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Matrix-Assisted Laser Desorption Ionization \(MALDI\) Time-of-flight Mass Spectrometry \(TOFMS\) for Direct Analysis of Bioactive Peptide an Protein

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Matrix-Assisted Laser Desorption Ionization (MALDI) Time-of-flight Mass Spectrometry (TOFMS) for Direct Analysis of Bioactive Peptide and Protein

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Final Report

Matrix-Assisted Laser Desorption Ionization (MALDI) Time-of-flight Mass Spectrometry (TOFMS) for Direct Analysis of Bioactive Peptide and Protein Mixtures

Running Title: MALDI TOFMS for Mixture Analysis

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Summary

The overall goal of this research was to develop a MALDI method of mixture analysis based on time-lag focusing matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). The specific objectives were 1) to develop sample/matrix preparation methods for mixture analysis and 2) to demonstrate the overall analytical performance of the MALDI method for mixture analysis. We have achieved these specific goals, as detailed in this report.

We have shown that direct analysis of peptide and protein mixtures by MALDI-TOFMS is strongly dependent on the sample and matrix preparation. A two-layer sample preparation method is demonstrated to be very effective for analyzing complex mixtures. In this method, the first layer on the MALDI probe is the densely packed matrix microcrystals formed by fast solvent evaporation of a matrix solution. A mixture solution containing both matrix and sample is then deposited onto the first layer to form uniform analyte/matrix micro-cocrystals. It is found that the addition of matrix to the second-layer sample solution proves to be critical in analyzing mixtures of peptides and proteins covering a broad mass range. The effect of solvent conditions for preparing the second-layer solution is discussed. The application of this method is demonstrated for the analysis of cow's milk where milk proteins as well as peptide fragments produced from proteins by indigenous proteinases are detected. Direct analyses of peptides and proteins from a bacteria extract and crude egg white are also illustrated.

The MALDI-TOFMS method developed from this research provides the foundation for future work in applying the mass spectrometric technique for bioactive reagent detection. We envision that it will also be widely used for protein identification and characterization.

Introduction

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) has a wide applicability and is moderately tolerant to buffers, salts, and other additives in the sample, at least for peptide and protein analysis. However, compared with electrospray, MALDI is difficult to interface to solution-based separation methods, such as liquid chromatography (LC) and capillary electrophoresis (CE) for on-line analysis of complex mixtures [1-3]. For many applications, off-line LC or CE fractionation, followed by MALDI analysis, is the method of choice. However, for applications where the speed of analysis is important, such as in the case of rapid identification of bacteria based on peptide and protein profiles, direct analysis of mixtures by MALDI offers the advantage.

Accurate analysis of a broad mass range of peptides and proteins in a complex mixture by MALDI-MS is an analytically challenging task. An excess amount of one component in a mixture may suppress the ion signals of the other components. The poor solubility of some peptides and proteins in a chosen solvent used for MALDI sample preparation may cause an incomplete detection of the species of interest. In addition, impurities in a complex mixture may interfere with analyte detection and reduce the sensitivity and detectability. Several studies have been reported on the effects of experimental parameters on MALDI analysis of peptide and protein mixtures [4-8]. It is clear that the performance of MALDI-MS for mixture analysis is very much dependent on the sample/matrix preparation method used. A major goal of MALDI method development is to optimize sample preparation tailored to specific applications.

We have developed a two-layer sample preparation method for the analysis of complex peptide and protein mixtures. Compared to other rapid sample preparation methods such as dried-droplet and fast evaporation method, the two-layer method is found to be the most effective in detecting a broad mass range of peptides and proteins.

Experimental

Mass spectra were recorded in a linear time-lag focusing (TLF) MALDI time-of-flight mass spectrometer that has been described previously [9,10]. Briefly, it features a four-plate source design with a grid inserted on the repeller side of the first extraction plate, pulsed ion extraction for time-lag focusing, and a one-meter linear flight tube. The ions are generated using the 337 nm laser beam from a nitrogen laser, having a pulse width of 3 ns (model VSL 337ND, Laser Sciences Inc., Newton MA). The laser flux used for MALDI is typically about 9 MW cm^{-2} for the analysis using sinapinic acid as the matrix and about 3 MW cm^{-2} for the analysis using α-cyano-4-hydroxycinnamic acid as the matrix [10]. A microchannel plate detector was used for ion detection and a Hewlett-Packard MALDI data system was used for mass spectral recording

and data processing. In general, mass spectra from 50 to 100 laser shots were summed to produce a final spectrum. No baseline correction was performed on the mass spectra shown in the figures.

