Recent Applications of Mass Spectrometry at Clarkson University

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Abstract

Mass spectrometry (MS) is a powerful technique that has various applications including the identification and characterization of proteins, protein-protein interactions and protein post translational modifications, as well as other molecules (i.e. metabolites, lipids, nucleotides and polynucleotides). However, not too many undergraduate students within the USA and around the world have access to (and are trained in) MS. The undergraduate students in our department are taught to analyze proteomics and metabolomics data obtained from MS analysis, including *de novo* sequencing of peptides and to interpret the MS and MS/MS data acquired in positive and negative ionization modes. Here, we give some examples of MS data analyzed in the Biochemistry I class and then examples of some independent research projects performed by students over the years in the Biochemistry and Biotechnology laboratory, where MS is used for both proteins, peptides and metabolites analysis, thus demonstrating the applicability of MS analysis in diverse fields. The projects discussed include analysis of the protein content present in yogurt, beer, protein shakes, contact lenses, or milk of animal or vegetal origin.

Keywords

Mass spectrometry · Proteomics · Metabolomics · Undergraduate students

Abbreviations

BCA	Bicinchoninic acid protein assay
ESI	Electron spray ionization
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometry
Pkl	Peaklist
PLGS	Protein Lynx Global Server
PMFA	Proteins migrating faster than albumin
QTof	Quadruple time of flight
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
TIC	Total ion chromatogram
UPLC	Ultra performance liquid chromatography

46.1 Introduction

MS is an extremely powerful method gaining popularity for its many applications in life science [1–4]. It allows identification and characterization of proteins (including protein-protein interactions and their post translational modifications), lipids, glycoproteins and small molecules. In addition, it facilitates the investigation of proteomes (proteomics), metabolomes (metabolomics), lipids (lipidomics) and oligosaccharides (glycomics). Although MS is versatile and powerful, it is not standardly taught in many universities. At Clarkson University, the students learn about MS and its applications in the Biochemistry I lecture, and then apply their theoretical knowledge in the Biochemistry and Biotechnology lab, where they obtain the hands-on experience in MS-based analysis, with a particular focus on proteomics. More advanced principled of MS are taught in the graduate-level Protein Chemistry and Proteomics or Advances in Mass spectrometry courses [1–12].

Biochemistry I course introduces students to amino acids, their physio-chemical properties, peptide bonds, all the

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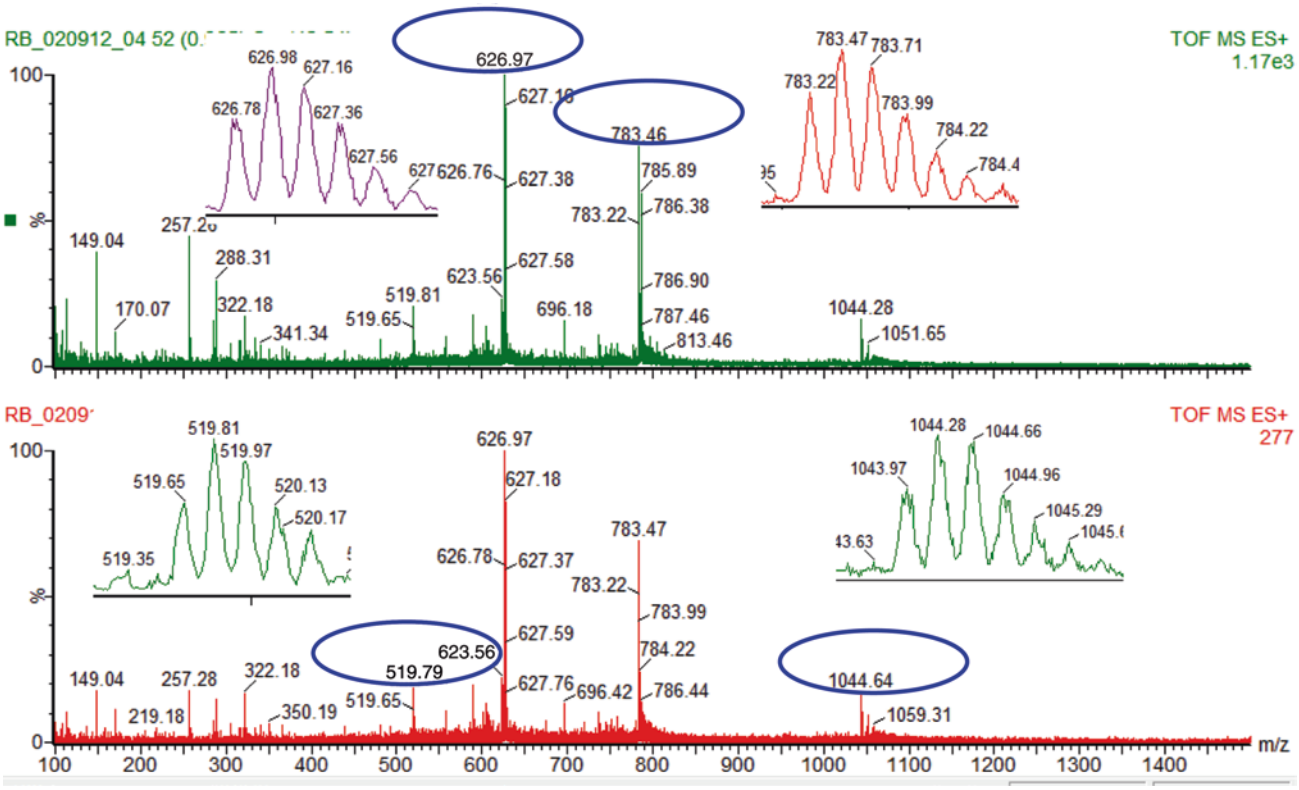

Fig. 46.1 ESI-MS analysis of a peptide with (3+), (4+), (5+) and (6+) charge state

simple biochemical structures and characteristics. Once the protein structure is understood, their interactions and post-translational modifications are taught. Further, the students are introduced to various principles of chromatography, electrophoresis and western blotting along with sequencing techniques (Edman, Sanger and *de novo* sequencing). Unique to this course at Clarkson is that the students are introduced to *de novo* sequencing using MS raw data along with being introduced to Matrix assisted laser desorption ionization (MALDI)- and electrospray ionization (ESI)-based MS. Here, the students learn to determine the charge and mass of peptides analyzed by ESI-MS and also learn to differentiate between positive and negative mode ionization. Examples of spectra in which the students are required to analyze are shown in Figs. 46.1, 46.2, 46.3, 46.4, 46.5, 46.6, and 46.7.

In the first example, the students are required to identify the charge state of some circled peaks and the relationship between them [Fig. 46.1]. Clearly, these spectra contain (3+), (4+), (5+) and (6+) ions of the same peptide.

In the second example, the students are required to identify relationship between the proteins from the two spectra shown in Fig. 46.2. In both spectra, the same protein (hemoglobin) was analyzed by direct infusion ESI-MS in both positive [ESI (+)] and negative [ESI (−)] modes.

In the third example, the students are required to determine the number of proteins and their masses in the spectrum shown in Fig. 46.3. The main protein is lysozyme and it has up to 7 visible modified protein isoforms.

In the fourth example, the students are given spectra of two proteins, wildtype and Trp to Phe mutant. They are then required to determine the charge state and the mass of the two proteins and then to identify the wildtype and the mutant spectrum. Since the mass of dehydrated Trp is 186 and of dehydrated Phe is 147, it is clear that the protein with the lower mass is the mutant protein.

