

Performance of VITEK mass spectrometry V3.0 for rapid identification of clinical *Aspergillus fumigatus* in different culture conditions based on ribosomal proteins

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Abstract: Fast and accurate discrimination of *Aspergillus fumigatus* is significant, since misidentification may lead to inappropriate clinical therapy. This study assessed VITEK mass spectrometry (MS) V3.0 for *A. fumigatus* identification using extracted fungal ribosomal proteins. A total of 52 isolates preliminarily identified as *A. fumigatus* by traditional morphological methods were inoculated in three different culture media and cultured at two different temperatures. The specific spectral fingerprints of different culture time points (48, 72, 96, and 120 h) were obtained. Of all strains, 88.5% (46/52) were discriminated as *A. fumigatus*, while the remaining 11.5% (6/52) produced results inconsistent with morphological analysis. Molecular sequencing, as a reference method for species identification, was used to validate the morphological analysis and matrix-assisted laser desorption/ionization time of flight MS. Chi-square tests (χ^2 test, $P=0.05$) demonstrated that the culture medium and incubation temperature had no effects on identification accuracy; however, identification accuracy of the strains in the 48-h group was lower than that in other groups. In addition, we found that ribosomal proteins extracted from *A. fumigatus* can be stored in different environments for at least 1 week, with their profiles remaining stable and strain identification results showing no change. This is beneficial for medical institutions with no mass spectrometer at hand. Overall, this study showed the powerful ability of VITEK MS V 3.0 in identifying *A. fumigatus*.

Keywords: VITEK MS V 3.0, *Aspergillus fumigatus*, identification, ribosomal protein, spectral fingerprints, fungal, matrix assisted laser desorption ionization-time of flight mass spectrometry, MALDI-TOF MS

Introduction

In recent years, with the overuse and misuse of antibiotics and non-standard use of chemotherapeutic drugs, humans are increasingly susceptible to infection by fungi,¹⁻⁴ especially *Aspergillus fumigatus*. Infection by this pathogen results in high morbidity and mortality. For clinical laboratory technicians, rapid identification of *A. fumigatus* is very significant for patient health. *A. fumigatus* is a multicellular fungus, composed of hyphae, spores, and mycelial elongation branches, intertwined into groups.⁵ Its identification in clinical laboratories is still performed by morphological methods,⁶ histopathology, and serology. However, such methods are cumbersome, relatively difficult to carry out, have long detection periods, and non-ideal sensitivity and specificity. To further improve *A. fumigatus* identification, sequencing techniques attract increasing attention, not requiring complex identification criteria for the clinical setting or

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long incubations of filamentous fungi used in conventional phenotypic methods.⁷ However, long detection cycles and high detection costs restrict the application to a relatively low number of institutions; therefore, it cannot be used as routine detection tools for *A. fumigatus*.

Fortunately, clinical microbiology laboratories have significantly shortened the identification time of *A. fumigatus* in recent years using a new technique, VITEK mass spectrometry (MS),^{8,9} which is matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) based. VITEK MS is a new identification tool that can directly quantify biomarkers (eg, ribosomal proteins) to identify viruses, bacteria, fungi, and other microorganisms,^{10–13} with high sensitivity, throughput, and speed. Importantly, this technology was used to identify *A. fumigatus* with quite satisfactory results^{8,9} and is clinically important for related infections caused by filamentous fungi (mainly *A. fumigatus*). However, whether culture conditions (eg, culture medium and incubation temperature) and time affect *A. fumigatus* identification results remains unclear.^{10,14–22} The current study addressed this issue.