For the two-layer sample preparation [11], the matrix solution is placed on a MALDI probe and allowed to dry to form a microcrystal layer. A solution containing *both* the analyte and the matrix is then added to the top of the matrix layer. For the analysis of standard peptide mixtures and *Escherichia coli*, α-cyano-4-hydroxycinnamic acid (HCCA) is used as the matrix. Sinapinic acid (SA) is used for the milk and crude egg white analysis. The concentration of the first-layer matrix solution is 5 mg/ml in 80% acetone/MeOH for HCCA and 6 mg/ml in 60% MeOH/acetone for SA. The concentration of the second-layer matrix solution is varied based on the solvents used and different applications. Milk samples were purchased from a local grocery store. Before testing, milk was diluted 1:50 (v/v) with water, unless specified, and then mixed 1:1 (v/v) with the second-layer matrix solution.

The bacterial sample (*Escherichia coli*, ATCC 9637) was a gift of Dr. Randy Long of Edgewood RDE Center, Aberdeen Proving Ground, MD. A solvent suspension method [12] was used for peptide and protein extraction from the bacteria. Briefly, about 1 to 1.5 mg of lyophilized bacteria were suspended in 250 μl 0.1% trifluoroacetic acid (TFA). The cell suspension was vortexed for about 2 min and then centrifuged. The supernatant solution was taken for MALDI analysis.

The crude egg white sample was purchased from Sigma (Catalog Number A-5253). It was dissolved in 0.1% TFA/water in a concentration of 2.1 mg/mL.

External calibration was used for mass calibration. For milk analysis, bovine insulin chain B, horse heart cytochrome c, and horse myoglobin were used as the calibrants. For bacteria analysis, lys-bradykinin and cytochrome c were used as the calibrants. For the analysis of crude egg white, trypsinogen and bovine serum albumin were used. All mass calibrants were purchased from Sigma.

Results and Discussion

Sample preparation plays an important role in MALDI analysis of peptide and protein mixtures. An optimal preparation method should provide good sensitivity, mass accuracy, and reproducibility. In addition, it should be able to detect analyte ions with a broad mass range. Other important considerations include the speed of sample preparation and feasibility for automation. There are a number of sample preparation methods developed for MALDI applications. These include dried-droplet [13], vacuum drying [14], crush crystals [15], slow crystal growing [16], active film [17,18], pneumatic spray [19], electrospray [20], fast solvent evaporation [21], sandwich [22,8], and two-layer method [11].

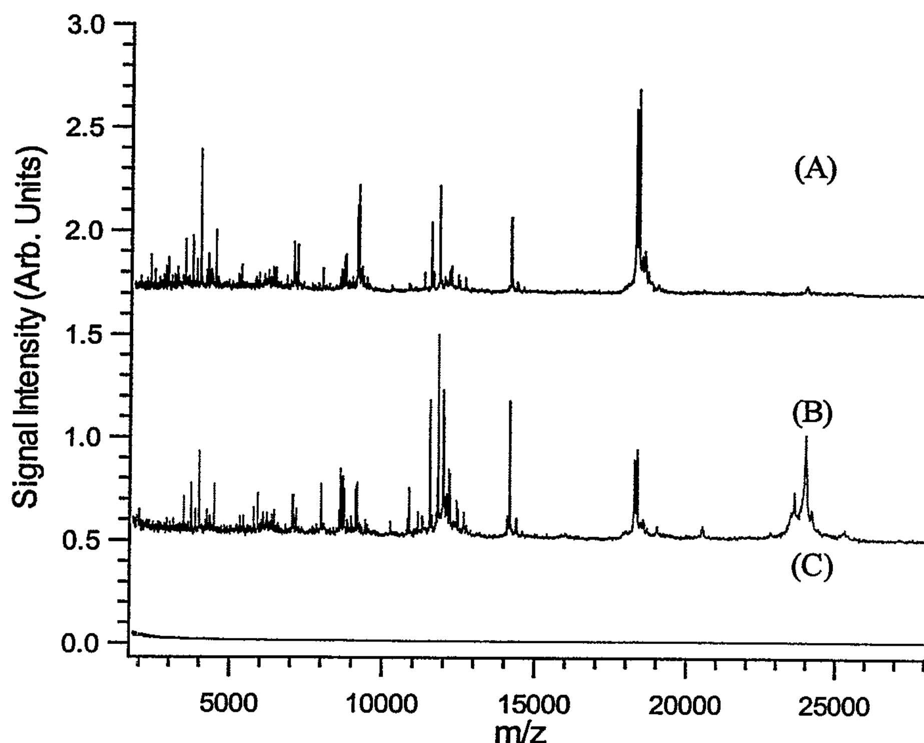

Figure 1. MALDI mass spectra of cow's milk containing 2% fat obtained by using (A) dried-droplet, (B) two-layer, and (C) fast evaporation sample preparation. In the dried-droplet method, the sample/matrix solution is the same as the second-layer solution used in the two-layer method. In the fast evaporation method, the second-layer solution contains the diluted milk only. The time-lag focusing conditions in MALDI-TOF were optimized to focus the ions at m/z~15,000.

