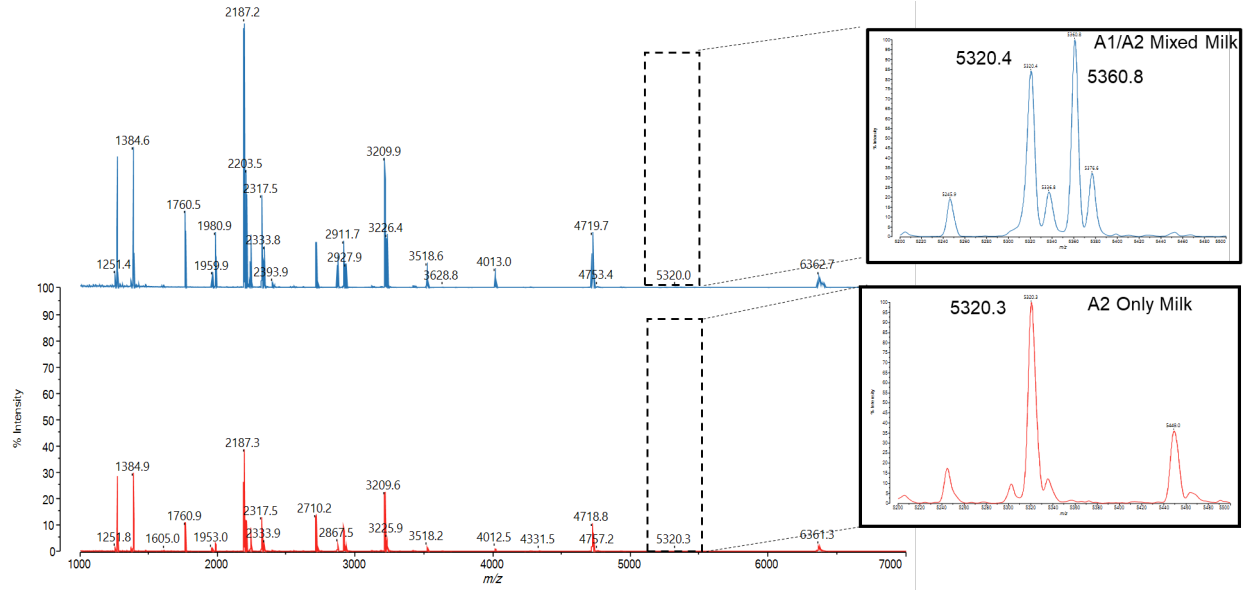

Figure 3: MALDI spectrum of on-plate digestion using extracted protein mixture of mixed A1/A2 milk and A2-only milk.

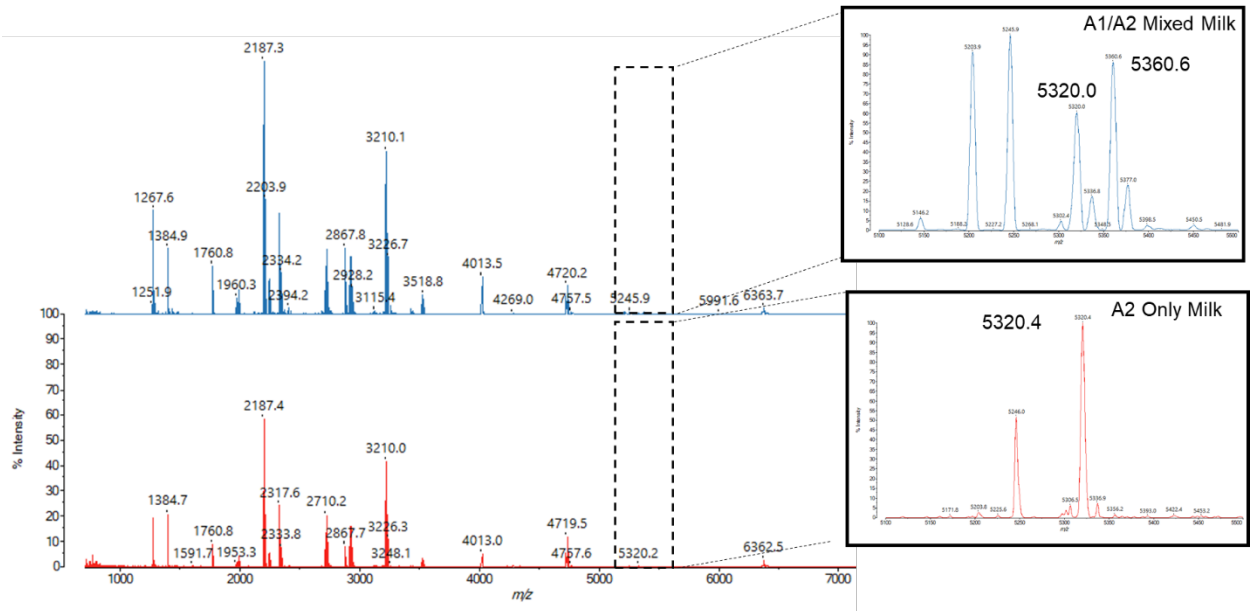

Figure 4: MALDI spectrum of on-plate digestion using diluted samples of mixed A1/A2 milk and A2-only milk.