In the next example (Fig. 46.5), the students are required to identify the amino acid sequence of the two spectra, identify the differences between them and explain the relationship between the two spectra (and peptides). They also have to discuss the ion pairs 249.10 and 277.10 (upper spectrum) and 306.11 and 334.12 (lower spectrum). Both spectra contain the same peptide: unmodified in the upper spectrum and modified by a 57 Da iodoacetamide (or glycine) at the N-terminus (note: the peptide is cysteineless). The key to identify the same unmodified and modified peptide is to look at the similarity of the fragmentation pattern, which suggests the spectra correspond to peptides that are related and then to look at the difference between the precursor ions, which could indicate which peptide is the modified one and by

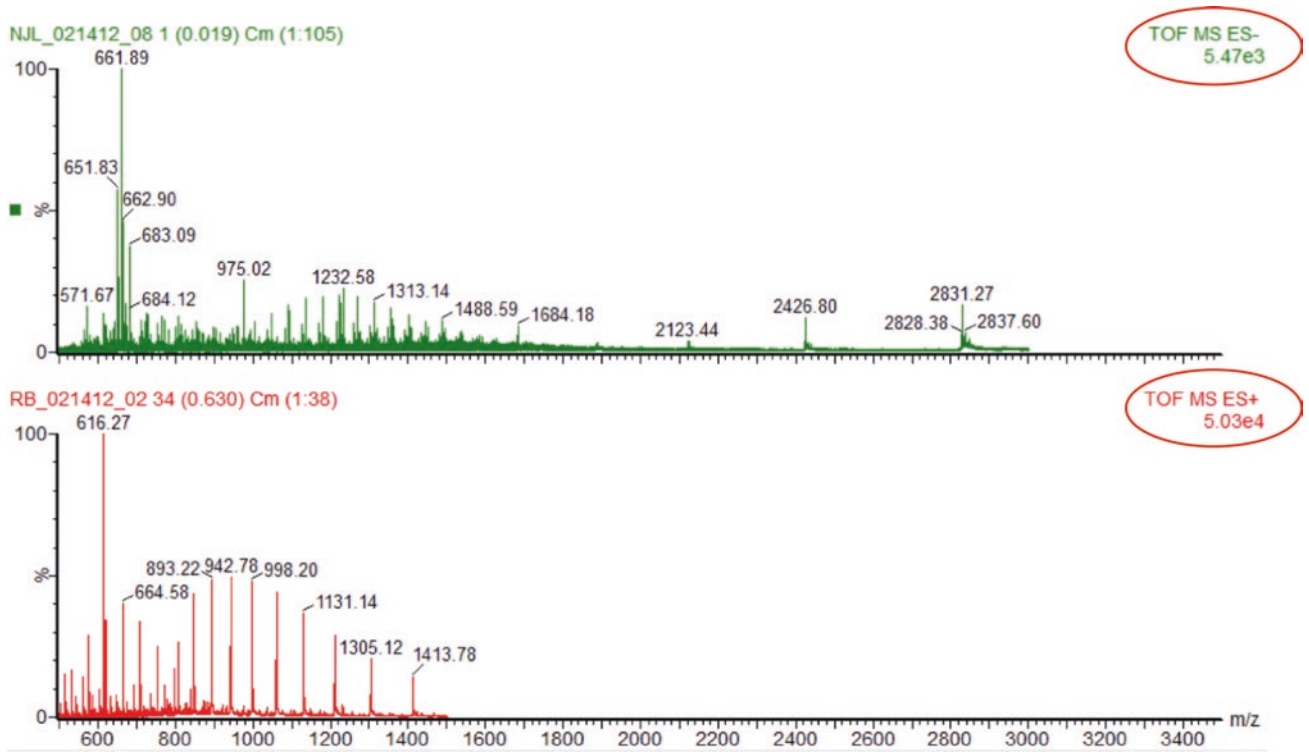

Fig. 46.2 ESI-MS analysis of a protein (hemoglobin) in positive ESI (+) and negative ESI (−) modes

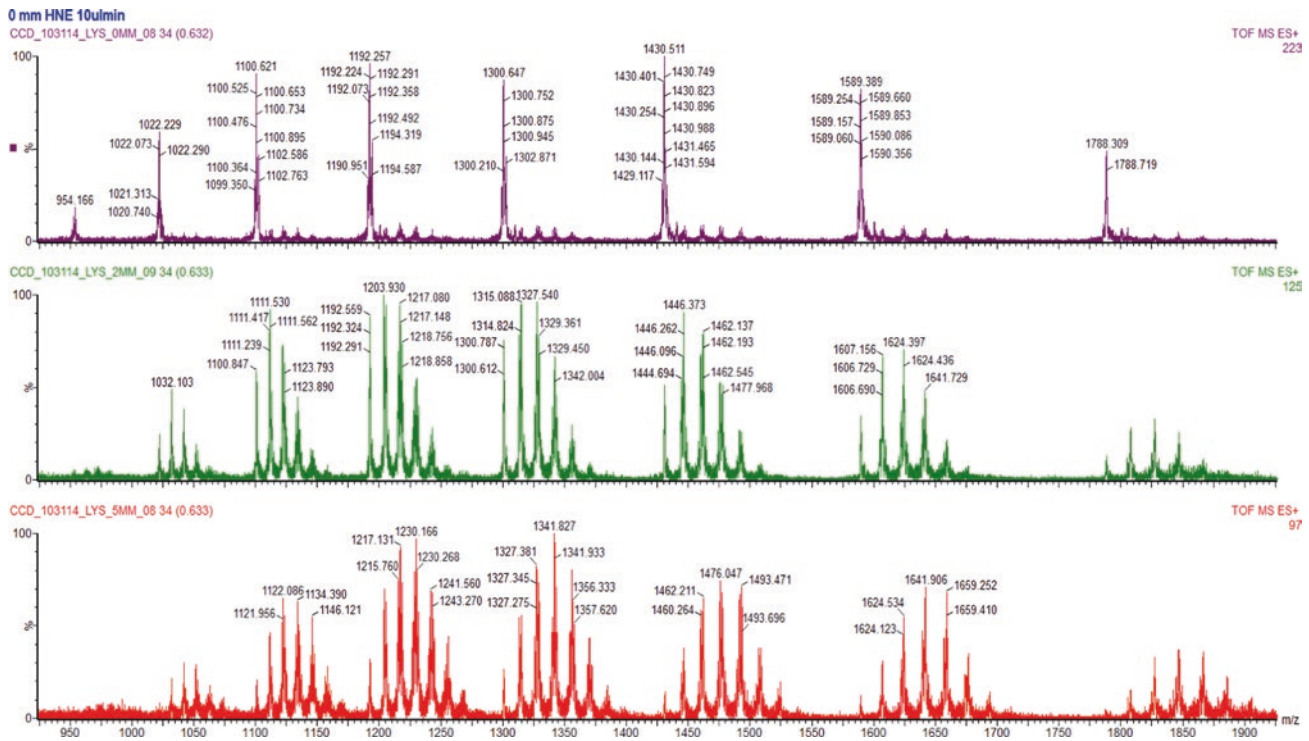

Fig. 46.3 ESI-MS analysis of a protein (lysozyme) in ESI (+) mode. The number of isoproteins increases from the top to the bottom spectra

