Chapter 4
Preparing Biological Tissue Sections for Imaging Mass Spectrometry

Yuki Sugiura and Mitsutoshi Setou

Abstract The process of making a thin slice section from frozen tissue blocks to the matrix application is described. Because the slice section created is served to direct mass spectrometry, there are some pivotal differences from other staining methods such as immunohistochemistry. In this chapter, the problems specific to mass spectrometry (MS) are introduced, and thereafter the ways of dealing with these problems are explained; last, the standard protocols are introduced.

4.1 Introduction

This section is devoted to the preparation of tissue sections for imaging mass spectrometry (IMS) i.e., the process of preparing thinly sliced sections from freshly frozen blocks of tissue to where these are served to the matrix application. The process of preparing sections for IMS measurement is essentially similar to that used in the preparation of frozen sections for immunostaining or dye-staining. However, because the sections created in this case are served to MS measurement, there are certain essential differences from other staining methods. A point to bear in mind is that IMS is an imaging technique based on MS, a microchemical analytical technique. In the preparation of such tissue sections, then, the use of an optimal cutting temperature compound (OTC) should be avoided, because contamination with such polymer

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Table 4.1 Major cautionary points at each step of slice preparation

Procedure for preparing a frozen section for IMS
Tissue extraction
Because of rapid metabolic turnover, tissues should be handled in a fixed time course, particularly for the analysis of small metabolite molecules
↓
Embedding
Avoid the use of polymeric compounds, such as optimal cutting temperature compound (OTC), for embedding [1, 2]
As an alternative, a precooled semiliquid gel of 2% sodium carboxymethylcellulose (CMC) can be used as an embedding compound, as it does not interfere with MS [3]
↓
Sectioning
Tissue thickness <20 μm improves spectrum quality especially for protein and peptide [4]
↓
Mounting
The use of conductive materials is recommended for supporting the tissue section [5][a]
↓
Washing
By removing small molecules such as lipids, the tissue-washing process improves the spectrum quality for protein/peptide detection [1, 6, 7]
↓
Drying
Failure to dry sections sufficiently may cause samples to peel off in the vacuum chamber of the mass spectrometer
↓
Sputtering
Sputtering of metals (e.g., gold) onto tissue sections before/after the matrix application process improves the spectrum quality [3, 8]

[a]Some instruments support the use of nonconductive materials [9]

molecules in MS causes a serious supression of biomolecule ionization [1]. The preclusion of OTC is a unique feature of the preparation protocol that distinguishes it from those used with immunohistochemical staining and other dye-staining methods. Table 4.1 summarizes such key points at each step in the section preparation process. In later paragraphs, problems specific to MS are introduced, as are techniques for handling these problems.

4.2 Embedding

In general, thin slices are prepared from a freshly frozen tissue block which has been penetrated with an embedding agent, such as the OTC and undergone freezing fixation. The tissues in the embedding agent are then cut into very thin slices. Embedding allows the sample to retain a good shape and facilitates the cutting process. However, in IMS experiments, it is known that the attachment and penetration of embedding agents (such as OTCs) in samples leads to a deterioration in the MS signals that are obtained [1, 2]. Such polymer-like resin compounds are commonly used as embedding agents and, in MS, these compounds have a high ionization efficiency; this leads to a decrease in the detection sensitivity of other molecules that would otherwise be observed. In particular, at analysis of small molecules with an *m/z* value of 1,000–2,000, contamination with OTC would lead to the emergence of extremely high polymer peaks in the mass spectra of positive ions, peaks that would virtually hide all the other smaller peaks (Fig. 4.1).

It also cause a decrease in the sensitivity of the signals when detecting higher molecular weight proteins [1, 2]. For all these reasons, OTC is used only for "supporting" the tissue blocks (as shown in Fig. 4.2a), such that it does not directly attach to the tissue sections. However, in the absence of the embedding process, difficulties may be encountered in cutting certain tissues into thin slice sections. Avoiding this problem, Stockli et al. used a precooled semiliquid gel of 2% sodium carboxymethylcellulose (CMC) as an alternative embedding compound that does not interfere with MS [3].