Materials and methods

Fungal strains and culture conditions

The 52 isolates used in this study were clinical specimens from patients hospitalized in the First Affiliated Hospital of Anhui Medical University and Anhui Provincial Hospital (isolated from sputum, urine, or tissue samples) between November 2016 and April 2017. When the same strain was detected more than once in a patient's specimens, only one of them was considered. The isolates were identified as *A. fumigatus* by morphological (macroscopic and microscopic) tools preliminarily, as described previously.²³ Pure cultures were preserved in 25% (v/v) glycerol at -80°C until use. *Escherichia coli* ATCC 8739 (gift from Biomerieux, France) was used as a quality control strain. Each clinical isolate was inoculated on three fungal media, including Sabouraud dextrose liquid, Sabouraud dextrose agar (SDA), and Chalet agar (Hangzhou Bai Si Biotechnology Co, Ltd, Hangzhou, People's Republic of China), and cultured at 28°C and 35°C, respectively.

Sample preparation for mass spectrometry, spectrum generation, and data analysis

Strains cultured for 48, 72, 96, and 120 h were assessed. For isolates grown on solid media (including Sabouraud- and Chalet-agar media), 1 µL of colonies (or ~2 cm in diameter)

were scraped with a sterile scalpel (or cotton swabs/disposable plastic inoculation rings) and resuspended in 300 µL of distilled water. After addition of 900 µL of anhydrous ethanol (AR), the samples were centrifuged at 10,000 rpm for 2 min. The resultant supernatant was removed, while the pellet was mixed with 40 µL of 70% formic acid; then, the same volume of acetonitrile was added, followed by centrifugation (10,000 rpm) for 2 min. The supernatant containing the proteins of interest (1 µL) was spotted on the mass spectrometer sample target (Biomerieux) and air-dried naturally. Next, 1 µL of α -hydroxy-4-cyanomethyl cinnamic acid (CHCA, Biomerieux) was used to cover the target plate before the assessment.

The procedures for strains grown in liquid media (Sabouraud liquid media) were as follows. Appropriate amounts of colonies were collected from the Sabouraud liquid medium and placed in 1.5 mL tubes. After high-speed centrifugation (10,000 rpm) for 10 min, the culture medium was removed, and the spores and hyphae were collected and resuspended in 1.2 mL of distilled water. After vortex mixing the sample, high-speed centrifugation (10,000 rpm) was performed for 2 min, discarding the supernatant. Two additional washes with distilled water were performed. The subsequent steps were carried out as described earlier for solid culture media.

Spectra were recorded in the linear positive mode at a laser frequency of 20 Hz within a mass range of 2000–20,000 Da by shooting each spot 108 times. Before analysis, the quality control strain *E. coli* ATCC8739 (Biomerieux) was used to calibrate the mass spectrometer.

Molecular sequence identification

To obtain accurate and reliable identification results, three genes (ITS, β -tubulin, and calmodulin) were used to characterize target strains. The base sequence of *Its1* was 5'-TCCGTAGGTGAACCTGCGG-3', and the base sequence of *Its4* was 5'-TCCTCCGCTTATTGATATGC-3'.²⁴ The following primers were used for β -tubulin: forward (F), 5'-TGACGGGTGATTGGGATCTC-3'; reverse (R), 5'-CGTCCGCTTCTTCCTTGTTT-3'.²⁵ The primers *cmd5* (5'-CCGAGTACAAGGAGGCCTTC-3') and *cmd6* (5'-CCGATAGAGGTCATAACGTGG-3') were used to amplify the calmodulin gene.²⁶ Strains were inoculated on SDA plates and incubated for 2 days at 28°C. Shanghai Biotechnology Company carried out DNA extraction, amplification, and sequencing, based on the above primers. Sequencing results were compared with GenBank by BLAST²⁷ and UniProt Knowledgebase (UniProtKB);²⁸ homology $\geq 97\%$ and $\geq 99\%$ reflected appurtenance to the same genus and species, respectively.

Ethical approval

This study was approved by the ethical committee of the First Affiliated Hospital of Anhui Medical University. Written informed consent was obtained from the patients whose samples were used.