The two-layer method was developed based on the crush crystal method [15] and the fast evaporation method [21]. It involves the use of fast solvent evaporation to form the first layer of small crystals (as in the first step of the fast evaporation method), followed by deposition of a *mixture* of matrix and analyte solution on top of the crystal layer (as in the sample/matrix deposition step of the crushed-crystal method). The key difference between the two-layer method and the fast evaporation method is the preparation of the second-layer solution. In the fast evaporation method, the analyte solution is directly deposited onto the first layer without premixing it with a matrix solution. This method is very effective for the analysis of peptides, but not proteins. In an earlier publication [11], we have shown that the two-layer method (originally called uniform submicron-crystal formation method) provides high detection sensitivity and excellent spot to spot reproducibility for peptides as well as proteins. The enhancement is likely due to the increase of the matrix to analyte ratio and improved isolation between analyte molecules as a result of depositing a solution containing both analyte and matrix molecules on top of the microcrystals, instead of using only the analyte solution. The two-layer

method is also different from the sandwich method in which the first and second layers are prepared as the fast evaporation method, followed by the deposition of a matrix layer [22,8]. In the sandwich method, the analyte is not pre-mixed with the matrix. As shown below, variation of solvent composition for preparing the analyte and matrix mixture is the key feature of the two-layer method and has a great effect on peptide and protein analysis.

In our previous work, we have shown that the two-layer method is effective for the analysis of simple peptide and protein standards [11]. With the optimization of the second-layer sample/matrix solution, the two-layer method is found to be very effective for the analysis of complicated mixtures containing both peptides and proteins. An application of this method and its comparison with other methods are shown in Figure 1. The MALDI mass spectra of 2% fat milk in Figure 1 were obtained by using dried-droplet, two-layer, and fast evaporation methods. The dried-droplet method provides good sensitivity, but the reproducibility from shot to shot is poor. The fast evaporation method does not produce any analyte signals. This is likely due to the presence of impurities such as milk fat in the milk that prevent the peptides and proteins from incorporating into the first-layer matrix crystals, or aggregation among protein molecules before incorporation into the matrix crystals. The two-layer method is found to provide the best overall results in terms of the mass range of detection, signal reproducibility, and sensitivity. In addition, mass accuracy, particularly with external calibration, is also improved over the dry-droplet method. This is attributed to the formation of a thin, flat crystal layer on the sample probe that can be readily reproduced from one sample deposition to another.

It should be noted that direct analysis of cow's milk by MALDI has been reported previously [16, 18, 23, 24]. However, the MALDI spectra shown in these studies only display 2 to 5 peaks corresponding to the major protein components in the milk. In addition, these studies used DC extraction MALDI-TOF systems. Thus, β-lactoglobulin B (MW 18277) and β-lactoglobulin A (MW 18363), as well as β-casein A2 (MW 23983) and β-casein A1 (MW 24023) were not resolved, which resulted in poor mass measurement accuracy. Compared to those results [16, 18, 23, 24], the mass spectrum shown in Figure 1A from the dried-droplet method displays about 63 peaks with signal (peak height) to background noise ratios above 5. Figure 1B from the two-layer method shows 68 peaks. Among these peaks, 20 peaks from the dried-droplet method and 24 peaks from the two-layer method can be assigned to the masses of the major protein components in the milk and peptide fragments from caseins. The three major proteins in milk are the water soluble α-lactalbumin (MW 14178) and β-lactoglobulin and the water insoluble caseins (α_{s1}, α_{s2}, and β). The casein proteins, present in milk as micelles, are susceptible to digestion by indigenous proteinases, especially plasmin [25]. Many of the peaks