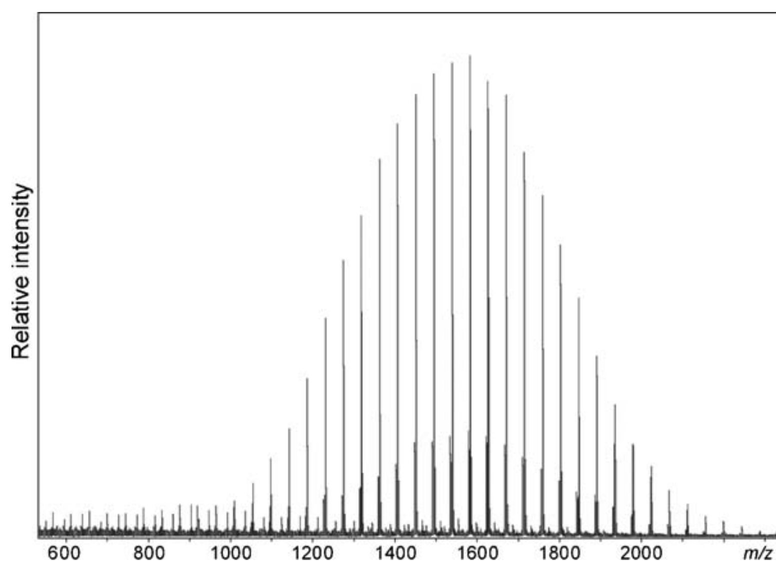

Fig. 4.1 Mass spectra of small molecules were obtained from a representative section that was completely embedded in optimal cutting temperature compound (OTC)

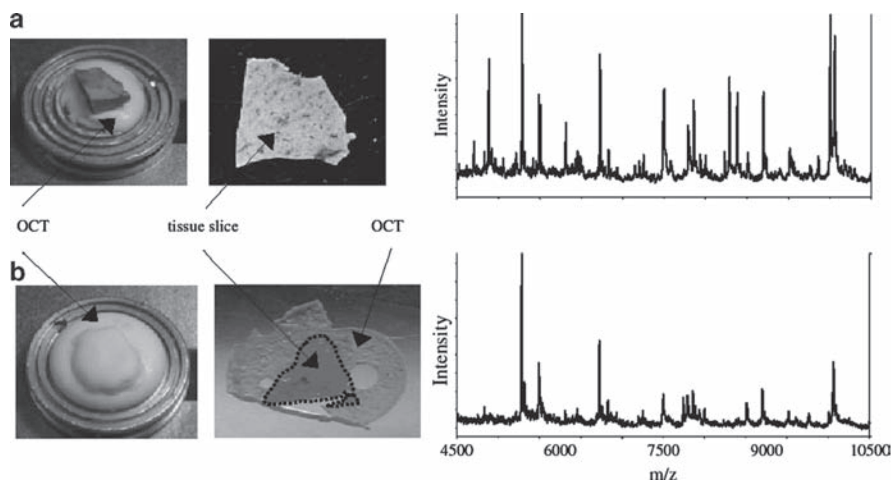

Fig. 4.2 Decrease in detection sensitivity of the ions originating from proteins because of contamination with OTC. OTC adhering to the tissue section diminishes the detectable peaks. (**a**) A case in which OTC was used only for supporting the tissue block. (**b**) A case in which the tissue block was completely embedded with OTC. (From [1]). (Reprinted from Schwartz et al., J Mass Spectrom 38:699–708.)

4.3 Excision of Thin Slices

Another important factor associated with the excision of thin slices for IMS is slice thickness. When the thickness of a slice is >15 μm, there is a deterioration in the detection sensitivity of IMS, particularly when analyzing high molecular weight proteins [4]. Figure 4.3 shows the mass spectra obtained from the cerebral cortex region in mouse brain slices with thicknesses of 2, 5, 10, 15, and 30 μm. A larger number of mass peaks with high signal-to-noise (S/N) ratios were observed in the spectra obtained from sections with 2-, 5-, and 10-μm thicknesses than in those obtained from sections with 15- and 30-μm thicknesses. This difference can be attributed to a phenomenon referred to as the "charging effect" [10]. Generally, biological tissue sections have low intrinsic electric conductivity, and this tendency is considered more apparent with thicker sections than with thinner ones. Therefore, on the surface of a thick slice, the surface of the sample is kept insulated from the support stage, which in turn has an electric conductivity. In this state, a surplus electric charge generated by laser irradiation is not lost through the sample stage, thus generating multiple charged ions on the sample; this ultimately leads to a significant loss of sample ions that would otherwise reach the detector [10]. However, in the case of frozen sections prepared without fixation, a high technical proficiency is required to stably create slices with thicknesses of several micrometers each. Currently, most samples are prepared with a slice thickness of 10–20 μm [11, 12]; these medium-thick sections appear to provide a good compromise between optimal IMS performance and experi-