Results

(I) A total of 52 *A. fumigatus* strains identified by morphological methods were also identified by VITEK MS, and the multi-gene detection method was employed to verify morphological data and VITEK MS findings. As shown in Table 1, VITEK MS identification showed 11.5% (6/52) inconsistent findings with morphological results, while 88.5% (46/52) were consistent. Most importantly, the VITEK MS technique was consistent with the molecular method.

(II) VITEK MS identified *A. fumigatus* in different culture conditions (Table 2).

For strains cultured in different culture media, the SPSS software revealed $\chi^2=1.660$ ($P=0.436$), indicating that the culture medium had no effect on the discrimination rate of *A. fumigatus*. When considering only temperatures, $\chi^2=1.029$ ($P=0.296$), indicating no effect of incubation temperature on the discrimination rate.

However, for strains grown at different time points, $\chi^2=18.677$ ($P=0.000$), suggesting that the MALDI-TOF-MS technique did not have exactly the same capacity for identifying *A. fumigatus* cultured at different time points. The 72 h, 96 h, and 120 h groups showed statistically significant differences compared with the 48-h group, which displayed the lowest discrimination rate.

The remaining six strains were identified as *A. flavus* or *A. oryzae* by VITEK MS, and these results were consistent with molecular findings.

(III) Identification of proteins stored at different times in various environments. In this study, we observed an interesting phenomenon: the proteins extracted by formic acid–acetonitrile were very stable in in vitro conditions and could be stored in different environments. The 72-h group cultured at 35°C (containing carbon dioxide) was assessed, selecting 15 *A. fumigatus* strains randomly; the extracted proteins were stored at 4°C, -20°C and -80°C for 48 h, 72 h, 120 h and 168 h. Identification results are listed in Table 3.

The above-mentioned results showed that the extracted proteins could be stored at 4°C, -20°C, and -80°C for at least 168 h (1 week), with identification results almost not affected by storage time or temperature; in addition, mass spectra at different time periods also showed no significant changes (Figures 1–3).

Discussion

This study demonstrated that for *A. fumigatus* grown either in liquid or on solid culture media, identification accuracy was stable after 72 h of culture and did not change with incubation time or culture conditions. These results addressed a question by Posteraro et al¹⁰ whether mold colonies cultured on solid media might be more heterogeneous than those grown in liquid media. Although *A. fumigatus* organisms have different components (including spores, fruiting bodies, surface hyphae, and basal mycelia) in different growth periods,⁹ identification results remained stable using VITEK MS after 72 h of culture and were not affected by product composition of the strain. These findings suggest that regardless of culture environment, *A. fumigatus* identification remains stable. Compared with 48-h cultures, the strains were more favorable for analysis after 72-h of incubation. The reason might be that a few strains cultured for 48 h have smaller colony products than those for 72 h, resulting in some ribosomal proteins not being extracted, which directly affects mass spectrometry data. Although identification after 72 h is more convenient, there is no significant difference in time compared with the traditional method; however, this technique is more accurate, and the patient can receive symptomatic treatment as soon as possible, with shortened hospitalization time and reduced treatment costs. VITEK MS is a relatively fast identification tool for *A. fumigatus*.

Importantly, in this study, we found that proteins extracted by formic acid–acetonitrile could be stored for at least 168 h (1 week) at 4°C, -20°C, or -80°C. Indeed, the proteins extracted from *A. fumigatus* strains were ribosomal proteins, which are typical housekeeping molecules abundantly present in microbial cells. Identification results did not vary significantly with storage time prolongation, suggesting that ribosomal proteins are very stable. There are ~78 ribosomal subunit proteins in eukaryotes. Nakamura et al selected and verified 26 kinds of ribosomal subunit proteins (RSPs) whose molecular weights were under 16000 Da that could be used as biomarkers for *A. fumigatus* strains.^{19,29} In conjunction with his study, we observed 20 kinds of RSPs that could be used as biomarkers when VITEK MS was used to identify our strains. Table 4 shows the RSP types selected in this study. Each strain expressed all 20 RSPs. Although some RSPs have the same m/z ratio in other aspergilli, we compared the RSPs of common aspergilli, such as *A. fumigatus*, *A. flavus*, *A. oryzae*, and *A. niger*. Among the 20 RSPs, *A. flavus* and *A. oryzae* could not be differentiated, because their genes are essentially the same. Assessing *A. fumigatus* and *A. flavus/A. oryzae*, we found that they had exactly the same L39, while *A. niger*