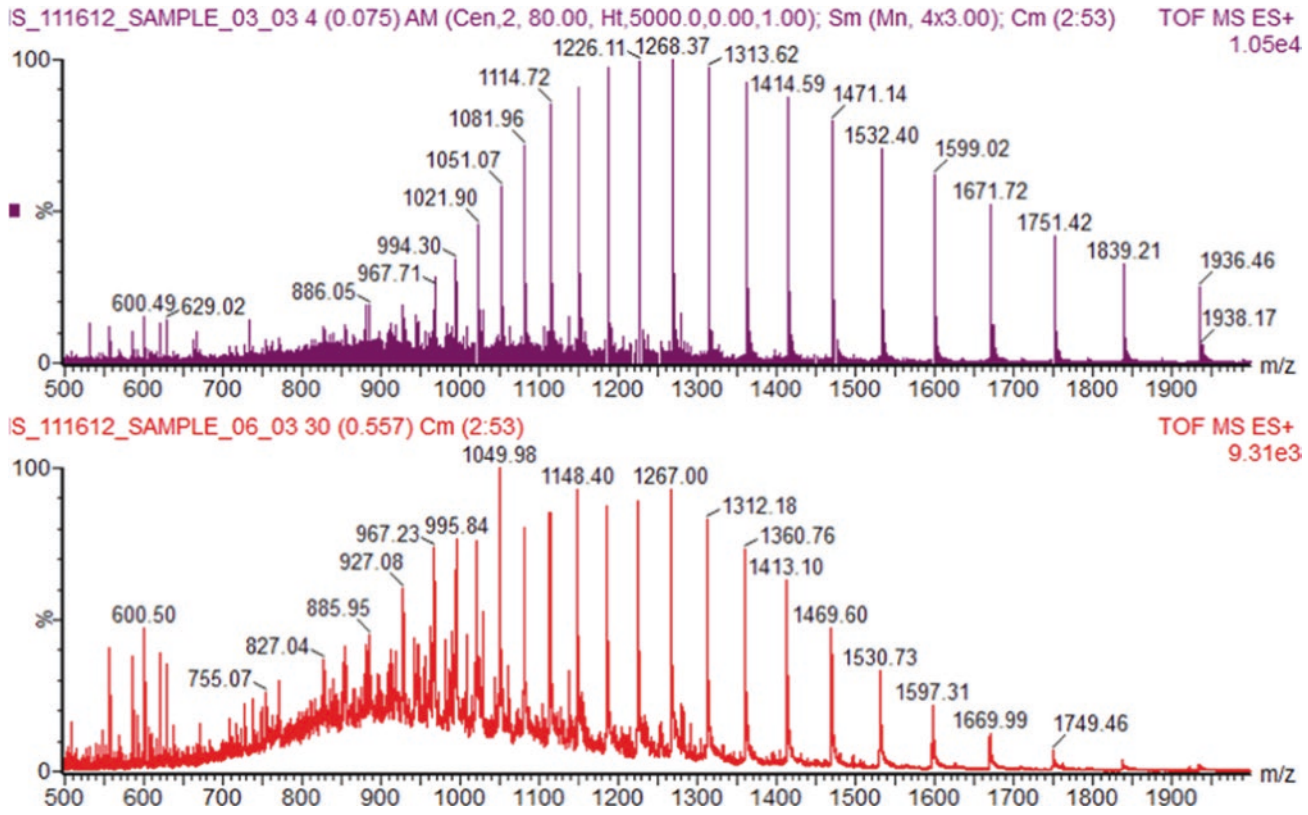

Fig. 46.4 ESI-MS analysis of a wildtype and a Trp to Phe mutant proteins

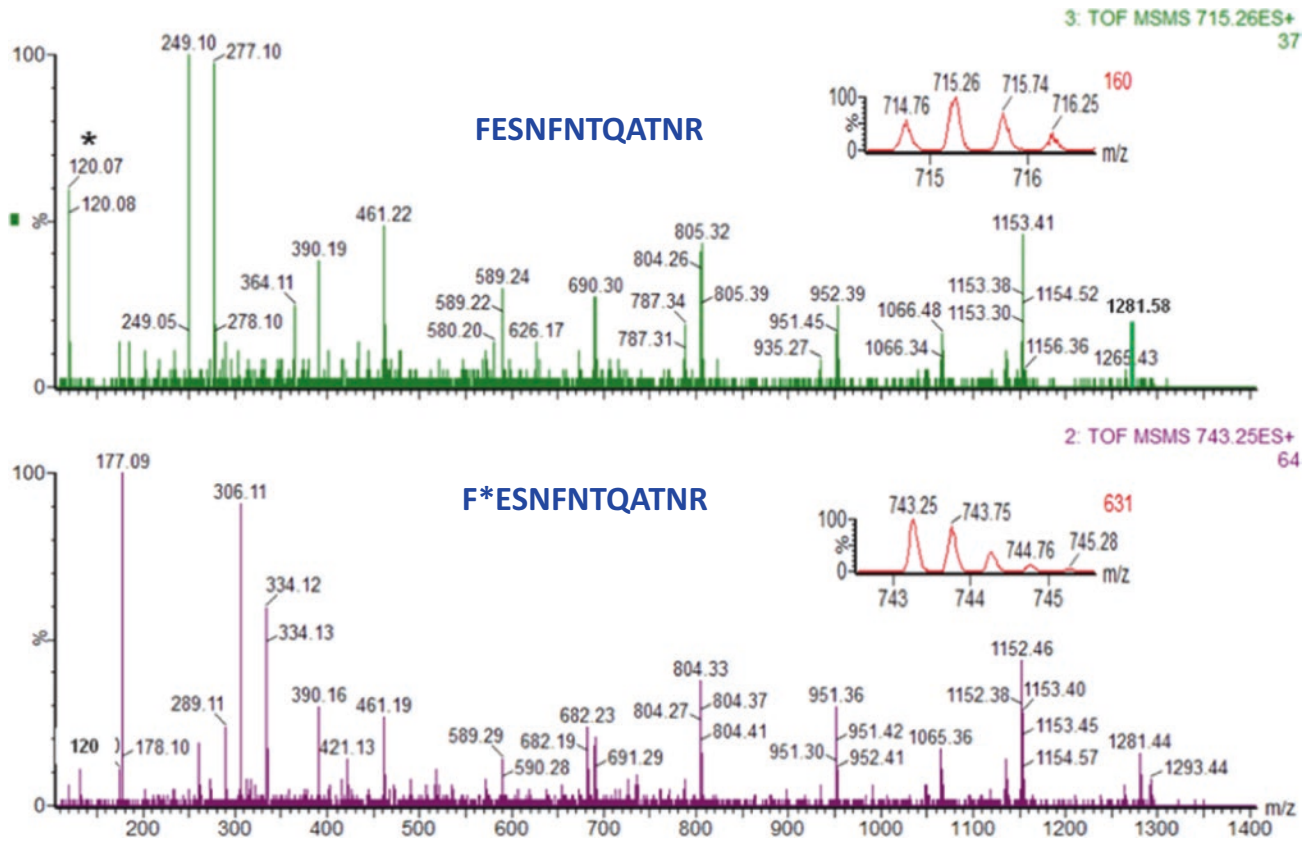

Fig. 46.5 MS and MS/MS of a peptide FESNFNTQATNR that is unmodified (top) or modified by IAA (57 Da; bottom)

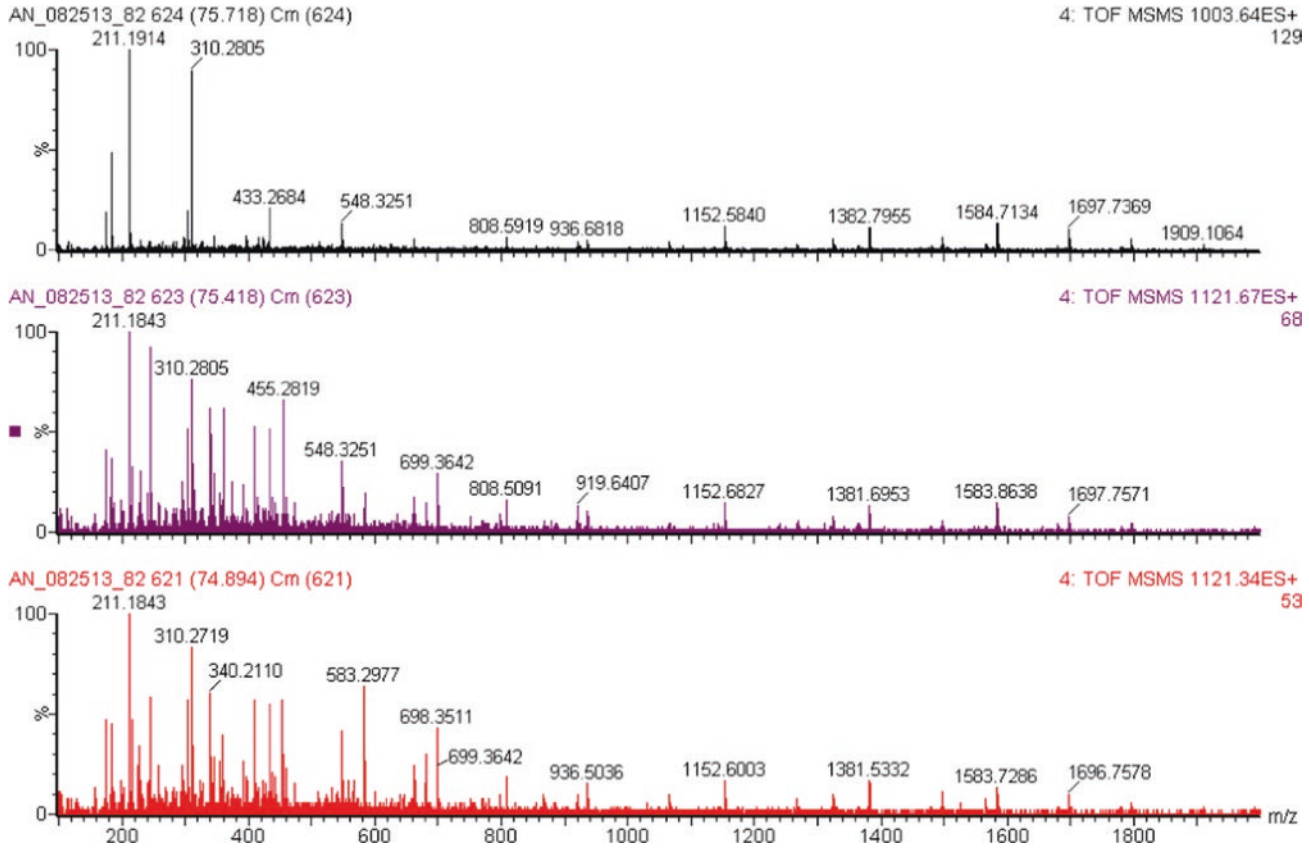

Fig. 46.6 MS/MS spectra have similar fragmentation pattern, suggesting that the peptides that correspond to these peptides are somehow related

