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Multi-atmospheric pressure ionisation interface for liquid chromatography–mass spectrometry

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Abstract

We describe the structure of a multi-atmospheric pressure ionisation (multi-API) interface for liquid chromatography–mass spectrometry (LC–MS). This interface includes five modes of atmospheric pressure spray with electron impact ionisation (APEI), atmospheric pressure chemical ionisation (APCI), atmospheric pressure spray ionisation (APSI), electrospray ionisation (ESI) and sonic spray ionisation (SSI). This LC–MS system was realised by developing an APEI interface which resembles other API interfaces and a highly sensitive API interface using advanced collision induced dissociation in a differential pumping region and an electrostatic ion guide. This system can deal with a wide variety of organic compounds from hydrocarbons with low polarity to proteins with high polarity by using the five modes. © 1998 Published by Elsevier Science B.V.

Keywords: Interfaces, LC–MS; Multi-atmospheric pressure ionisation interface; Mass spectrometry

1. Introduction

Direct coupling between a liquid phase separation technique such as liquid chromatography (LC) and mass spectrometry (MS) has been recognised as a powerful tool for mixture analyses. However, this combination remains a challenging target for many reasons. One of those reasons is that measurable organic compounds by liquid chromatography–mass spectrometry (LC–MS) are very limited compared with separable compounds by LC alone. Our idea to overcome this difficulty was to develop plural LC–MS interfaces which could easily replace one another and for which measurable samples were complementary to one another. An LC–MS system equipped with atmospheric pressure ionisation (API) interfaces seemed to be one of the most promising candidates for this strategy. This was because several different API interfaces have been previously reported including an atmospheric pressure chemical ionisation (APCI) by Henion's and our groups [1,2], atmospheric pressure spray ionisation (APSI) by our group [3], electrospray ionisation (ESI) or ion spray (IS) by Fenn's and Henion's groups [4–6] and sonic spray ionisation (SSI) by our group [7,8]. These four ionisation methods are so-called API methods, which means that ions produced at atmospheric pressure are introduced into a mass analysing region through a differential pumping region.

In order to realise our idea, we had to develop (1) an LC–MS interface for electron impact ionisation (EI) which was similar to those available for the other API interfaces, and (2) a highly sensitive API interface with a simple structure. Organic compounds with low polarity such as hydrocarbons and aromatic compounds cannot be measured with APCI,

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APSI, ESI or SSI methods. Although one possibility to measure these compounds is a particle beam (PB) method developed by Browner et al. [9], the interface structure and pumping system of this method are very different from those of the API methods described above.

In API–MS, protonated molecules or cationised molecules are produced at atmospheric pressure and introduced into a vacuum region through sampling apertures in a differential pumping region. Charged droplets appear to be formed by clustering following adiabatic expansion in the intermediate pressure region, and these flow into the vacuum region where a mass spectrometer is located. When these droplets travel through the mass spectrometer, they are a major source of noise. This reduces the sensitivity of the instrument. Therefore, to improve the sensitivity of API–MS, it is important to eliminate the charged droplets. To accomplish this, the mass spectrometer is located far from the ion sampling aperture that introduces the ions and droplets into the vacuum region, and an ion transport system is constructed between the aperture and the mass spectrometer. Rf-only quadrupoles or octapoles are currently used for ion transport. However, a high precision configuration of the many electrodes and a rf generator are needed for these ion transport systems.

This paper reports on the multi-API LC–MS interface which realises the combination of EI mode with APCI, APSI, ESI and SSI modes in one LC–MS system, and two key techniques to provide a highly sensitive API interface. The applications of these five modes are also described.

2. Experimental section

2.1. Mass spectrometer

A Hitachi M-1200 LC–MS system with a quadrupole mass spectrometer and Hitachi M-4100 LC–MS system with a magnetic sector type mass spectrometer (Tokyo, Japan) equipped with originally designed API interfaces were used. The mass ranges of these systems are 2000 for the quadrupole type and 6000 for the magnetic sector type.

2.2. Liquid chromatograph

A Hitachi Model L-6200 HPLC system (Tokyo, Japan) was employed for all chromatographic separations described in this work that included 4.6 mm I.D. and 2.1 mm I.D. HPLC columns. A flow-rate of HPLC was set to 1 ml min^{-1} for 4.6 mm I.D. column and 0.2 ml min^{-1} for 2.1 mm I.D. column under isocratic and gradient conditions without a post column split. A Rheodyne Model 7125 injector (Cotati, CA) equipped with a 10 μl loop was used to inject samples onto a HPLC column.

2.3. HPLC separation conditions

HPLC separation condition in each mode was as follows. These conditions are summarised in Table 1.

2.3.1. APEI mode

The separation of a mixture of 30 ng each of testosterone and progesterone was accomplished using a 4.6 mm I.D.×250 mm HPLC ODS column (Hitachi, Tokyo, Japan). The eluent composition was methanol/water (90/10) at a flow-rate of 1.0 ml min^{-1}.