Table 1 Identification results of the sample strains using different methods

Strains	Traditional morphological	MALDI-TOF MS	Molecular sequencing results		
			ITS	β -Tubulin	Calmodulin
001	Afu	Afu	Afu	Afu	Afu
002	Afu	Afl/Aor	Afl/Aor	Afl/Aor	Afl/Aor
003	Afu	Afu	Afu	Afu	Afu
004	Afu	Afu	Afu	Afu	Afu
005	Afu	Afu	Afu	Afu	Afu
006	Afu	Afu	Afu	Afu	Afu
007	Afu	Afu	Afu	Afu	Afu
008	Afu	Afu	Afu	Afu	Afu
009	Afu	Afu	Afu	Afu	Afu
010	Afu	Afu	Afu	Afu	Afu
011	Afu	Afl/Aor	Afl/Aor	Afl/Aor	Afl/Aor
012	Afu	Afu	Afu	Afu	Afu
013	Afu	Afu	Afu	Afu	Afu
014	Afu	Afu	Afu	Afu	Afu
015	Afu	Afu	Afu	Afu	Afu
016	Afu	Afu	Afu	Afu	Afu
017	Afu	Afu	Afu	Afu	Afu
018	Afu	Afu	Afu	Afu	Afu
019	Afu	Afl/Aor	Afl/Aor	Afl/Aor	Afl/Aor
020	Afu	Afu	Afu	Afu	Afu
021	Afu	Afu	Afu	Afu	Afu
022	Afu	Afu	Afu	Afu	Afu
023	Afu	Afu	Afu	Afu	Afu
024	Afu	Afu	Afu	Afu	Afu
025	Afu	Afu	Afu	Afu	Afu
026	Afu	Afu	Afu	Afu	Afu
027	Afu	Afl/Aor	Afl/Aor	Afl/Aor	Afl/Aor
028	Afu	Afu	Afu	Afu	Afu
029	Afu	Afu	Afu	Afu	Afu
030	Afu	Afu	Afu	Afu	Afu
031	Afu	Afu	Afu	Afu	Afu
032	Afu	Afu	Afu	Afu	Afu
033	Afu	Afl/Aor	Afl/Aor	Afl/Aor	Afl/Aor
034	Afu	Afu	Afu	Afu	Afu
035	Afu	Afu	Afu	Afu	Afu
036	Afu	Afu	Afu	Afu	Afu
037	Afu	Afu	Afu	Afu	Afu
038	Afu	Afu	Afu	Afu	Afu
039	Afu	Afu	Afu	Afu	Afu
040	Afu	Afu	Afu	Afu	Afu
041	Afu	Afu	Afu	Afu	Afu
042	Afu	Afu	Afu	Afu	Afu
043	Afu	Afu	Afu	Afu	Afu
044	Afu	Afu	Afu	Afu	Afu
045	Afu	Afl/Aor	Afl/Aor	Afl/Aor	Afl/Aor
046	Afu	Afu	Afu	Afu	Afu
047	Afu	Afu	Afu	Afu	Afu
048	Afu	Afu	Afu	Afu	Afu
049	Afu	Afu	Afu	Afu	Afu
050	Afu	Afu	Afu	Afu	Afu
051	Afu	Afu	Afu	Afu	Afu
052	Afu	Afu	Afu	Afu	Afu

Abbreviations: Afu, *Aspergillus fumigatus*; Afl, *A. flavus*; Aor, *A. oryzae*; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry.

