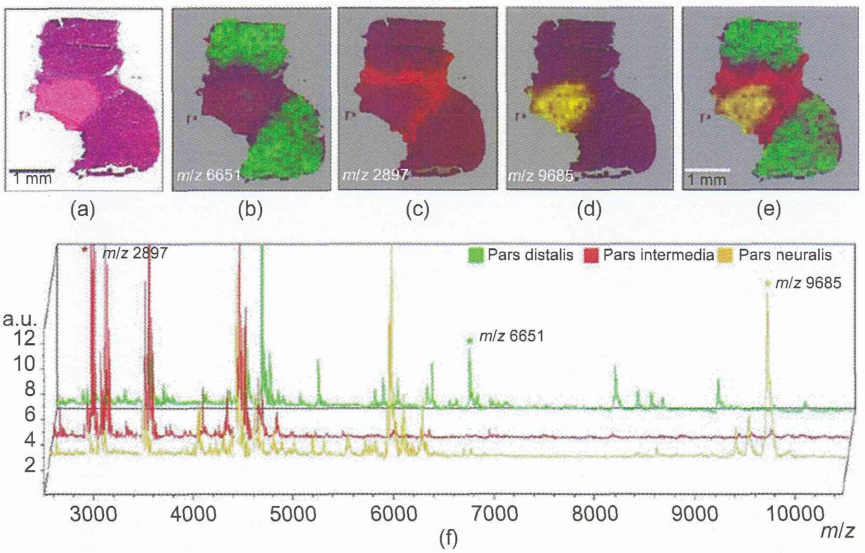

FIGURE 5 MALDI-IMS of a tissue section of rat pituitary gland. (a) Optical microscopic image of an H&E-stained tissues section. The staining was done after the MALDI measurement of the tissue section. (b)–(d) Visualized selected m/z species representing features to pars distalis (m/z 6,651; green), pars intermedia (m/z 2,897; red), and pars neuralis (m/z 9,685; yellow). (e) Merge of (a–d). (f) MALDI-TOF-MS spectra obtained from this case from pars distalis (green), pars intermedia (red), and pars neuralis (yellow) showing the molecular differences between the histological regions. Reprinted from Walch *et al.* (2008) with permission from Springer.

and another section is cut for histological staining. They can then be superimposed on each other and provide an absolute value of the molecular distribution (Figure 5). Recently a new approach for tissue section staining after the MALDI measurement has been reported. The results obtained from IMS analysis were correlated with the H&E staining of the tissue section (Schwamborn *et al.*, 2007).

3.2. Choice of Matrix

The choice of a suitable matrix for MALDI-IMS analysis depends on the mass range and analyte of interest. The main function of the matrix is to absorb laser energy from the source and transfer it to the analyte (Dreisewerd, 2003). The matrix thus ensures that efficient desorption and ionization occur and protects the tissue section from the disruptive energy of the laser. Sinapinic acid is generally used for the analysis of higher-molecular-weight proteins, and α-cyano-4-hydroxycinnamic acid (CHCA) is used for lower-molecular-weight molecules such as peptides (Schwartz *et al.*, 2003). 2,4-dihydroxybenzoic acid (DHB) and 2,6-dihydroxyacetophenone (DHA) are generally used for analysis of

TABLE 1 Commonly used MALDI matrices for imaging of biomolecules in tissue samples

Matrix	Applications	References
2,5-Dihydroxybenzoic acid (DHB)	Lipids, Sugars, peptides, nucleotides, glycopeptides, glycoproteins, and small proteins	Fournier *et al.* (2003); Herring *et al.* (2007); Tholey and Heinzle (2006)
α-Cyano-4-hydroxycinnamic acid (CHCA)	Peptides, small proteins and glycopeptides	Schwartz *et al.* (2003); Tholey and Heinzle (2006)
2,6-Dihydroxyacetophenone (DHA)	Phospholipids	Jackson *et al.* (2005); Seeley *et al.* (2008); Tholey and Heinzle (2006)
2,4,6-Trihydroxyacetophenone (THAP)	Lipids	Stuebiger and Belgacem (2007)
p-nitroaniline (PNA)	Phospholipids	Estrada and Yappert (2004); Rujoi *et al.* (2004)
2-mercaptobenzothiazole (MBT)	Phospholipids	Astigarraga *et al.* (2008)
Sinapinic acid (SA)	Peptides and large proteins	Schwartz *et al.* (2003)
CHCA/aniline, ionic matrix	Peptides	Lemaire *et al.* (2006b)
CHCA/n-butylamine, ionic matrix	Phospholipids	Shrivas *et al.* (2010)

phospholipids (Herring *et al.*, 2007; Seeley *et al.*, 2008). A great variety of matrices are used for the analysis of biomolecules, some of which are listed in Table 1.