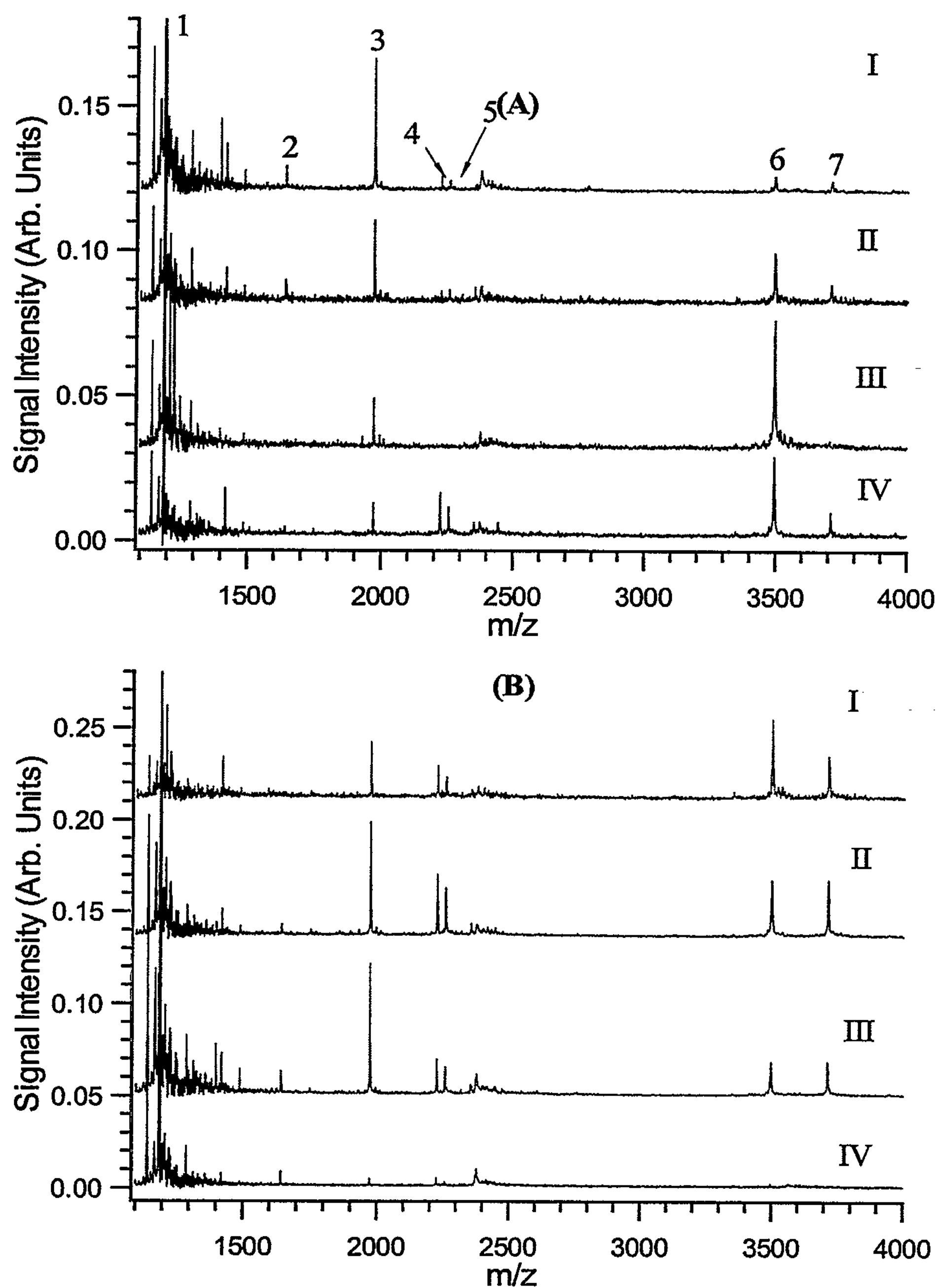

Figure 2. MALDI mass spectra of a peptide mixture analyzed by using different solvents for preparing the second-layer solutions. (A) The solvent mixture used contains a different organic solvent: (I) 50% isopropanol/0.2% formic acid in water, (II) 50% ethanol/0.2% formic acid in water, (III) 50% methanol/0.2% formic acid in water, and (IV) 50% acetonitrile/0.2% formic acid in water. (B) The solvent mixture contains a different amount of formic acid: (I) 50% isopropanol/10% formic acid in water, (II) 50% isopropanol/5% formic acid in water, (III) 50% isopropanol/1% formic acid in water, (IV) 50% isopropanol/water. The time-lag focusing conditions were optimized to focus the ions at m/z~3,000.

identified in the spectrum correspond to known plasminolysis peptides produced from digestion of α_{s1} and β-casein [25]. The ability to rapidly detect a large number of peptides and proteins in milk opens the possibility of using MALDI-MS as a tool for monitoring the protein compositional changes during milk processing. One can also envision the application of MALDI-MS to the direct analysis of therapeutic peptides and proteins from cow's milk produced through genetic engineering.