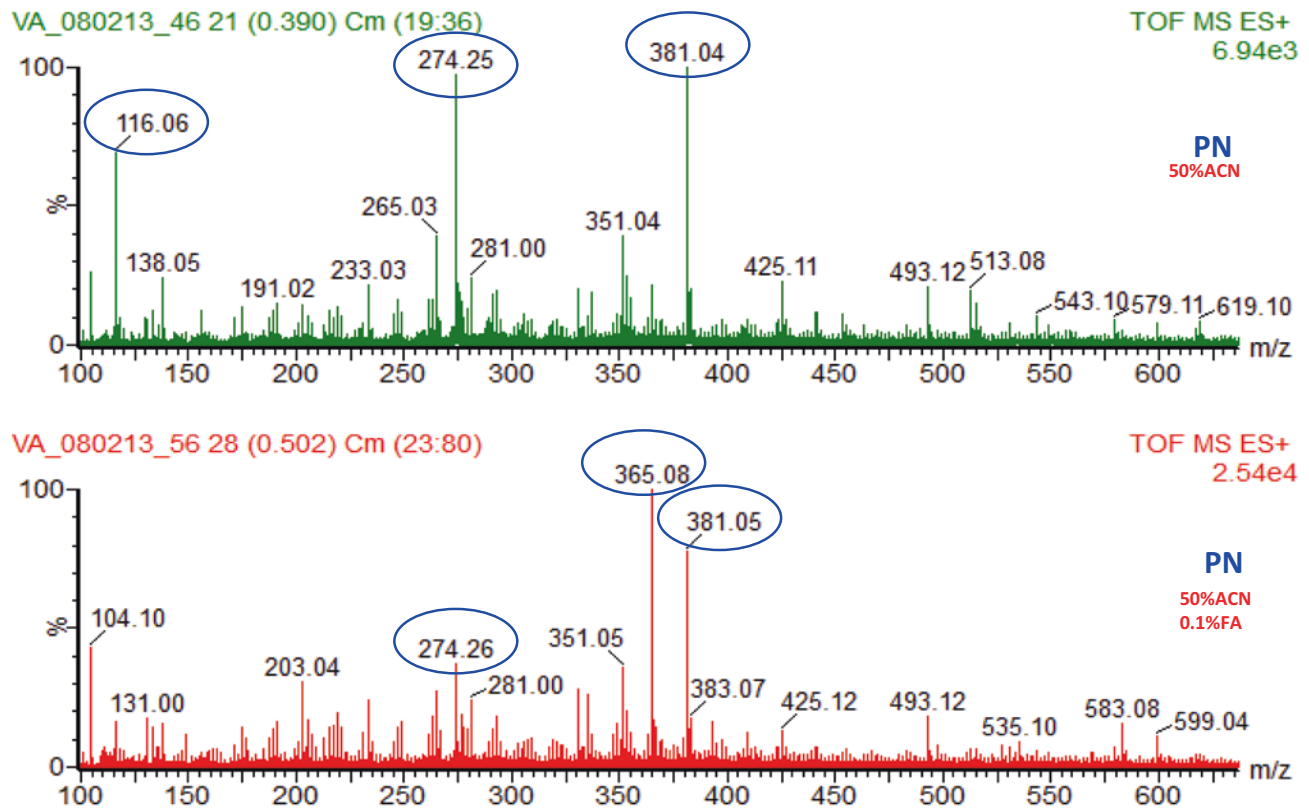

Fig. 46.7 ESI-MS analysis of a Pinot Noir (PN) wine samples under neutral pH (50% acetonitrile/ACN) and acidic (0.1% formic acid/FA and 50% ACN)

what. The ion pairs 249.10 and 277.10 (upper spectrum) and 306.11 and 334.12 (lower spectrum) are unmodified and modified b2 and a2 ion pairs.

A somewhat similar example to the one from Fig. 46.5 is shown in Fig. 46.6. Here, the students are required to identify the relationship between the three spectra. Since they are MS/MS spectra and the fragmentation is similar, it also suggests that the peptides that correspond to these MS/MS spectra are similar or related. Here, the key in solving this problem is to look at the *m*/*z* of the precursor ions. The precursor ions from the bottom two spectra differ by 0.3 Da, suggesting that the precursor ion is (3+), which it will allow us to identify the mass of the peptide and to suggest that the precursor ion from the bottom MS/MS spectrum is the 12C peak and the precursor ion from the middle spectrum is 13C peak. The difference between the mass of the precursor ions from the bottom two MS/MS spectra and the top MS/MS spectrum will reveal the difference between the peptide which corresponds to the bottom two MS/MS spectra and the truncated peptide which corresponds to the bottom top MS/MS spectrum.

In the last example, the students investigate wine (PN: Pinot Noir) by ESI (+) using different pH (neutral pH and low pH, i.e., with formic acid). The students are required to discuss the pKa of the compounds that correspond to circled peaks. As an example, peak with *m*/*z* 381.04 is protonated at both low and neutral pH, therefore, the pKa of this compound in equal or higher than pH 7.0. Conversely, peak 365.08 is not observed at neutral pH, suggesting that the pKa of this compound is somewhere in the acidic pH range.

Although this course is highly informative, helping students learn about all the diverse techniques in Biochemistry with the main focus on MS, it is the Biochemistry and Biotechnology lab course that helps students perform these techniques hands on and helps them do their independent project by combining a variety of techniques and analyzing their samples in the MS to identify any compounds they want. Therefore, the Biochemistry and Biotechnology lab reinforces the students' theoretical knowledge implemented in the Biochemistry I lecture class into an experimental setting [1].

One important component of the Biochemistry and Biotechnology lab is the independent project, where the students are required to propose, conduct, write up, present and defend their project. Before beginning their independent projects, the students learn techniques that they are familiar with from their lecture class such as chromatography for albumin purification, quantification of proteins using Bradford, Lowry, BCA and Biuret methods, SDS-PAGE followed by different staining conditions such as Coomassie Brilliant Blue, Ponceau S and Zinc transient staining. In addition, they perform a classic proteomics experiment, which includes the digestion of peptides using trypsin and LC-MS/MS analysis and subsequent identification of proteins using online database searches [1]. Finally, they are introduced to MS by demonstrating a series of direct infusion of peptide fragments using ESI-MS. The data obtained from that identifies the peptides using Masslynx software and further using Mascot database search and scaffold software analysis, the proteins are identified. The spectrum obtained from MS allows students to identify the charge, mass and peptide sequence using *de novo* sequencing and other bioinformatics tools [1]. Thus reinforcing them to implement the sequencing techniques learnt in their lecture class. They further validate their results obtained from the database searches by comparing their raw data. Once the students have performed all of these experiments, they are given the opportunity to choose their independent project.

The unique independent projects in the Biochemistry and Biotechnology lab allows students to design, budget, propose, conduct and perform their experiments and analyze their outcomes. This is an excellent opportunity for students to apply the knowledge they learned theoretically and practically to their projects. Most of the students utilize MS in their experiments because of its diverse applicability to identify proteins and small molecules [1]. Below are examples of several projects performed by students in Biochemistry and Biotechnology lab over the years using MS.

46.2 Mass Spectrometry Applications

46.2.1 Analysis of Proteins in Contact Lenses

Contact lenses are often contaminated with the accumulation of debris such as proteins, causing discomfort and damage of the lenses. Previous studies show the presence of lactoferrin, IgG, IgA, subunits of protein G and protein migrating faster than albumin (PMFA) [5–7]. These proteins are 'tear film proteins' and are found in the aqueous layer of the eye. They cleave the cell wall constituents to kill bacteria (i.e. lysozyme) or are secreted in tears as an immune response (i.e. lipocalin). When these (and other) proteins accumulate on the lenses, there is severe discomfort. Hence, the goal of this study was to determine the presence of these proteins and their relative concentrations using Bradford assay and identify the most abundant proteins using nanoLC-MS/MS analysis, which allows the determination of amino acid sequences of peptides and proteins.