3.2.1. Ionic Matrices for IMS

Ionic matrices (IMs) constitute a new class of organic matrices reported for the analysis of a number of different molecules in MALDI-MS. IMs are good for MALDI-MS imaging studies due to the fact that the process solubulizes several analytes, has vacuum stability, and forms homogenous crystals with analyte molecules. IMs have been used to obtain enhanced sensitivity and good reproducibility in the analysis

of biomolecules (Armstrong *et al.*, 2001; Laremore *et al.*, 2007). IMs such as 2,5-dihdroxybenzoic acid butylamine (DHBB) and α-cyno-4-hydroxycinnamic acid butyl amine (CHCAB) render good crystal formation, signal intensity, and reproducibility compared with conventional matrices such as DHB and CHCA (Shrivas *et al.*, 2010). The results are

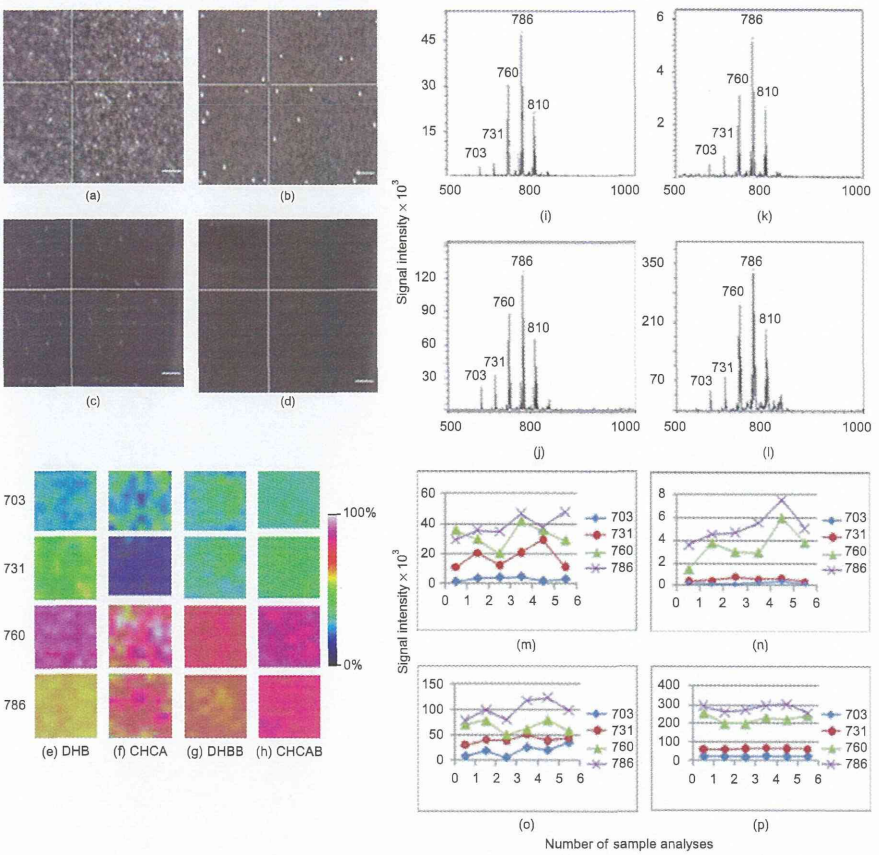

FIGURE 6 The crystal formation of (a) DHB, (b) CHCA, (c) DHBB, and (d) CHCAB matrixes with phospholipids on to a MALDI target plate. The pictures were taken with an Ultraflex II TOF/TOF. The scale bar (white color line) is 100 μm; images (e) to (h) show the ion image of phospholipids reconstructed obtained by using (e) DHB, (f) CHCA, (g) DHBB, and (h) CHCAB matrix at *m/z* 703, 731, 760, and 786. Images (i) to (l) show the signal enhancement: 3- to 7-fold enhancement of signal intensity when DHBA IM (image i) is used as a matrix compared with DHB matrix (image j) and 50- to 100-fold improvement of signal intensity using CHCAB IM (image k) compared with CHCA matrix (image l). Graphs (m) to (p) show the six replicate analyses of samples with ± relative standard deviation, % by using (m) DHB: ± 20.5–40.8%, (n) CHCA: ± 29.5–45.8%, (o) DHBB: ± 14.5–21.8%, and (p) CHCAB: ± 7.5–10.0%. Reprinted from Shrivas *et al.* (2010) with permission from American Chemical Society.

shown in Figure 6. Direct tissue analyses of peptides in rat brain tissue sections using IMs improved the ionization efficiency and increased the signal intensity of ion images of molecules compared with the conventional matrix (Lemaire *et al.*, 2006a). IMs were also used for imaging and identification of gangliosides in mouse brain (Chana *et al.*, 2009). DHB and CHCAB IMs in MALDI-IMS were also used for analysis of mouse liver and cerebellum tissues to identify the different species of lipids; results with CHCAB were better than with conventional matrices (DHB and CHCA).

3.2.2. Nanoparticles as Matrices for IMS