The use of a mixture of matrix and analyte with different solvent compositions and proportions adds versatility to the two-layer method. In this method, the selection of appropriate solvent conditions for preparing the second-layer solution is found to be critical in analyzing peptide and protein mixtures. As an example, Figure 2 shows several MALDI mass spectra of a peptide mixture obtained by using different solvent compositions for the preparation of the second-layer matrix solution. The list of peptides in the mixture and their relative amounts along with the peak number labeled in Figure 2 are shown in Table 1. Lys-bradykinin was purposely added in an excess amount, compared to other peptides, to mimic a real sample where relative amounts of different analytes can vary considerably. As shown in Figure 2A, the type of organic solvents used in the mixture can affect the signal intensities of the peptides. In this case, the use of 50% isopropanol provides the best overall detection for all seven peptides. There are a few other peaks present at the low mass region likely from the impurities in the sample and the matrix cluster ions. The spectra shown in Figure 2B were obtained by using the mixture solvents with different formic acid contents. It is clear that the acid content can also affect the mass spectral patterns. There seems to be no direct correlations between the molecular weight or pH or hydrophobicity of the peptide and its detectability in samples prepared under different solvent conditions. Thus it is necessary to vary the second-layer solvent conditions including the type of organic solvents, pH, and pH modifier as a part of the MALDI optimization process for the detection of peptides and proteins in a mixture.

Table 1. List of seven peptides used in Figure 2.

Peak number	Peptide	Amount (pmol)*
1	Lys-bradykinin	500
2	EAEALKKEIEALKK	0.5
3	EEAQAQAEAEAKAKAEEK	0.5
4	ELEKLLKELEKLLKEAEK	1
5	ELEKLLKECEKLLKELEK	1
6	Insulin Chain B (oxidized)	1.5
7	KCEALEGKLGAVEEKLGAVEEKLGAVEEKLEALEG	1.5

*The amount of matrix in the second layer is 5.3 nmol.

The effect of solvent composition on the detection of peptides and proteins in a complex mixture such as cow's milk is illustrated in Figure 3. The MALDI mass spectra of skim milk containing 0.5% milk fat in Figure 3 were obtained by using different solvents for the preparation of the second-layer matrix. In this case, the use of 50% acetonitrile/50% water (Figure 3D) provides the best detection, particularly for caseins. Note that the effect of the type of organic solvent on mass spectral patterns is also dependent on the pH of the second-layer solution. For example, when milk was first diluted with 0.2 M formic acid (pH~2.2) or 0.0123 M trifluoroacetic acid (pH~2.1), followed by mixing it with the second-layer matrix solution prepared by a solvent containing 50% acetonitrile/50% water or 50% ethanol/50% water, similar spectral patterns were observed. This is shown in Figure 4. In all cases, both β-lactoglobulin B and β-lactoglobulin A signals were suppressed compared with Figure 3.

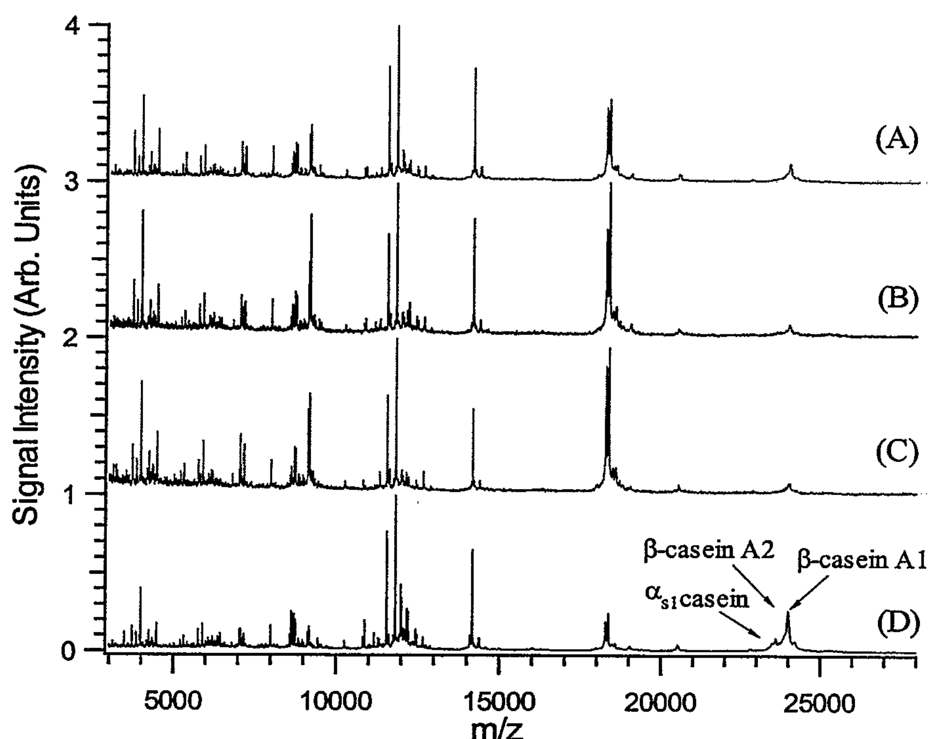

Figure 3. MALDI mass spectra of skim milk obtained by using different second-layer solvents. (A) 50% isopropanol/water, (B) 50% ethanol/water, (C) 50% methanol/water, (D) 50% acetonitrile/water. The time-lag focusing conditions were optimized to focus the ions at m/z~15,000.