In order to identify these proteins, the samples were subjected to fractionation by SDS-PAGE. They were stained with Coomassie brilliant blue and further, the gel pieces were excised, digested by trypsin and the resulting peptides were subjected to nanoLC-MS/MS analysis [5]. The MS analysis identified several proteins such as lysozyme, keratin, tryptophan 5-hydroxylase and lipocalin-1. Lysozyme

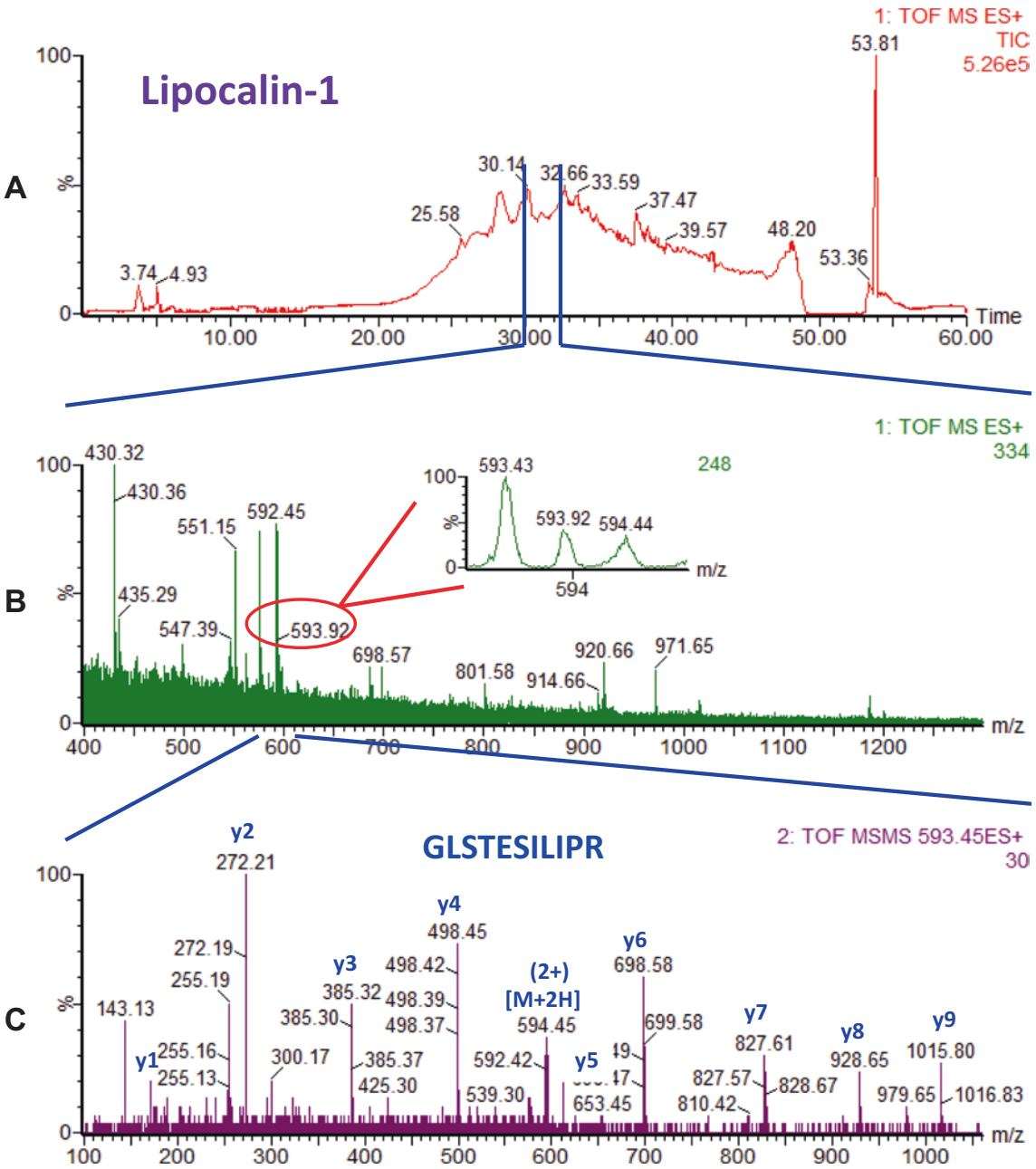

Fig. 46.8 NanoLC-MS/MS analysis of proteins bound to contact lenses. (**a**) Total Ion Chromatogram (TIC) of a 60 min HPLC gradient. (**b**) At elution time point ~31 min, a MS spectrum was recorded. One of the doubly charged peaks (circled) with *m/z* of 593.43 (insert), was selected for fragmentation and produced a MS/MS spectrum (**c**). Analysis of the b and y fragment ions led to identification of peptide GLSTESILIPR, which is part of human lipocalin-1 protein. Reproduced under Creative Commons from M. Beglinger et al., Mod. Chem. Appl. 01. 2013 [5]

and Lipocalin-I identification was significantly relevant as both proteins are part of the immune system, found in the tear film of the eye and also found on the skin. Tryptophan 5-hydroxylase and Keratin are mainly found on the skin, although Keratin is considered as contamination, it often works as a good positive control. Overall, LC-MS/MS analysis successfully identified proteins in contact lenses that are usually present in the aqueous layer of the eye, suggesting that these proteins tend to accumulate in the contact lenses and cause discomfort. The total ion chromatogram, MS and MS/MS spectra of lipocalin-1 is shown in Fig. 46.8.

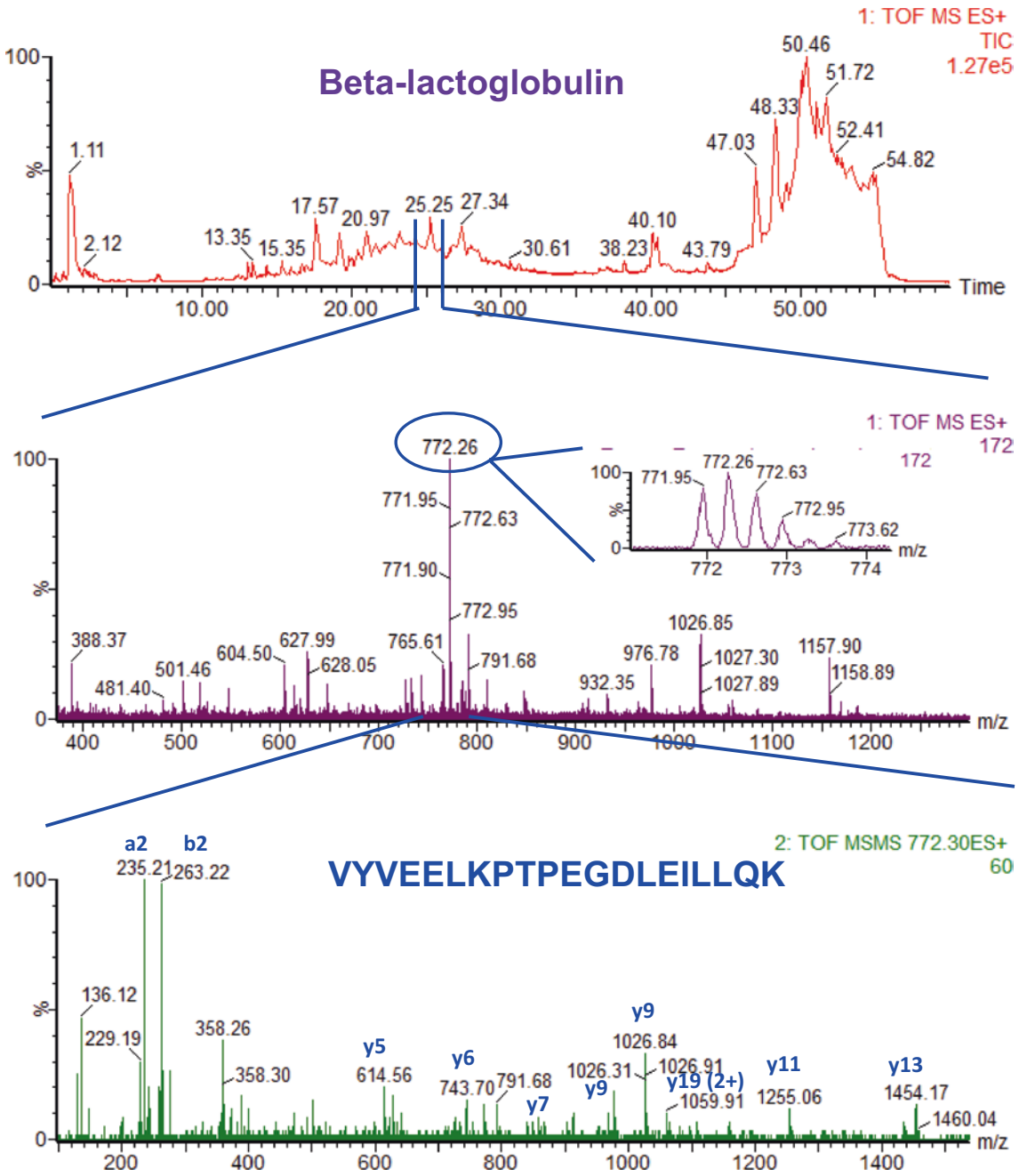

Fig. 46.9 NanoLC-MS/MS analysis of yogurt samples. Top: TIC. The middle panel shows MS of a series of multiply charged ions eluted at minute 25. The circled peak is (3+) charged peak with a mass-to-charge ratio (*m/z*) of 771.95 that was selected for MS/MS fragmentation shown in the lower panel. The MS/MS analysis led to the identification of the amino acid sequence of beta-lactoglobulin which is VYVEELKPTPEG DLEILLQK. Reproduced under Creative Commons from Gooding, R.C., et al., *What's in Your Yogurt? A Proteomic Investigation.* Modern Chemistry & Applications, 2014 [8]