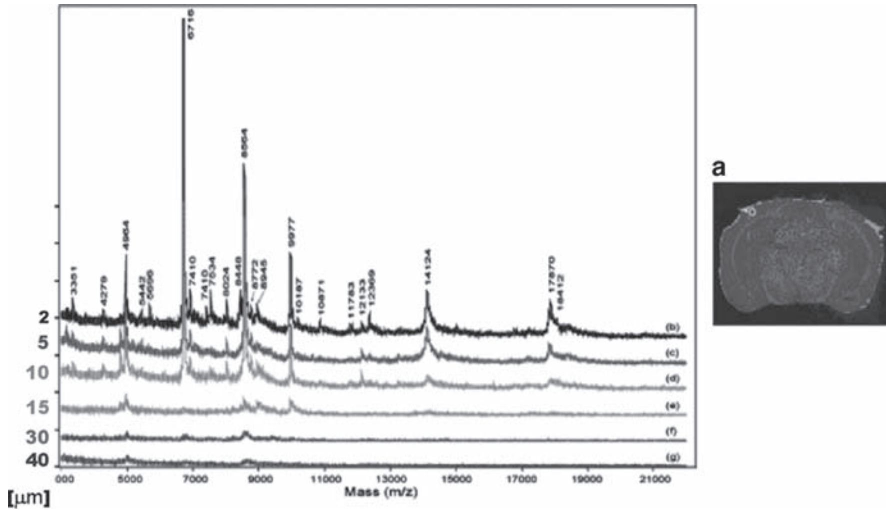

Fig. 4.3 Mass spectra obtained from the cerebral cortex region (see inset *a*) of mouse brain. Signals with good S/N ratios were obtained from thin slices. (From [1], modified). (Reprinted from Sugiura et al., J. Mass Spectrom Soc Jpn 54:45–48.)

mental efficiency [13], particularly when a large number of samples need to be analyzed [14]. The "metal sputtering" technique enhances the signal intensity and thus image quality [3, 8], presumably by avoiding the "charging effect."

4.4 Section Support Materials

Care should also be exercised in the selection of section support materials, i.e., the materials on which the tissue sections are laid. In IMS, in principle, support materials of which their surfaces are coated with conductive materials are used. In particular, when a tandem time-of-flight (TOF/TOF)-type instrument is used a high voltage is applied to the sample stage; therefore, omitting the use of conductive materials can lead to a significant reduction in detection sensitivity as a result of the aforementioned "charging effect." Exceptionally, a certain ion trap-type instrument makes it possible to measure the sample even on nonconductive materials [9].

The simplest technique involves placing the tissue slices directly onto a metal target plate [1]; however, in this case, when multiple measurements are made, the target plate must be thoroughly cleaned after each measurement. Currently, the method commonly used involves preparing samples on a disposable plastic sheet or a glass slide that is coated with a series of conductive materials [5]. Plastic sheets (ITO sheets) [15] or glass slides [5] (ITO glass slides; available from Bruker Daltonics or Sigma), coated with indium tin oxide (ITO), are particularly useful because they have a superior optical transparency that is suitable for microscopic