In addition, nanoparticles (NPs) can be used as a matrix instead of organic matrices for the analysis of low-molecular-weight molecules (< 500 Da). One problem with the organic matrix ions is that they themselves produce an intense peak in the mass spectrum and hence suppress detection of the analyte of interest, which then obviously decreases the sensitivity of the method. To circumvent this disadvantage, nanomaterials and inorganic compounds have been introduced. The Tanaka and Sunner groups investigated the application of cobalt powder (NPs) and graphite microparticles, respectively, suspended in glycerol to analyze proteins and/or peptides in MALDI-MS analyses. The use of NPs as a matrix in MALDI-MS allows for efficient absorption of laser energy as well as efficient subsequent desorption and ionization of molecules from the sample surface (Sunner *et al.*, 1995; Tanaka *et al.*, 1988). Desorption/ionization on porous silicon (DIOS) is another matrix-free method that is produced by etching of the silicon surface. Small molecules can be efficiently ionized using DIOS as an effective surface (Wei *et al.*, 1993). Today nanomaterial surfaces are also applied for the direct analysis of tissue samples in MALDI-IMS. Northen's group introduced a new nanostructure surface for imaging of biomolecules in tissue samples known as ionization nanostructure-initiator mass spectrometry (NIMS) (Northen *et al.*, 2007). Several other sample preparation procedures, such as graphite-assisted laser desorption/ionization (GALDI) (Cha and Yeung, 2007), nano-assisted laser desorption/ionization (NALDI) (Vidova *et al.*, 2010), and DIOS have been proposed for imaging of biomolecules in tissue samples. Taira *et al.* (2008) developed another matrix-free method called nanoparticle-assisted laser desorption/ionization imaging mass spectrometry (Nano-PALDI-IMS) that can be used to visualize peptides, phospholipids, and metabolites in tissue sections. Recently silver (Hayasaka *et al.*, 2010) and gold (Goto-Inoue *et al.*, 2010a) NPs were applied for imaging and identification of fatty acids and glycosphingolipids, respectively, an analysis that could be difficult to perform by conventional MALDI-MS using DHB as a matrix. Figure 7 demonstrates imaging and identification of fatty acids from mouse liver sections using silver NPs as a matrix (Hayasaka *et al.*, 2010). More recently, TiO_2 NPs were applied for the analysis of low-molecular