46.2.2 Proteomic Investigation of Yoghurt

In a different study, the students were interested in a proteomics analysis of different types of yogurt [8]. The goal was to verify whether they can identify milk and non-milk proteins in yogurt. Different yogurts such as Stonyfield soy, Stonyfield greek, Activia, Yoplait, Dannon, Chobani were analyzed, first by SDS-PAGE and then by nanoLC-MS/MS. Indeed, the most abundant soluble proteins were found to be caseins (alpha S1 and kappa caseins) and lactoglobulins (alpha and beta lactoglobulins). No non-milk proteins were identified. The TIC, MS and MS/MS data that corresponds to peptides that are part of alpha-lactoglobulin, beta-lactoglobulin and alpha-S1-casein are shown in Figs. 46.9 and 46.10 [8].

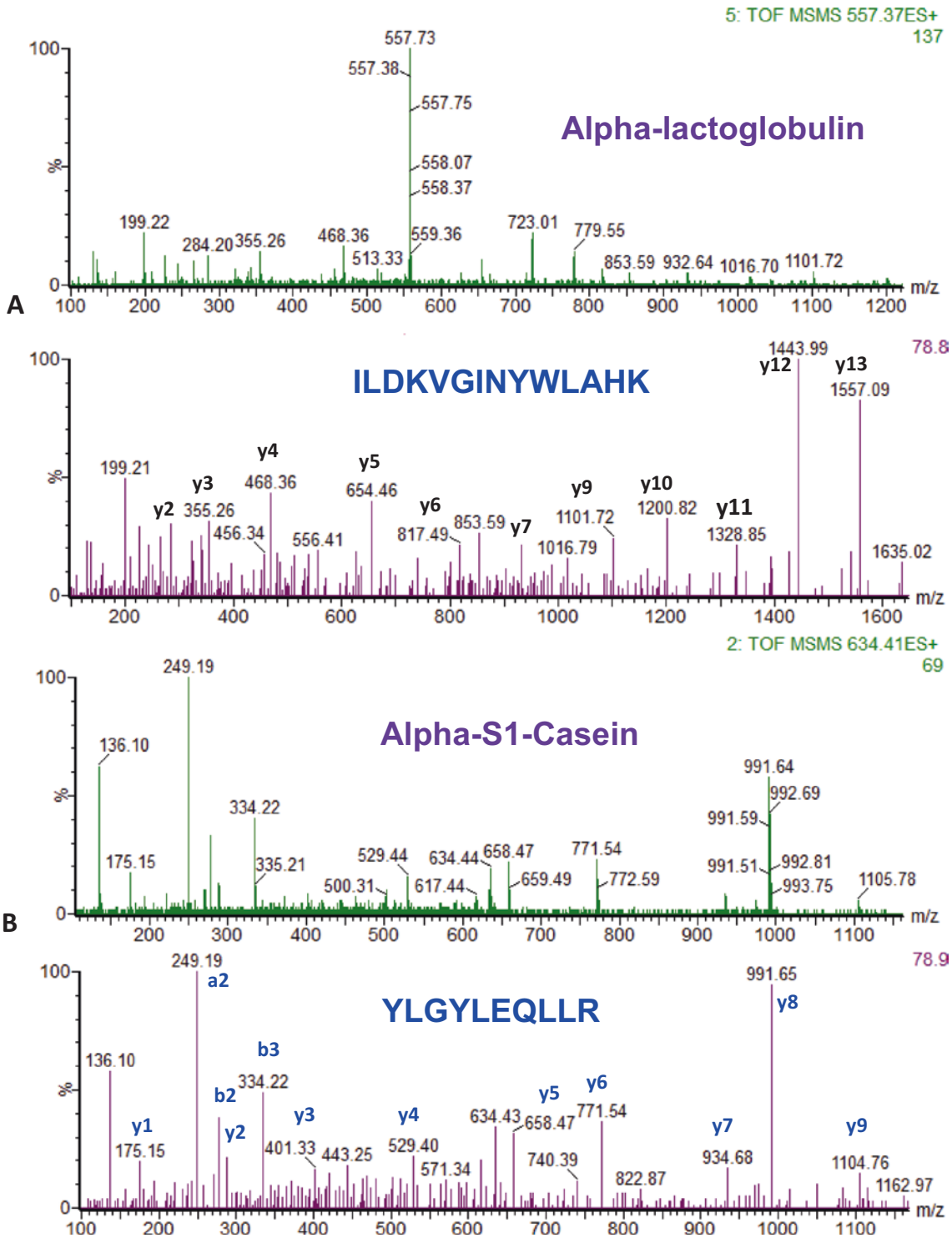

Fig. 46.10 NanoLC-MS/MS analysis of yogurt samples. Data analysis of MS/MS spectra identifying amino acid sequences (**a**) ILDKVGINYWLAHK and (**b**) YLGYLEQLLR which are part of Alpha-lactoglobulin and Alpha-S1-Casein, respectively. (**a**) and (**b**) shows the MS/MS spectrum at the top and deconvoluted spectrum at the bottom. (Deconvolution processes the multiple charged peaks into single charged peaks, allowing easier data analysis). Reproduced under Creative Commons from Gooding, R.C., et al., What's in Your Yogurt? A Proteomic Investigation. Modern Chemistry & Applications, 2014 [8]