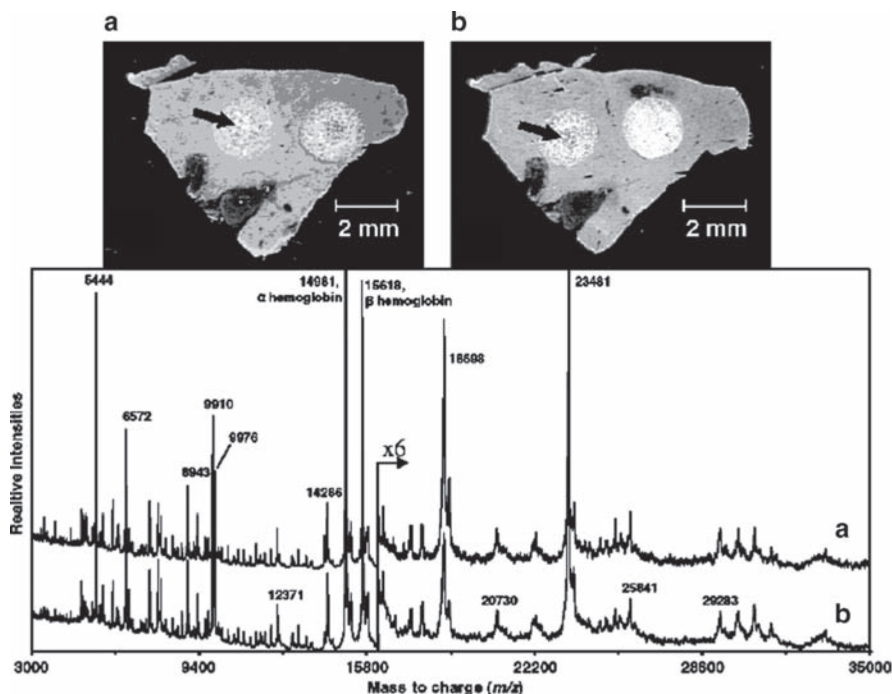

Fig. 4.4 Mouse liver sections (*upper panel*) on glass slides coated with gold (**a**) and ITO (**b**), and the corresponding mass spectra (*lower panel*). (Reprinted from Chaurand et al., Anal Chem 76(4):1145–1155.)

observations. In addition, they provide mass spectra with a high quality that is comparable to that obtained with materials coated with gold – the most conductive material (Fig. 4.4) [1, 5, 15]. ITO sheets are sufficiently thin (i.e., 125 μm) to be cut into appropriate shapes with scissors, and they also provide good light transmission [~85% visible light ($500<\lambda<900$ nm)]. In addition, the technique does not require the use of a specific adapter for introduction of glass slides into the mass spectrometer. Moreover, these materials are also versatile in that they can be attached to existing metal plates with conductive double-sided tape, which can then be introduced into any mass spectrometer [15, 16].

4.5 Postmortem Degradation

Another issue is the postmortem degradation of analyte molecules. In the sample preparation process, care needs to be exercised during both the organ extraction and section dehydration stages [17–19]. In both stages, a time-dependent appearance and a loss of signals may be observed [19], indicating that particular care is needed to ensure good sample-to-sample reproducibility.

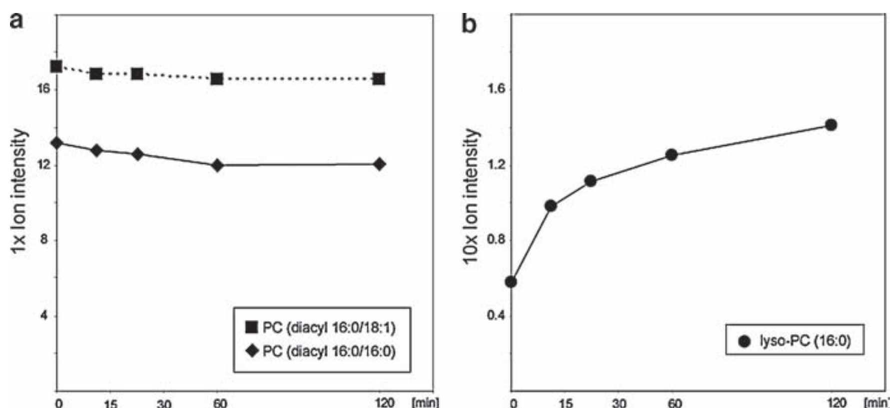

Fig. 4.5 Postmortem degradation of phosphatidylcholines and increase in lyso-PCs. We performed IMS on a series of mouse brains extracted at different time points after the mice had been sacrificed (<1, 15, 30, 60, and 120 min). After IMS, the ion intensities of the PCs were averaged over the entire section. As postmortem events, the degradation of PCs and an increase in lyso-PCs were observed within 15 min. These events were presumably caused by stimulation of the activity of phospholipases under ischemic conditions (Rehncrona et al. [20], Umemura et al. [21]). In this study, mouse brains were extracted within a minute (typically in 40 s) after sacrifice. (Reprinted from Sugiura et al., J Lipid Res 50:1776–1788.)

The first point is the time taken to extract organs. This is a critical parameter, particularly for the analysis of small molecules, owing to their rapid metabolic turnover [17, 18]. In our study involving an series of mouse brains extracted at different times after death (within 1, 15, 30, and 120 min), the postmortem degradation of phospholipids was observed within 15 min (Fig. 4.5). As postmortem events, the degradation of phospholipids and an increase in lyso-phospholipids were observed within 15 min, and they were presumably caused by a stimulation of the activity of phospholipases under ischemic conditions [20, 21]. The same applies to other metabolic molecules, such as dopamine and serotonin, which are metabolized into different molecules within a very short time period (within a few minutes) [17, 18]. All these factors suggest that the extraction of tissues should be performed as rapidly as possible. As an alternative method, the use of microwave heating devices is a relatively effective means of inactivating endogenous enzymes, thus eliminating postmortem degradation [22].