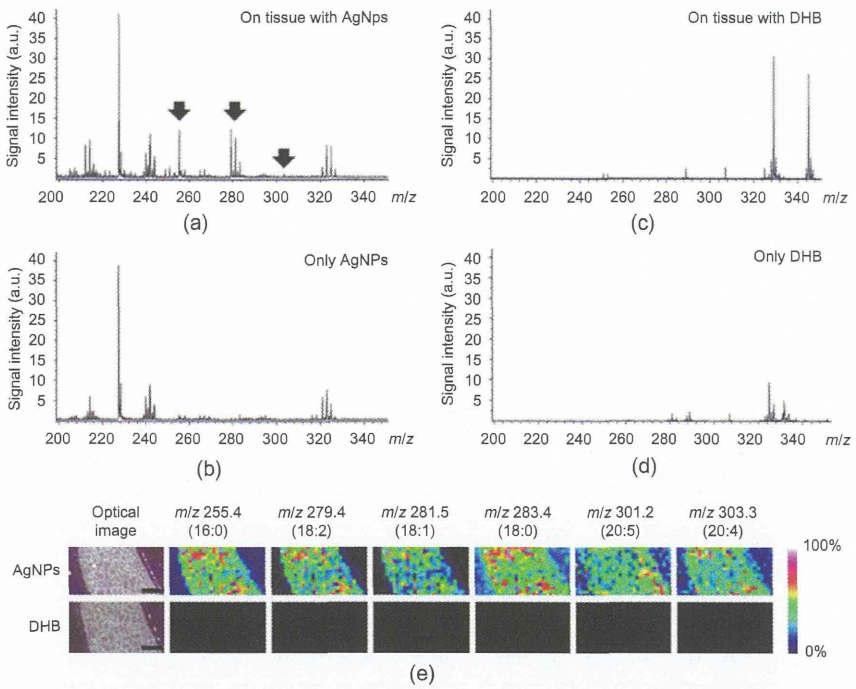

FIGURE 7 Identification of fatty acids from mouse liver sections in nano-PALDI-IMS. The serial sections were sliced to a thickness of 10 μm. Silver nanoparticles (NPs) or DHB matrix solution was sprayed on the surface of the mouse liver sections, respectively. Their sections were measured with a scan pitch of 100 μm by nano-PALDI-IMS analysis in negative-ion mode. The mass spectra were obtained from the sections sprayed with silver NPs (a) on tissue section, (b) only silver NPs or DHB matrix, (c) on tissue section, and (d) only DHB solution. The peaks used to reconstruct the ion image are indicated by arrows. (e) In the analysis using silver NPs and DHB, the ion signals at m/z 255.4 (16:0), 279.4 (18:2), 281.5 (18:1), 283.4 (18:0), 301.2 (20:5), and 303.3 (20:4) were detected. The scale bars are 500 μm. Reprinted from Hayasaka *et al.* (2010) with permission from Springer.

weight-biomolecules in mouse brain without observing any NP-related peaks. More individual signals and higher intensity were obtained when TiO_2 NPs were used as a matrix compared with a DHB matrix (Shrivas *et al.*, 2011). Thus we can conclude that the use of a nanomaterial surface is efficient and effective for desorption and ionization of molecules; the process yields images with higher resolution.

3.3. Application of Matrix Solution

The deposition of matrix solution on the surface of a tissue section is another important step in obtaining homogeneity, reproducibility, and good resolution of the biomolecule. The matrix solution consists of three

components—organic solvent, matrix, and trifluoroacetic acid (TFA). Crystal formation is affected by the concentration of matrix and the ratio of organic solvent to water; organic solvent is used to dissolve the solid matrix and extract the molecules from the tissue section. This extraction is followed by crystal formation on the surface of the tissue section. The addition of TFA provides free protons for effective ionization of the analytes, and typically, singly charged $[M + H]^+$ ions are formed. A number of devices are useful for the deposition of matrix solution on the surface of tissue sections—for example, chemical inkjet printer spotter (Baluya *et al.*, 2007), robotic spotting depositors (Aerni *et al.*, 2006), electrospray depositors (Altelaar *et al.*, 2007), and airbrush sprayers (Hayasaka *et al.*, 2009). The sublimation (Hankin *et al.*, 2007) and stainless steel sieve (Puolitaival *et al.*, 2008) methods have demonstrated good signal intensity and sample reproducibility. Figure 8 shows a thin layer chromatography (TLC) sprayer (image a), sublimation apparatus (b), air brush sprayer (c), and a chemical inkjet printer (d) used for matrix deposition. The goal of these matrix deposition approaches is to improve the homogeneity of the sample surface and enhance the signal intensity for the identification of biomolecules compared with direct deposition of the matrix.