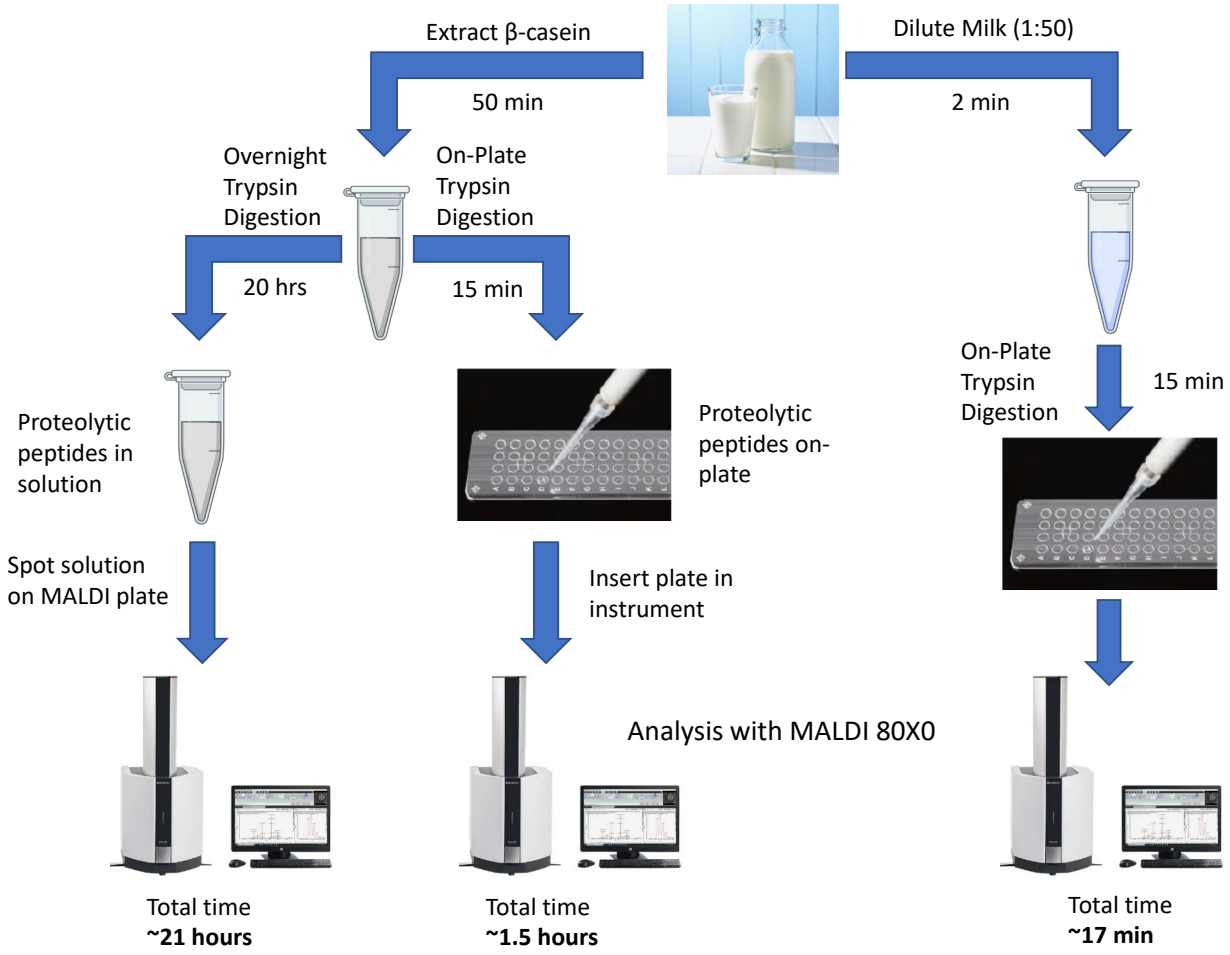

Figure 5: Sample preparation overview with overall time for each procedure.

■ Conclusion

The use of trypsin digestion and MALDI-TOF-MS analysis in tandem has the potential to be a powerful tool for the determination of mixed A1/A2 milk from A2-only milk. The addition of on-plate trypsin digestion and minimal sample preparation makes for a rapid detection method.

Here, we have shown that MALDI-TOF-MS can be used to determine the difference between mixed A1/A2 milk and A2-only milk using a variety of sample preparation techniques and trypsin digestion protocols.

■ References

Available Upon Request

First Edition: July 2023

SHIMADZU Corporation
www.shimadzu.com/an/

SHIMADZU SCIENTIFIC INSTRUMENTS
7102 Riverwood Drive, Columbia, MD 21046, USA
Phone: 800-477-1227/410-381-1227, Fax: 410-381-1222
URL: www.ssi.shimadzu.com