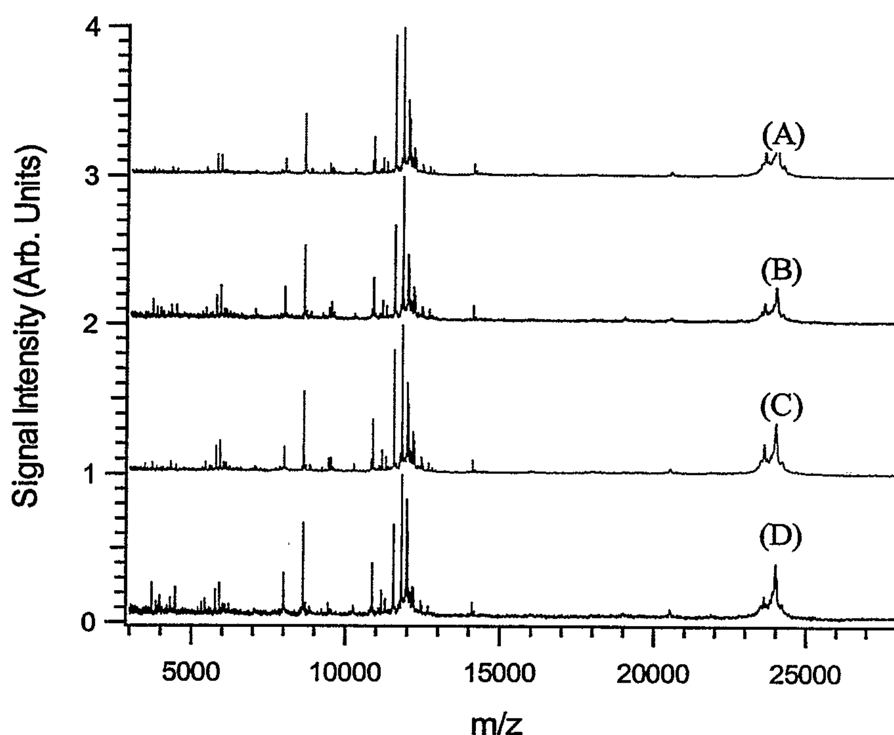

Figure 4. MALDI mass spectra of skim milk diluted with 0.0123 M trifluoroacetic acid (A, B) and 0.2 M formic acid (C, D). The solvents used for the preparation of the second-layer matrix are 50% acetonitrile/water (A, C) and 50% ethanol/water (B, D). The time-lag focusing conditions were optimized to focus the ions at m/z~15,000.

In analyzing real-world samples, other impurities of non-peptide or protein nature may affect the detection of peptides and proteins. In the case of analyzing cow's milk, the presence of milk fat does not seem to affect the analysis of peptides and proteins. This can be seen by comparing the spectra of 2% fat milk (Figure 1B) and skim milk (Figure 3D). In addition, the mass spectrum of homogenized milk (3.25% fat content) obtained by using the two-layer method (not shown) is also very similar to those of 2% fat milk and skim milk.

To illustrate the applicability of the two-layer method to other complex mixtures, Figure 5 shows the MALDI mass spectra of a bacteria sample and crude egg white. As it can be seen from Figure 5A, there are a number of peaks at the m/z range up to 12,000 observed for *E. coli* (ATCC 9637). The chemical nature of some of these peaks has recently been identified to be proteins expected to be present in *E. coli*. A detailed investigation of chemical identification on bacteria proteins is reported elsewhere [26]. In the MALDI spectrum of egg white (see Figure 5B), several proteins and glycoproteins are detected. Egg white proteins are the principal solutes present in egg white, making up about 10% of its weight [27]. Many of them are glycoproteins with carbohydrate contents ranging from 2 to 58% [27]. Four major proteins are ovalbumin