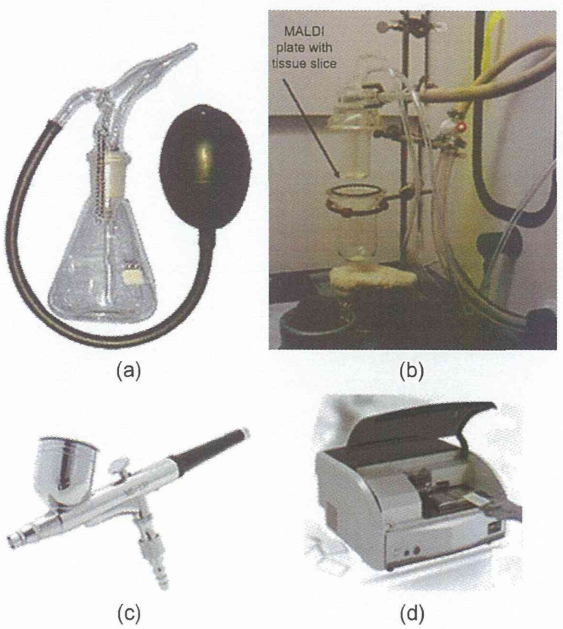

FIGURE 8 Apparatus used to deposit matrix on the tissue section. (a) Thin-layer chromatography sprayer, (b) sublimation apparatus, (c) air brush sprayer, and (d) chemical inkjet printer. Reprinted from Hankin *et al.* (2007) with permission from Springer.

4. INSTRUMENTATION

4.1. Quadrupole Mass Analyzer

A quadrupole mass analyzer is made from four parallel rods maintained at fixed direct current (DC) with an alternating radiofrequency (RF). With this setup molecular ions formed at the source pass through the middle of the quadrupoles in the electric field region and the ions of a specific m/z have a stable trajectory path and may pass all the way to the detector, while the remaining ions collide with the electrodes and never reach the detector (Gross, 2004). Using a continuous and controlled manner to change the frequency and potential, the quadropole transmits molecules at certain m/z values. Figure 9a shows a diagram of quadrupole mass analyzer. The sensitivity of the instrument can be enhanced by increasing the number of qaudupoles from two to three (triple quadrupole) in series. In triple-quadrupole analyzers, the first (Q_1) and third (Q_3) quadrupoles act as filters, and the second (Q_2) quadrupole functions as a collision cell. The third (Q_3) quadrupole is worked at normal RF/DC or in the linear ion trap (LIT) mode (Douglas *et al.*, 2005). Hopfgartner *et al.* (2009) demonstrated the fast imaging of complete rat sections using MALDI coupled with a triple-quadrupole LIT where the precursor ion mode can be used to monitor the presence of the parent drug in the tissue section.

4.2. Time-of-Flight Mass Analyzer

The TOF-MS analyzer has become valuable for direct analysis of biomolecules from tissue samples. In TOF-MS, the different masses of ions are separated based on their differences in travel time through a drift region. The lighter ions produced from the source travel faster at the end of the drift region compared with heavier ions in the tube (see Figure 9b). However, TOF-MS has disadvantages in mass accuracy, resolving power, and its inability to perform tandem MS experiments (Goto-Inoue *et al.*, 2011; Gross, 2004). This drawback has been overcome by the introduction of an orthogonal geometry (oTOF)-MS analyzer to extract pulsed ions from a continuous ion beam. Huang *et al.* (2011) investigated the use of oTOF-MS for imaging and simultaneous detection of metal and nonmetal elements in tissue section with spatial resolution of 50 μm.

Ion mobility (IM) spectrometry can also be coupled with the TOF-MS system for direct analysis of tissue samples. The instrument has oTOF-MS and is equipped with an IM spectrometer located between the quadrupole and the TOF-MS analyzer. The IM spectrometer separates ions based on their IM (i.e., their shape) and TOF-MS separates ions according to their m/z ratio in the MS (Verbeck *et al.*, 2002). Separation of structurally similar