Second, the dehydration of sections before rinsing with organic solvents can also lead to changes in the signals that would otherwise be obtained [19]. Goodwin et al. experienced the time-dependent appearance and loss of signals when frozen tissue slices were brought rapidly to room temperature over a period of 30 s to 3 h. Sections of mouse brain were cut on a cryostat microtome, placed on a MALDI target, and allowed to warm to room temperature for the indicated period. Even within 1 min, protein/peptide signals were altered, in both an increasing and decreasing manner. Figure 4.6 shows three replicate experiments and time-courses of 0, 1, 2, 3, 4, 5, 6, and 7 min at room temperature; it demonstrates the typical changes observed over the

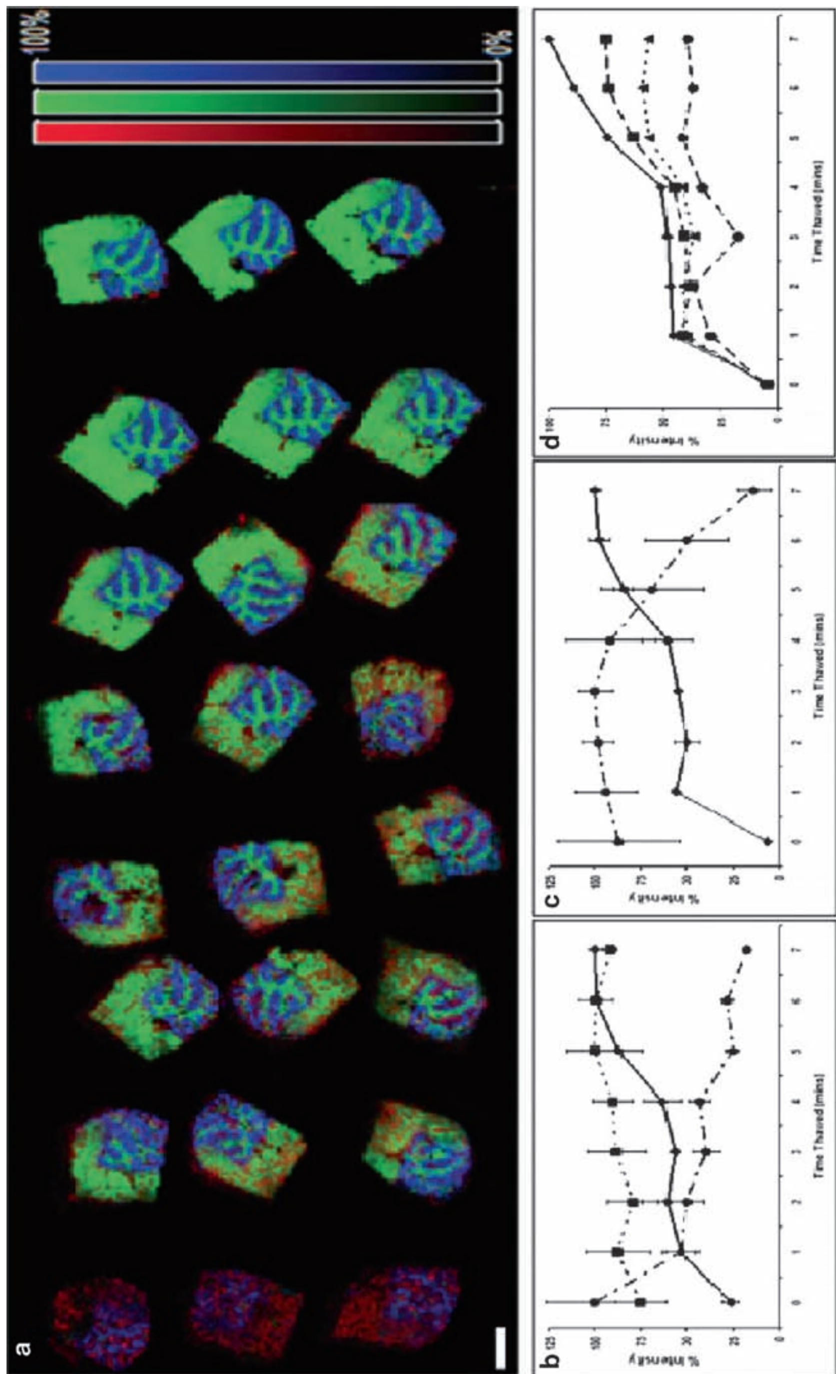

Fig. 4.6 MALDI-IMS images and plots of relative normalized intensities for a number of representative marker masses for 14-mm-thick mouse sections of the cerebellum thaw-mounted onto ITO-coated slides. Samples were manually spray-coated with 10 mg ml^{-1} cyano-4-hydroxy cinnamic