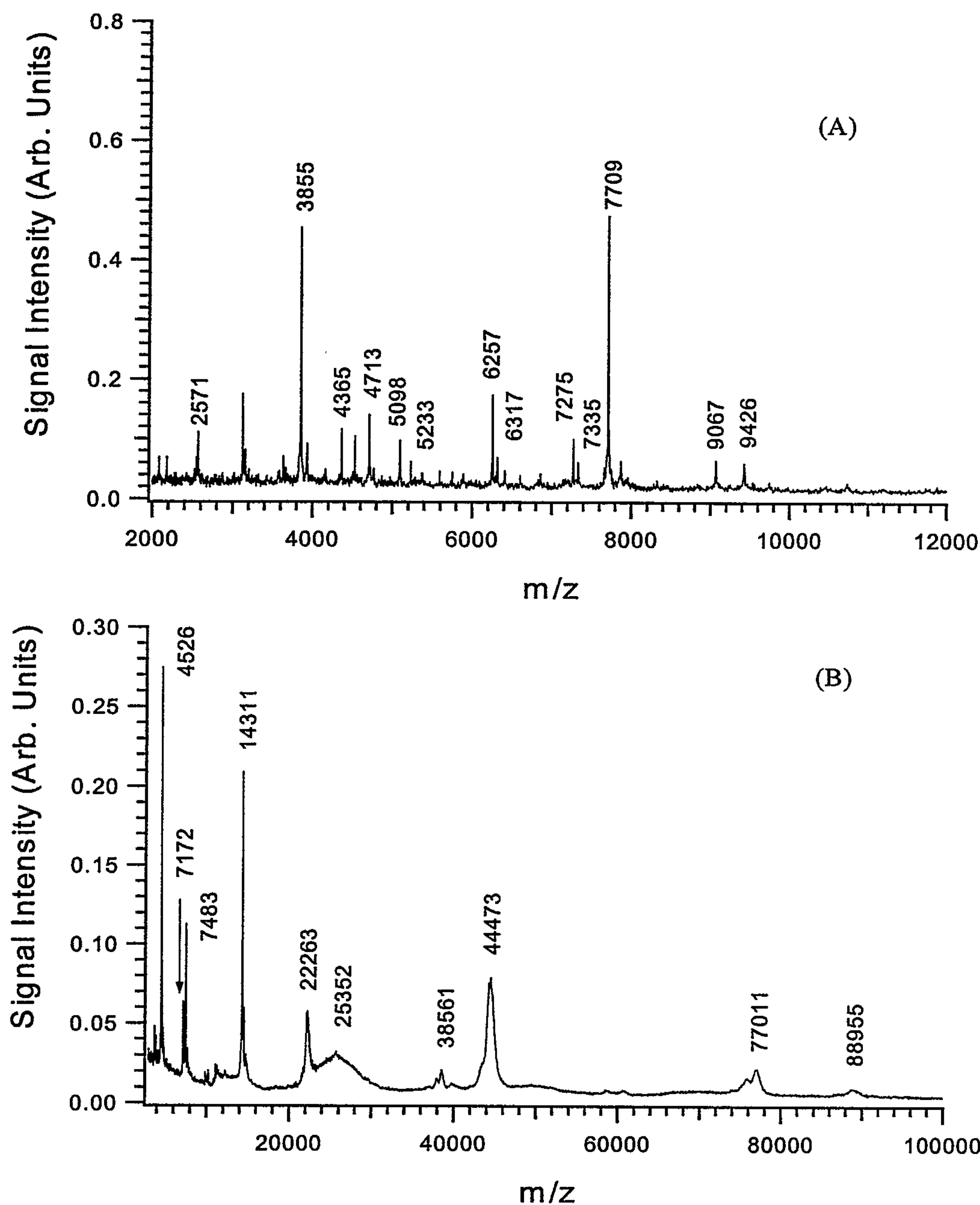

Figure 5. MALDI mass spectra of (A) *Escherichia coli* (ATCC 9637) and (B) crude egg white obtained by the two-layer sample preparation method. For *E. coli* analysis, the second-layer solution consists of saturated HCCA in 33% acetonitrile/67% water and the sample solution (1:1 v/v). The time-lag focusing conditions were optimized to focus the ions at m/z~8,500. For crude egg white, the second layer solution consists of saturated SA in 25% acetonitrile/0.1% TFA in water and sample solution (9:1 v/v). The time-lag focusing conditions were optimized to focus the ions at m/z~44,000. For (A), the sample solution is the bacteria extract using 0.1% TFA as the extraction solvent. For (B), the sample solution is egg white dissolved in 0.1% TFA in water with a concentration of 2.1 mg/mL.

(MW~45,000; comprising 54% of the total proteins in egg white), ovotransferrin (MW~76,600; 12%), ovomucoid (MW~28,000; 11%), and lysozyme (MW=14,306; 3.4%) [27]. In Figure 5B, the peak at m/z 77,011 is likely from ovotransferrin. The peak at m/z 44,473 is from ovalbumin. The broad peak centered at m/z 25,352 is likely from ovomucoid, a glycoprotein with carbohydrate contents ranging from 16.5 to 32.6% of its weight. The peak at m/z 14,311 is from lysozyme.