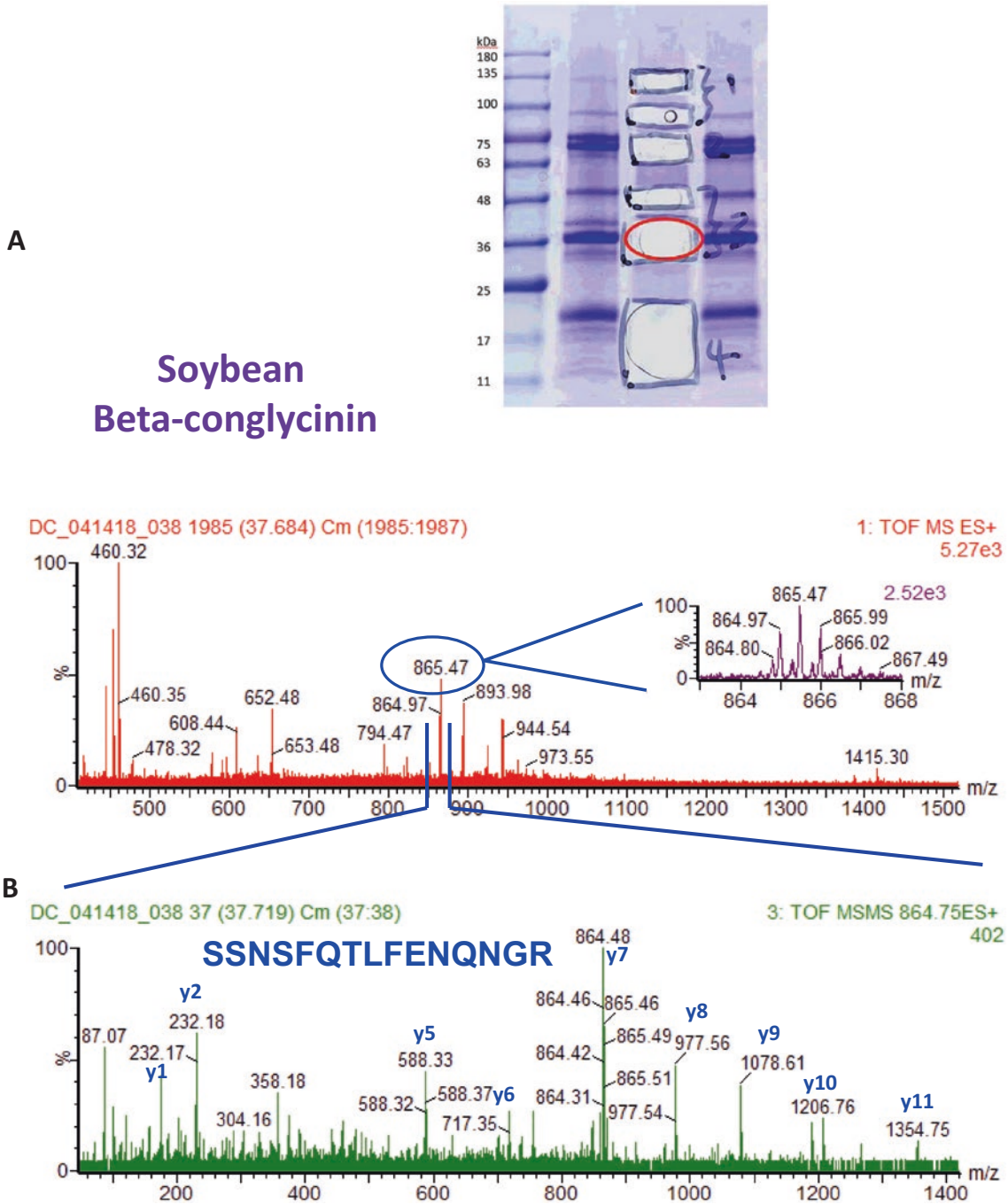

Fig. 46.11 Proteomics analysis of soymilk samples. (**a**): SDS-PAGE of soymilk proteins. The gel piece circled in red was digested by trypsin and analyzed by LC-MS/MS, shown in (**b**). (**b**) MS and MS/MS of a peak with *m/z* of 864.80 (2+), whose fragmentation produced a series of y ions that led to identification of peptide SSNSFQTLFENQNGR, which is part of Soybean Beta-conglycinin

46.2.3 Proteomics Analysis of Cow Milk and of Plant Based Milk Proteins

One of the goals in this project was to identify the proteins from cow milk and plant-based milks. The major proteins in cow's milk are caseins and lactoglobulins, while the major proteins in soy milk are β-conglycinin (7S) and glycinin (11S) [9, 10]. Proteomics analysis of cow milk indeed identified caseins and lactoglobulins as the major proteins (data not shown). Proteomics analysis of soymilk indeed showed the presence of soybean proteins. One such protein was β-conglycinin (Fig. 46.11).

46.2.4 Investigation of Proteins in Beer

Another independent study aimed study of proteins from different types of beer. There have been many experiments that have tested the amount of proteins in beer using different chromatography techniques. These techniques allowed scientists to tell the differences in protein and amino acid content in various beers [11–15]. Therefore, an effort was made to identify proteins in beer and characterize them using MS. A total of four beer samples were taken: Labatt Blue Light (BL), Guiness (G), UFO Hefeweizen (UFO) and Heineken (H). UFO and Guinness brands had the highest protein concentration. The beer samples were then separated by SDS-PAGE under non-reducing (−DTT) and reducing (+DTT) conditions. No difference between the protein pattern was observed between (−DTT) and (+DTT) samples (Fig. 46.12a), suggesting that (1) disulfide linkages in these samples either do not exist or are very few and (2) there are no disulfide-linked homo- or heterodimers/polymers in beer proteins. After the gel bands were digested by trypsin and analyzed by LC-MS/MS, the database search indeed found in one of the bands (sample #4 in Fig. 46.12a) protein z-type serpin previously found by others [16]. The MS and MS/MS spectra that correspond to a peptide that is part of protein z-type serpin is shown in Fig. 46.12b. This protein was from barley (*Hordeum vulgare L*) [17]. Because one of the common ingredients in every beer is barley, it was not surprising that this protein was found in the analysis. Overall, MS offered a good approach to analyze the protein content in beer.

46.2.5 Analysis of Proteins and Protein Content in Protein Enhanced Shakes

This experiment aimed to analyze some of the dietary supplement shakes that are on the market advertising high protein content and to determine if the proteins present in them were safe [18–24]. The samples tested were Boost, Equate, Ensure, Special K, Slimfast, Myoplex, and Muscle Milk shakes. These samples were run on SDS-PAGE after protein quantification. The selected bands were excised, digested with trypsin and analyzed by LC-MS/MS. The primary protein in the shakes found by MASCOT database search was casein (data not shown), found in cow's milk and contains important biological roles. This was one of the most abundant proteins found in all the samples. Therefore, MS allows us to conclude that protein shakes are mostly bovine milk.

46.2.6 MS Analysis of Coffee Components Based on Different Brewing Methods

LC-MS method was used to determine the compounds present in coffee and to analyze how these compounds vary under different brewing conditions. In this study, coffee was brewed in four different ways: a standard countertop coffee machine, a Moka pot, a French press as well as a cold brew method. The samples were analyzed using LC-MS/MS method. It was found that all the coffee samples obtained from different brewing methods contained quinic acid (Fig. 46.13a), citric acid, and caffeine (Fig. 46.13b). In addition, the table top sample was found to have the highest concentrations of quinic acid, citric acid, and caffeine, while the cold brew sample had the lowest concentration of each of the compounds. Chromatograms of table top and moka pot are shown in Fig. 46.13c, d. Therefore, the application of MS successfully facilitated the detection of different compounds present in coffee obtained via different brewing methods and their respective concentrations.

46.3 Conclusions

MS is a successful method to investigate proteomes in a variety of samples including yogurt, cow milk, plant-based milk, beer, protein powders or protein shakes, as well as metabolomes in samples like coffee, tea or soda. MS also provides a comprehensive understanding of analyzing any sample that is ionized or that can be ionized (i.e. by co-crystallization or derivatization).

Acknowledgement We would like to thank all the students, who did their independent research projects in Biochemistry and Biotechnology lab course using MS method for analysis and other students in the lab for creating a pleasant environment. Part of this chapter was partial credit for an extra credit assignment for the Biochemistry I class.