time course (*red*, decreasing; *blue*, no significant change; *cyan*, increasing) [19]. Therefore, tissue slices are preferably served at the next step – the washing step for protein measurement and the matrix application step for small molecule measurement – as rapidly as possible. Considerable care is required at these stages to facilitate comparisons among the biomarkers of different IMS experiments.

4.6 Rinsing of Tissue Sections with Organic Solvents

As mentioned in Chap. 2 of this book, the rinsing of tissue sections with organic solvents is required when detection targets include peptides and proteins. For the measurement of small molecules, this process is usually eliminated [23]. The rinsing step promotes the ionization of peptides and proteins, mainly by washing out phospholipids from the sections [1, 6]. It also plays a role in flushing out salts that could interfere with the crystallization of the matrix [1].

In earlier studies, several rinsing methods using ethanol solutions were described. The following are representative procedures:

- 70%:30% (v/v) ethanol/water (30-s immersion) × 2 times (Fig. 4.7) [1]
- 70%:30% (v/v) ethanol/water (30-s immersion), 100% ethanol (15-s immersion) [24]
- 70%:30% (v/v) ethanol/water (30-s immersion), 100% ethanol (15-s immersion) [24]
- 90%:9%:1% (v/v/v) ethanol/water/glacial acetic acid (30-s immersion) [25]

On the other hand, Lemaire et al. examined washings with five different organic solvents – chloroform, xylene, toluene, hexane, and acetone – by dropping each of the solvents on a tissue sample (200 µl solvent per cm^2 of tissue), removing the solvent with a syringe, and then repeating the procedure. In each case, they found that the detection of peaks originating from proteins increased, compared to

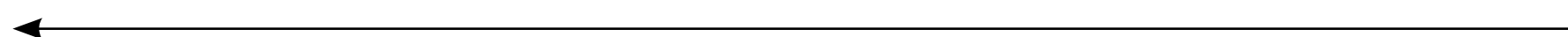

Fig. 4.6 (continued) acid (CHCA) matrix. The figure represents three replicate experiments and a time course of 0, 1, 2, 3, 4, 5, 6, and 7 min at room temperature. Error bars on the graphs are SDs for $n = 3$. **a** MALDI-IMS image of the replicate experiments vertically and time course horizontally. Intensity plots for three markers (*red*, decreasing; *blue*, no significant change; *cyan,* increasing) are shown, demonstrating typical changes observed over the time course. *Bar* 2 mm. **b** Graph of relative normalized intensity for three markers in the total tissue area showing a decrease (1007.8 6 0.5 Th, *filled circles*), increase (2407.3 6 0.5 Th, *filled diamonds*), or no significant change (1315.9 6 0.5 Th, *filled squares*). **c** Graph of relative normalized intensity for two markers in the total tissue area, one showing a decrease with a significantly different overall profile to that in (**b**) (950.9 6 0.5 Th, *filled circles*), and the other an increase very similar in rate to that in (**b**) (1835.5 6 0.5 Th, *filled diamonds*). **d** The apparent relative change in a marker (1835.5 6 0.5 Th) averaged across the whole tissue (*filled squares*), the cerebellum (*filled triangles*), the adjacent brainstem and neocortical tissue (*filled diamonds*), and molecular layer of the cerebellum (*filled circles*), showing how significant differences in the rate of change in specific regions can be masked when analyzing the whole tissue [19]. (Reprinted from Goodwin et al., Proteomics 8:3801–3808.)