In conclusion, we have shown that two-layer sample preparation is a very effective method for the detection of a broad mass range of peptides and proteins in a mixture. It provides excellent reproducibility from shot to shot and from one sample deposition to another. The method is rapid and amenable to automated sample deposition for high throughput experiments. The possibility of varying the solvent conditions of the second-layer solution adds another dimension for fine tuning of the sample/matrix preparation to achieve optimal performance.

References

1. Li, L.; Wang, A.P.L.; Coulson, L.D. *Anal. Chem.*. **1993**, *65*, 493-495.
2. Nagra, D.S.; Li, L. *J. Chromatogr. A* ., **1995**, *711*, 235-245.
3. Whittal, R.M.; Russon, L.M.; Li, L. *J. Chromatogr. A* ., **1998**, *794*, 367-375.
4. Billeci, T.M.; Stults, J.T. *Anal. Chem.* **1993**, *65*, 1709-1716.
5. Perkins, J.R.; Smith, B.; Gallagher, R.T.; Jones, D.S.; Davis, S.C.; Hoffman, A.D. *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 670-684.
6. Gusev, A.J.; Wilkinson, W.R.; Proctor, A.; Hercules, D.M. *Anal. Chem.* **1995**, *67*, 1034-1041.
7. Cohen, S.L.; Chait, B.T. *Anal. Chem.* **1996**, 68, 31-37.
8. Kussmann, M.; Nordhoff, E.; Rahbek-Nielsen, H.; Haebel, S.; Rossel-Larsen, M.; Jakobsen, L.; Gobom, J.; Mirgorodskays, E.; Kroll-Kristensen, A.; Palm, L.; Roepstorff, P. *J. Mass Spectrom.* **1997**, *32*, 593-601.
9. Whittal, R.M.; Li, L. *Anal. Chem.* **1995**, *67*, 1950-1954.
10. Whittal, R.M.; Russon, L.M.; Weinberger, S.R.; Li, L. *Anal. Chem.*, **1997**, *69*, 2147-2153.
11. Dai, Y.Q.; Whittal, R.M.; Li, L. *Anal. Chem.* **1996**, *68*, 2494-2500.
12. Wang, Z.P.; Russon, L.; Li, L.; Roser, D.C.; Long, S.R. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 456-464.
13. Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299-2301.
14. Weinberger, S.R.; Boernsen, K.O.; Finchy, J.W.; Robertson, V.; Musselman, B.D. in *Proceedings of the 41st ASMS Conference on Mass Spectrometry and Allied Topics;* San Francisco, CA, May 31- June 4, **1993**, pp 775a-b.

15. Xiang, F.; Beavis, R.C. *Rapid Commun. Mass Spectrom.* **1994,** *8*, 199-204.

16. Xiang, F.; Beavis, R.C. *Org. Mass Spectrom.* **1993,** *28*, 1424-1429.

17. Mock, K.K.; Sutton, C.W.; Cottrell, J.S. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 233-238.

18. Bai, J.; Liu, Y.H.; Cain, T.C.; Lubman, D.M. *Anal. Chem.* **1994,** *66*, 3423-3430.

19. Köchling, H.J.; Biemann, K. in *Proceedings of the 43rd ASMS Conference on Mass Spectrometry and Allied Topics;* Atlanta, Georgia, May 21- 26, **1995,** p 1225.

20. Hensel, R.R.; King, R.; Owens, K.G. in *Proceedings of the 43rd ASMS Conference on Mass Spectrometry and Allied Topics;* Atlanta, Georgia, May 21- 26, **1995,** p 947.

21. Vorm, O.; Roepstorff, P.; Mann, M. *Anal. Chem.* **1994,** 66, 3281-3287.

22. Li, L.; Golding, R.E.; Whittal, R.M. *J. Am. Chem. Soc.* **1996**, 118, 11662-11663.

23. Catinella, S.; Traldi, P.; Pinelli, C.; Dallaturca, E. *Rapid Commun. Mass Spectrom.* **1996,** *10*, 1123-1127.

24. Catinella, S.; Traldi, P.; Pinelli, C.; Dallaturca, E.; Marsilio, R. *Rapid Commun. Mass Spectrom.* **1996,** *10*, 1629-1637.

25. Fox, P.F.; Singh, T.K.; McSweeney, P.L.H. In Chemistry of structure-function relationships in cheese; Malin EL, Tunick MH, Ed.; Plenum Press: New York, 1995; Vol. 367; pp. 59-98.

26. Dai, Y.Q.; Li, L.; Roser, D.C.; Long, S.R. *Rapid Commun. Mass Spectrom*. **1999**, 13, 73-78.

27. Stevens, L. *Comp. Biochem. Physiol.* **1991**, 100B, 1-9.

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