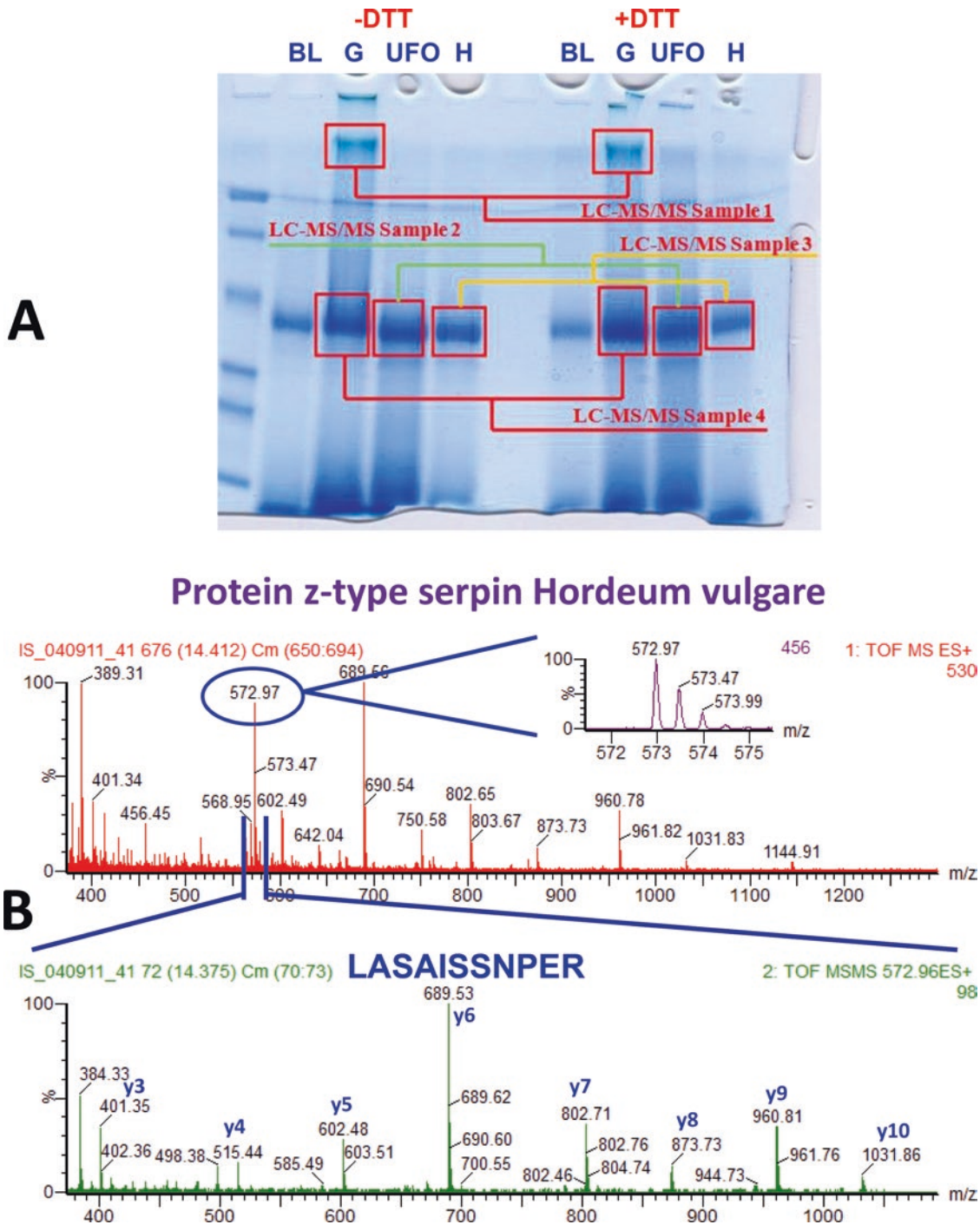

Fig. 46.12 Proteomics analysis of beer samples. (**a**): SDS-PAGE of different types of beer. A total of four beer samples were analyzed: Labatt Blue Light (BL), Guiness (G), UFO Hefeweizen (UFO) and Heineken (H). UFO and Guinness brands had the highest protein concentration. The beer samples were analyzed under non-reducing (–DTT) and reducing (+DTT) conditions. (**b**): LC-MS/MS analysis of gel band #4 from (**a**) led to identification of Protein z-type serpin Hordeum vulgare as the main protein component of all beer types. MS and MS/MS of a peak with *m/z* 572.97(2+) led to identification of peptide LASAISSNPER, which is part of Protein z-type serpin

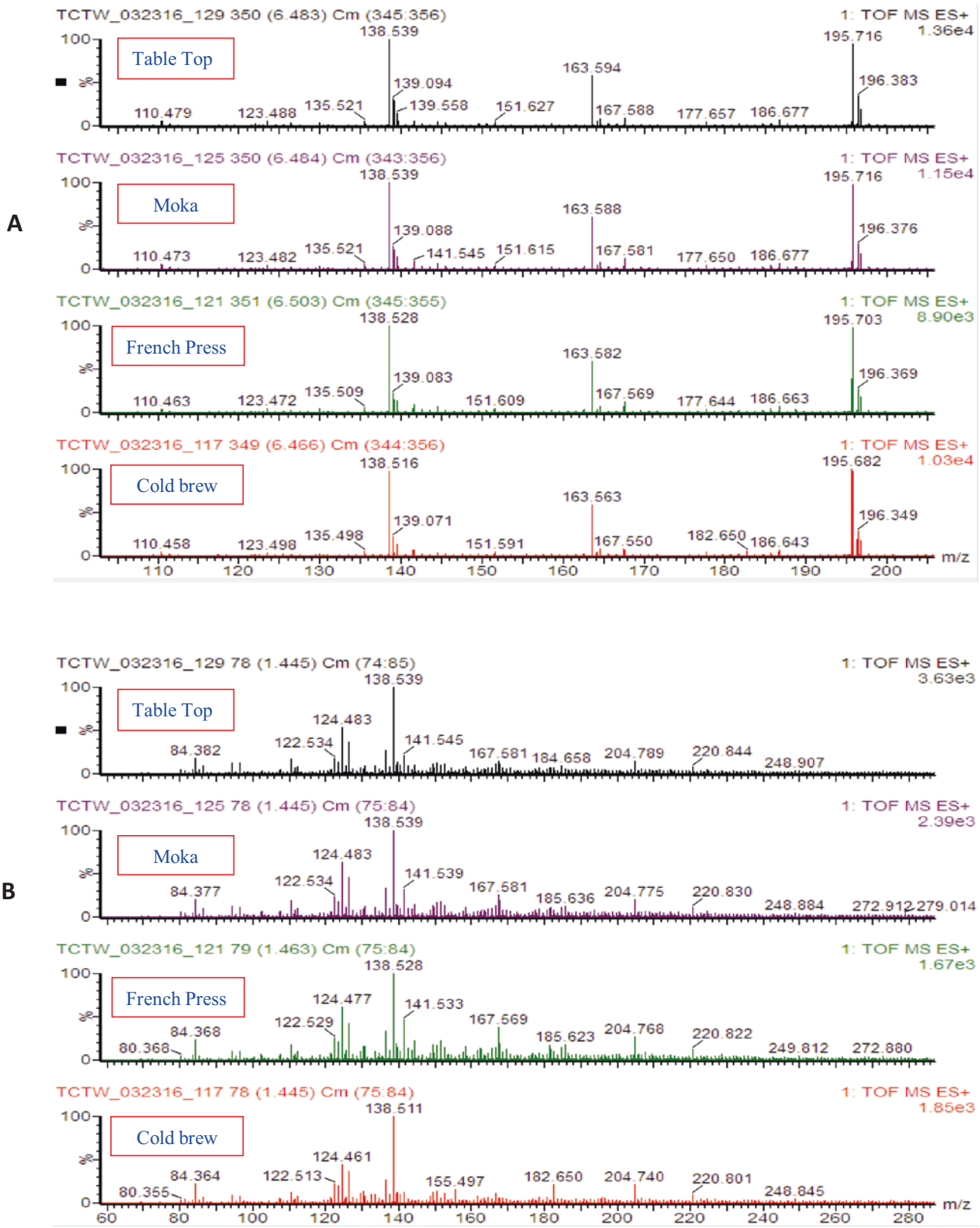

Fig. 46.13 (**a**) LC-MS spectra of caffeine (peak 195) in all four brewing methods. (**b**) LC-MS spectra of quinic acid (peak 138.5) in all four brewing methods. (**c**) HPLC chromatogram for table top coffee sample. (**d**) HPLC chromatogram of moka pot coffee sample

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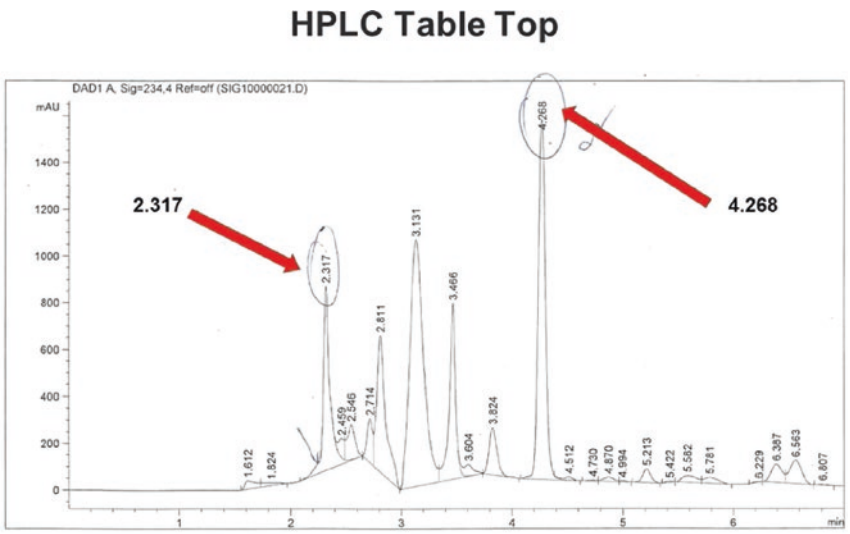

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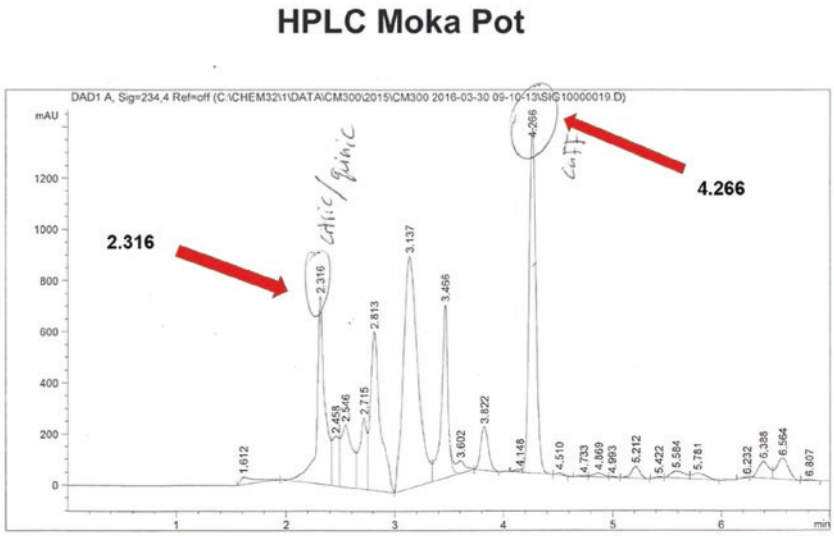

Fig. 46.13 (continued)

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