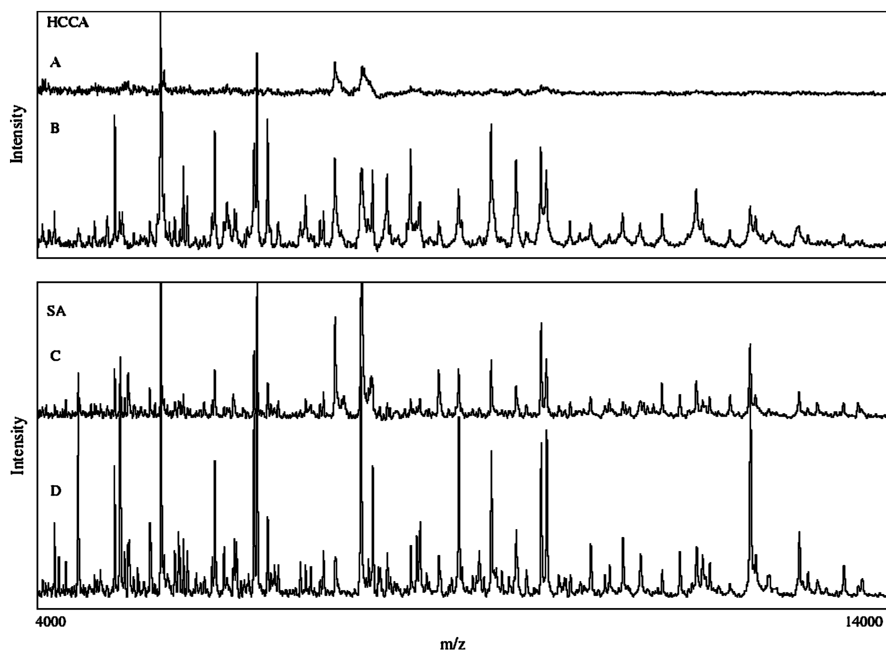

Fig. 4.7 Mass spectra obtained from mouse liver tissue before (**a**, **c**) and after (**b**, **d**) the washing process was completed [70%:30%, v/v, ethanol/water (30-s immersion) × 2]. Cyano-4-hydroxycinnamic acid (CHCA) and sinapic acid (SA) were used as the matrix in the *upper* and the *lower panels*, respectively. (Reprinted from Schwartz et al., J Mass Spectrom 38:699–708.)

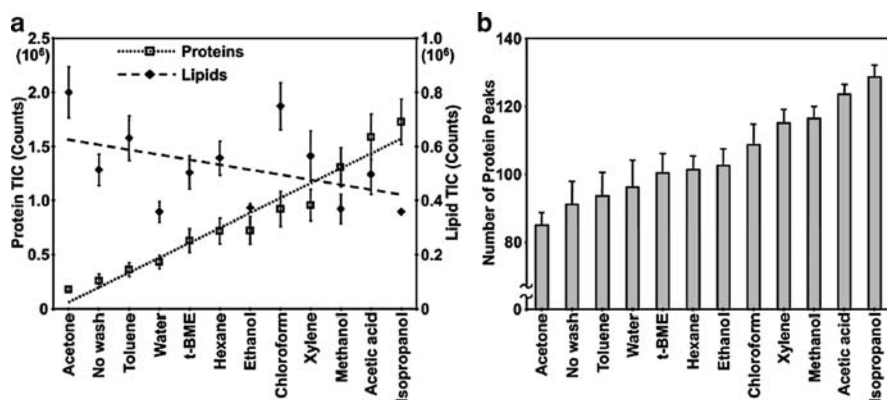

Fig. 4.8 (**a**) Total ion count (TIC) variations recorded from the MALDI-TOF MS protein profiles acquired from serial mouse liver tissue sections not washed or washed with different solvent systems in the *m/z* range 500–1,100 (lipid component) and the *m/z* range 2,000–25,000 (protein component). (**b**) Number of peak variations as a function of the same washes for the protein component [7] (Reprinted from Seeley et al., J Am Soc Mass Spectrom 19:1069–1077.)

untreated samples [6]. More recently, Seeley et al. demonstrated that the rinsing step enhances protein detection in terms of both the number of observed peaks and ion counts, and that this trend was inversely correlated with lipid detection (Fig. 4.8) [7]; after examining 12 different rinsing solvents, these authors established the following conditions as the most effective for protein analysis, with respect to the S/N ratio of protein signals, matrix crystal forms, and histological integrity of the tissues:

70%:30% (v/v) isopropanol/water (30-s immersion), followed by 90%:10% (v/v) isopropanol/water (30-s immersion) [7]

4.7 Staining of Tissue Sections Before IMS Measurement

As mentioned earlier, the use of ITO-coated materials makes microscopic observation possible. Invaluable expertise is acquired by examining the ion distribution images of IMS in conjunction with light microscopic images; however, simply viewing a frozen section under a microscope would merely reveal the indistinct outlines of the tissue's internal structural organization. When there are certain particular target areas or specific lesion sites to be analyzed by an experimenter, it is preferable that the analyzed sites are, initially, selectively determined by using appropriate dye methods. Chaurand et al. examined the mass spectra of tissue sections stained with certain different staining dyes and introduced staining methods that can be used in concert with IMS (Fig. 4.9) [5].