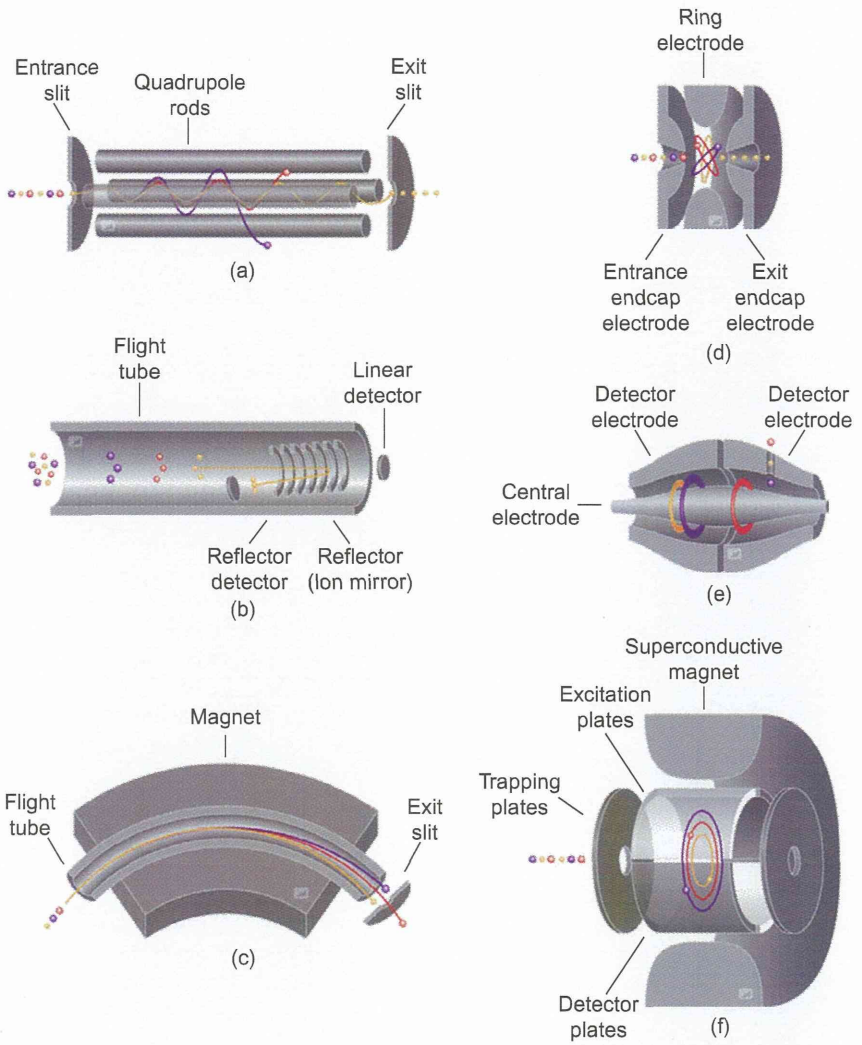

FIGURE 9 Schematic description of six mass analyzers used in mass spectrometers. (a) Quadrupole, (b) time-of-flight, (c) magnetic sector, (d) ion trap, (e) orbitrap, (f) ion cyclotron resonance. Reprinted from Pol *et al.* (2010) with permission from Springer.

ions and ions of the same charge state is thus possible through their different mobility in the IM spectrometer. The combined techniques of IM and TOF-MS were used for imaging and identification of digested proteins. IM separates isobaric ions that cannot be distinguished by MALDI-TOF alone, providing mass- and time-selected ion images of biomolecules in tissue samples (Stauber *et al.*, 2010).

In addition, the combination of a quadrupole (Q) mass analyzer with a TOF-MS is called a Q-TOF-MS system and is used for structural analysis with tandem MS. The localization of a xenobiotic substance in skin has been reported by applying a Q-TOF-MS (Bunch *et al.*, 2004). Another approach for imaging and identification of molecules is the combination of two TOF mass analyzers; this hybrid is called TOF/TOF. First, TOF-MS separates precursor ions using a velocity filter; second, TOF-MS analyzes the fragment ions (Gross, 2004). MALDI-TOF/TOF is a simple, rapid, and sensitive technique for MALDI imaging of biomolecules in tissue sections (Hayasaka *et al.*, 2010; Sugiura *et al.*, 2009).