Table 2 *Aspergillus fumigatus* identification by VITEK MS V 3.0 (a total of 46 strains)

Media	28°C								35°C							
	48 h		72 h		96 h		120 h		48 h		72 h		96 h		120 h	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Sabouraud liquid media	41	5	44	2	46	0	45	1	42	4	46	0	45	1	44	2
Sabouraud agar media	43	3	46	0	45	1	46	0	46	0	46	0	46	0	45	1
Chalet agar media	41	5	44	2	46	0	45	1	44	2	46	0	45	1	43	3

Note: “+”, identified; “-”, not identified (not wrong identification result).

Abbreviation: MS, mass spectrometry.

Table 3 Identification results for proteins stored at different temperatures for various time periods

Temperature (°C)	Storage time (h)			
	48	72	120	168
4	100% (15/15)	100% (15/15)	100% (15/15)	100% (15/15)
-20	100% (15/15)	100% (15/15)	100% (15/15)	100% (15/15)
-80	100% (15/15)	100% (15/15)	100% (15/15)	100% (15/15)

Table 4 Types of RSP used in this study

Types of RSP	Different strains		
	<i>Aspergillus fumigatus</i>	<i>A. flavus/A. oryzae</i>	<i>A. niger</i>
L40	6002.3	6043.3	6016.3
L39	6151.2	6151.2	6123.2
S29	6646.7	6582.6	6793.1
S30	6789.1	6691.9	6745
L29	7456.6	7414.5	7368.4
S28	7710	7708	7681.9
S31	9134.9	9090.8	9101.8
L38	9153.8	9173.9	9073.7
S21	10038.1	10050.1	10027.1
L43	10025.8	9997.7	9997.7
L37	10386.9	10436.9	10451.9
L30	11171.1	11223.1	11210.1
L36	11869.8	11847.8	11831.8
L42	12028.3	12029.2	12025.2
L33	12215.1	12299.2	12250.1
L34	13164.5	13178.5	13147.4
S26	13338.7	13381.7	13381.7
L35	14532	14536.9	14384.8
L26	14979.4	14999.3	14877.2
S24	15226.6	15157.5	15163.5

Abbreviation: RSP, ribosomal subunit protein.

and *A. flavus/A. oryzae* had the same L43 and S26. However, this did not affect identification results, because each strain has a set of unique ribosomal proteins, with a unique mass spectrum fingerprint; even if individual ribosomal protein identical in some strains, they have no significant effect on mass spectrum fingerprints.

For clinical laboratory technicians, providing accurate results of the patient's isolates as soon as possible is of outmost importance. The MALDI-TOF MS technology has been developed rapidly and successfully applied in routine identification of clinical microorganisms. The stability of

ribosomal proteins is beneficial to many medical institutions. As mass spectrometers are still expensive, only a few large medical institutions can afford them; therefore, those without mass spectrometer can extract ribosomal proteins from clinical isolates and send to relevant agencies for mass spectrometry identification. Many laboratories used MALDI-TOF MS to identify common isolates quickly and accurately. However, compared with bacteria, the MALDI-TOF MS technology is seldom used to identify fungi, and especially rare for the analysis of *Aspergillus*, *Penicillium*, and other filamentous fungi. This can be explained by many reasons. The first and most important point is that the cell wall of filamentous fungi is very thick and hard, requiring a special treatment for ribosomal protein extraction; although the formic acid–acetonitrile extraction method is quite useful, it remains complex and time-consuming for daily clinical application, particularly for strains grown in liquid media or positive blood cultures, since culture media and other nutrients may interfere with mass spectrometry analysis; therefore, colonies must be washed as much as possible before the test.²⁰ Second, the current database covers a limited number of strains and does not allow the detection of all strains; for rare strains, the identification rates are generally low, and sometimes they are not correctly identified. For example, the current method cannot be used to detect *Mucor*, which is not included in the library.