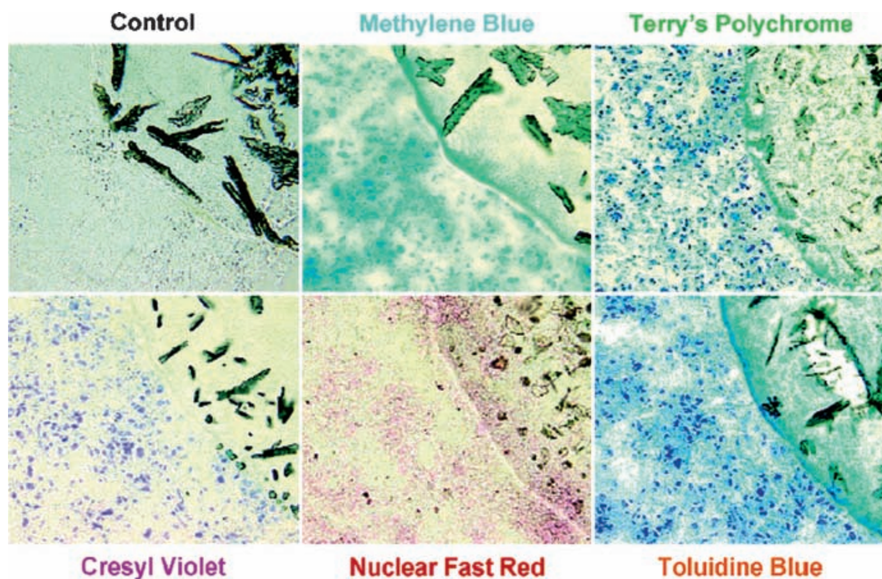

Fig. 4.9 Microscopic image (×400) of human glioma tissue sections that were stained with different dyes and onto which matrix was subsequently deposited. (From [3]) (Reprinted from Chaurand et al., Anal Chem 76(4):1145–1155.)

4.8 A Standard Procedure for Preparing Frozen Sections

4.8.1 Materials

The following items are needed when preparing frozen tissue sections:

- Cryostat
- Blades for cryostat
- OTC compound
- ITO glass slides

4.8.2 Methods

To prepare frozen tissue sections, execute the following procedure:

1. Place a glass slide inside the cryostat
2. Wipe off any oil remaining on the blade with ethanol and attach the blade to the cryostat
3. Pre-cool the forceps and brushes by placing them inside the cryostat
4. After the conditions for thin sectioning have been determined, i.e., the position of the anti-roll, temperature, and thickness (Note 1), perform thin sectioning of the sample
5. Keep the slice under the anti-roll for a while, and hold the end of slice with forceps or brushes when it no longer curls
6. Hold up the glass slide in the cryostat, and immediately place the slice on it
7. Keep performing thin sectioning and pasting
8. Cool a container filled with nitrogen gas in the cryostat, and immediately transfer the section to this container, and then store at −80°C (Note 3)
9. When the sections are to be analyzed, take them out of the container, and then dry them in a stream of nitrogen gas (Note 4)
10. Serve the slice to the next process (washing process for protein analysis or matrix application for small molecule analysis)

4.8.3 Notes

1. Sections tend to curl up when the temperature is too high or if the anti-roll is placed inappropriately.
2. For the analysis of small molecules that might be oxidized, it is recommended to serve the section to the matrix application step immediately after it has been excised.
3. Insufficient drying causes the sample to peel off.

4.9 Conclusion

In this chapter, we briefly introduced sample preparation methodologies, particularly those used in the preparation of tissue sections. Because there are a number of key points in this process that can determine the success of an IMS experiment, we identified each of these and described the techniques used to handle them. However, there are still many aspects of these techniques to be investigated: for example, lipid-rich organs, including the brain, require a stringent washing protocol, whereas such a treatment might even result in the removal of proteins in other organs. We proposed a protocol that summarizes the aforementioned key points. The optimization of tissue preparation protocols is an ongoing initiative; thus, the inquisitive reader would be directed not only to the excellent work referenced in this chapter but also to the most recent literature.

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