4.3. Sector-Type Mass Analyzer

The sector mass spectrometer consists of large electromagnetic ("B" sector) and electrostatic focusing devices ("E" sector) that, depending on the different manufacturers' use, differ in their geometries (Cottrell and Greathead, 1986). The motion of the ions in the trajectory pathway depends on the strength of electric and magnetic field where each ion (m/z) travels with different speeds (see Figure 9c). Magnetic sectors are used for high-resolution elemental imaging and identification of samples in combination with dynamic SIMS. The magnetic sector and several movable detectors allow a simultaneous detection of several elements or small molecules (within a narrow mass range) with higher sensitivity. Slodzian *et al.* (1992) used a SIMS coupled with a magnetic sector double-focusing mass spectrometer for simultaneous imaging of several elements in tissue sample.

4.4. Ion Trap Mass Analyzer

A quadrupole ion trap (QIT or 3D-IT) operates in a 3D quadrupole field maintained at constant DC and RF fields to trap the moving ions of m/z range. A QIT consists of three hyperbolic-shaped electrodes: the central ring electrode and two adjacent end cap electrodes (entrance and exit) (see Figure 9d). A 3D-IT is a small, relatively inexpensive instrument for sensitive analysis; it can also be used for MS^n analysis of molecules in the tissue samples (Gross, 2004; Hopfgartner *et al.*, 2004). Shimma *et al.* (2008) reported their use of a MALDI-QIT–TOF-MS instrument for imaging of phospholipids, glycolipids, and tryptic-digested proteins. MS analyses were performed to confirm their presence. Recently a mass microscope coupled with a high-resolution atmospheric pressure-laser desorption/ionization (AP-MALDI) and QIT-TOF was used for imaging and identification of volatile substances in ginger (Harada *et al.*, 2009). This instrument allows researchers to precisely determine the

specific tissue section prior to IMS and has spatial resolution (10 μm) higher than the naked eye.

In a linear quadrupole ion trap (LIT) or 2D traps (2D-IT), the ions are trapped in a 2D quadrupole field and then pass axially. The 2D-IT ion trap produces reasonable mass accuracy, mass resolution, and sensitivity (Schwartz *et al.*, 2002). LIT has a better ion storage capacity and a higher trapping efficiency compared with 3D-IT. However, the disadvantage of LIT is the relatively narrow mass range of small molecule analysis. Garrett *et al.* (2007) described a new MALDI-LIT-MS for imaging of tissue samples and also used for MS^n analyses to confirm the molecules. Enomoto *et al.* (2011) demonstrated the visualization of phosphatidylcholine (PC), lysophosphatidylcholine, and sphingomyelin in mouse tongue using LTQ (linear trap quadrupole)-MALDI-IMS (Enomoto *et al.*, 2011).

4.5. Orbitrap Mass Analyzer

In an orbitrap mass analyzer, the ions are rotated around a central electrode by applying an appropriate voltage between the outer and central electrodes. Hence, the ions of a specific m/z ratio cycle in rings that oscillate around the central spindle and then pass through the detector (Makarov *et al.*, 2006). Figure 9e shows the overview of the orbitrap mass analyzer. LTQ-Orbitrap has been used to analyze compounds with high resolving power and excellent mass accuracy that appreciably decrease false-positive peptide identifications in the sample (Adachi *et al.*, 2006; Makarov *et al.*, 2006). Verhaert *et al.* (2010) demonstrated the use of LTQ-orbitrap for imaging of neuropeptides in neural tissue samples. In addition, it has also been used for identification and sequencing of neuoropeptides from neural tissue using MALDI-MS with an ion trap–orbitrap hybrid instrument. Landgraf *et al.* (2009) showed the high resolution and accurate measurement of ion images of lipids in spinal cord using MALDI-LIT–orbitrap-MS. Manicke *et al.* (2010) demonstrated imaging of lipids in rat brain tissue section with a high-resolving power instrument of DESI-LTQ–orbitrap-MS.

4.6. Ion Cyclotron Resonance Mass Analyzer

In an ion cyclotron resonance (ICR)-MS analyzer, the ions of a particular m/z ratio are isolated based on the cyclotron frequency of the ions in a constant magnetic field. The oscillation of ions in ICR induces an alternating current that is equivalent to their m/z ratios. Figure 9f shows the schematic for an ICR analyzer. Fourier transforms (FT)-ICR-MS continues to deliver the highest mass-resolving power and mass measurement accuracy (Gross, 2004). The combination of MALDI-TOF-MS with the FT-ICR-MS technique is useful for high–spatial resolution analysis and