Conclusion

Overall, MALDI-TOF MS can be used to identify *A. fumigatus* quickly and accurately. Moreover, this technology has many advantages, such as stability, high-throughput potential, and relatively low cost. In clinical microbiological

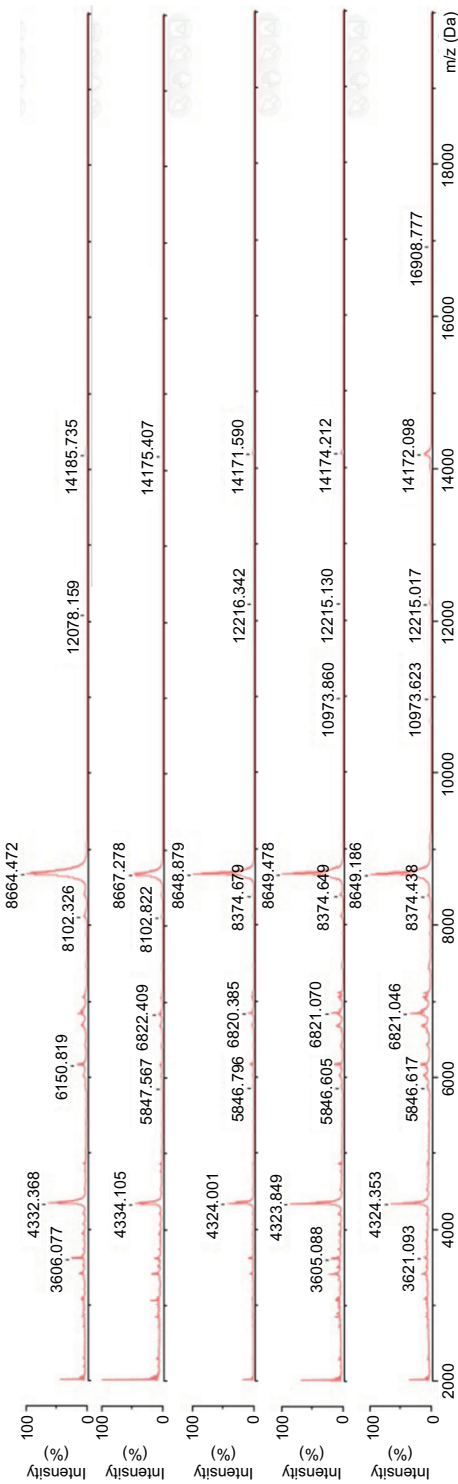


Figure 1 Proteins stored at 4°C.
Note: The 0 h, 48 h, 72 h, 120 h, and 168 h groups, respectively, are presented from bottom to top.

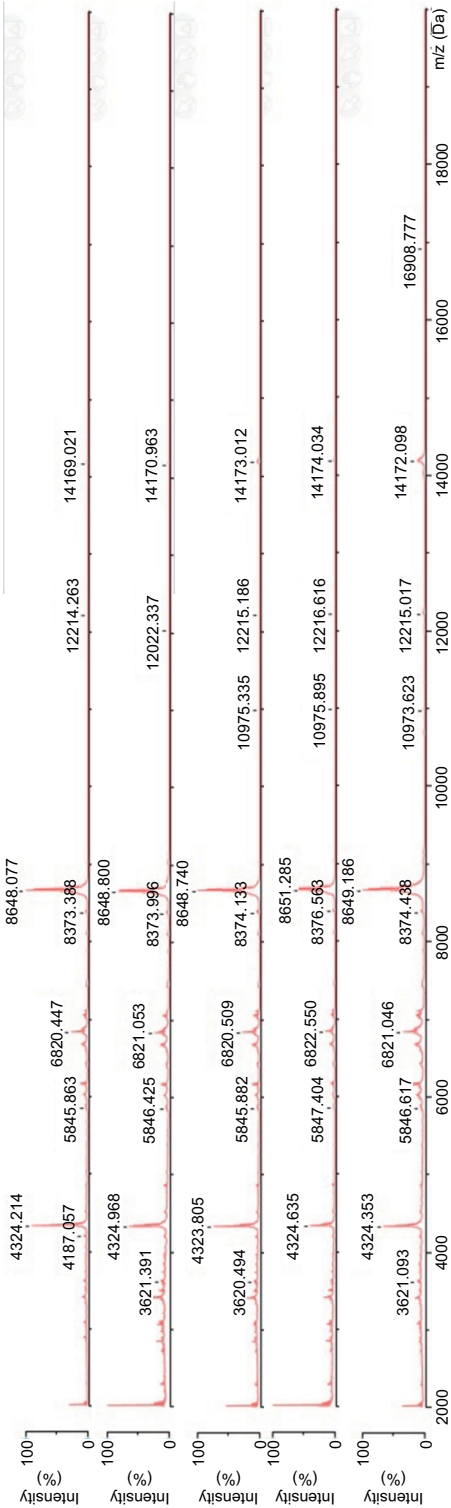


Figure 2 Proteins stored at -20°C.
Note: The 0 h, 48 h, 72 h, 120 h, and 168 h groups, respectively, are presented from bottom to top.

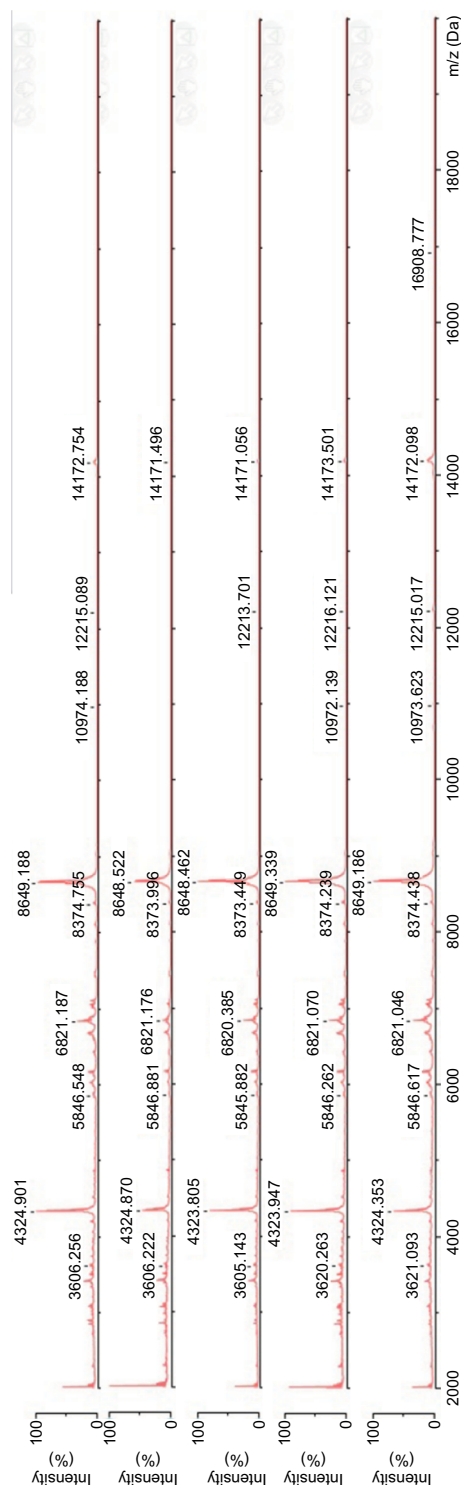


Figure 3 Proteins stored at -80°C .
Note: The 0 h, 48 h, 72 h, 120 h, and 168 h groups, respectively, are presented from bottom to top.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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