2.3.2. APCI mode

A mixture analysis of 100 pmol each of oxytetracycline, tetracycline, chlorotetracycline and deoxycycline was performed using a 4.6 mm I.D.× 150 mm HPLC ODS column (Hitachi, Tokyo, Japan). The eluent composition was acetonitrile–2*M* acetic acid (30:70) at a flow-rate of 1.0 ml min^{-1}.

2.3.3. APSI mode

The separation of a mixture of 100 pmol each of glucose, sucrose and raffinose was carried out on a 4.6 mm I.D.×250 mm HPLC ODS column (Hitachi, Tokyo, Japan). One hundred percent water was utilised as a mobile phase at a flow-rate of 1.0 ml min^{-1}.

2.3.4. ESI mode

An aliquot of the tryptic digests of human haemoglobin A_0 or S was loaded onto a 2.1 mm I.D.×250 mm HPLC ODS column (TOSO, Tokyo, Japan). Solvent A was 0.5% formic acid–acetonitrile (90:10); Solvent B was 0.5% formic acid–acetoni-

Table 1
HPLC separation condition in each mode

Mode	Samples	Column	Solvent
APEI mode	testosterone progesterone	4.6 mm×250 mm ODS column	methanol–water (9:1) 1 ml min^{-1}
APCI mode	oxytetracycline tetracycline chlorotetracycline deoxycycline	4.6 mm×150 mm ODS column	acetonitrile– 2 *M* acetic acid (1:3) 1 ml min^{-1}
APSI mode	glucose sucrose raffinose	4.6 mm×250 mm ODS column	water 1 ml min^{-1}
ESI mode	tryptic digests of human haemoglobin A_0 and S	2.1 mm×250 mm ODS column	gradient of 0–100% B over 60 min A; 0.5% formic acid–acetonitrile (90:10) B; 0.5% formic acid–acetonitrile (40:60) 200 μl min^{-1}
SSI mode	simazine thiuram thiobencarb	2.1 mm×250 mm ODS column	first 10 min water–methanol (60:40) gradient of 0–100% B over 10 min A; water–methanol (60:40) B; methanol (100) 200 μl min^{-1}

trile (40:60). Separation of the tryptic digests was effected with a gradient of 0–100% B over 60 min. The column effluent was directly introduced into an ESI interface without split.

2.3.5. SSI mode

Sample mixture of simazine, thiuram and thiobencarb with each sample amount of 50 pg was separated with a semi-micro LC ODS column (TOSO, Tokyo, Japan). The mobile phase was pumped at a flow-rate of 200 μl min^{-1}. The mobile phase was water–methanol (60:40) for the first ten minutes, and then a linear gradient from 60% water–40% methanol to 100% methanol over ten minutes was used for separation.

2.4. Enzymatic digestion

Human haemoglobin A_0 and S was digested with trypsin treated with L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK) for 16 h at 37°C with a substrate-to-enzyme ratio of 50:1 (w/w) in 50 m*M* ammonium bicarbonate buffer solution at pH 8.1 (the pH of the buffer solution was adjusted with 1 *M* ammonium hydroxide). The digestion solution was then lyophilised and redissolved in water containing 0.5% formic acid with an approximate sample concentration of 0.5 mg protein per milliliter solvent.

3. Results and discussions

3.1. Multi-API interface

3.1.1. APEI interface

Although an LC–MS system using EI mode has received much less attention than other methods suitable for thermolabile compounds, HPLC analysis of small and volatile compounds remains an important target. In EI mode, sample molecules must be introduced into an EI source under low pressure. If sample solutions can be efficiently nebulised at atmospheric pressure and vaporised sample molecules can be efficiently introduced into the EI source through a differential pumping region, even the interface structure for the EI mode becomes similar to those of other API methods. Here, we call this method the atmospheric pressure spray with electron impact ionisation (APEI) mode. Under this idea

described here, we designed the APEI interface as shown in Fig. 1(a).

In this mode, sample solutions are vaporised by the nebuliser consisting of a stainless steel capillary brazed to a stainless steel block and the desolvator consisting of a bored stainless steel block. The two stainless steel blocks can be heated up to 450°C and temperature controlled. These nebuliser and desolvator structures are exactly the same as those of the APCI interface that we previously reported except for a corona discharge needle. [1] The fine droplets produced are directly introduced into a low pressure region through the regions located between a sampling aperture (I.D. 0.3 mm, 30 mm long, heated to 120–130°C), a middle aperture (I.D. 0.3 mm, 0.5 mm long) and a skimmer (I.D. 0.3 mm, 0.5 mm long, heated to 120–130°C). The region between the middle aperture and the skimmer was evacuated to approximately 1 Torr using a mechanical pump. Block temperatures were usually set to 300–350°C to

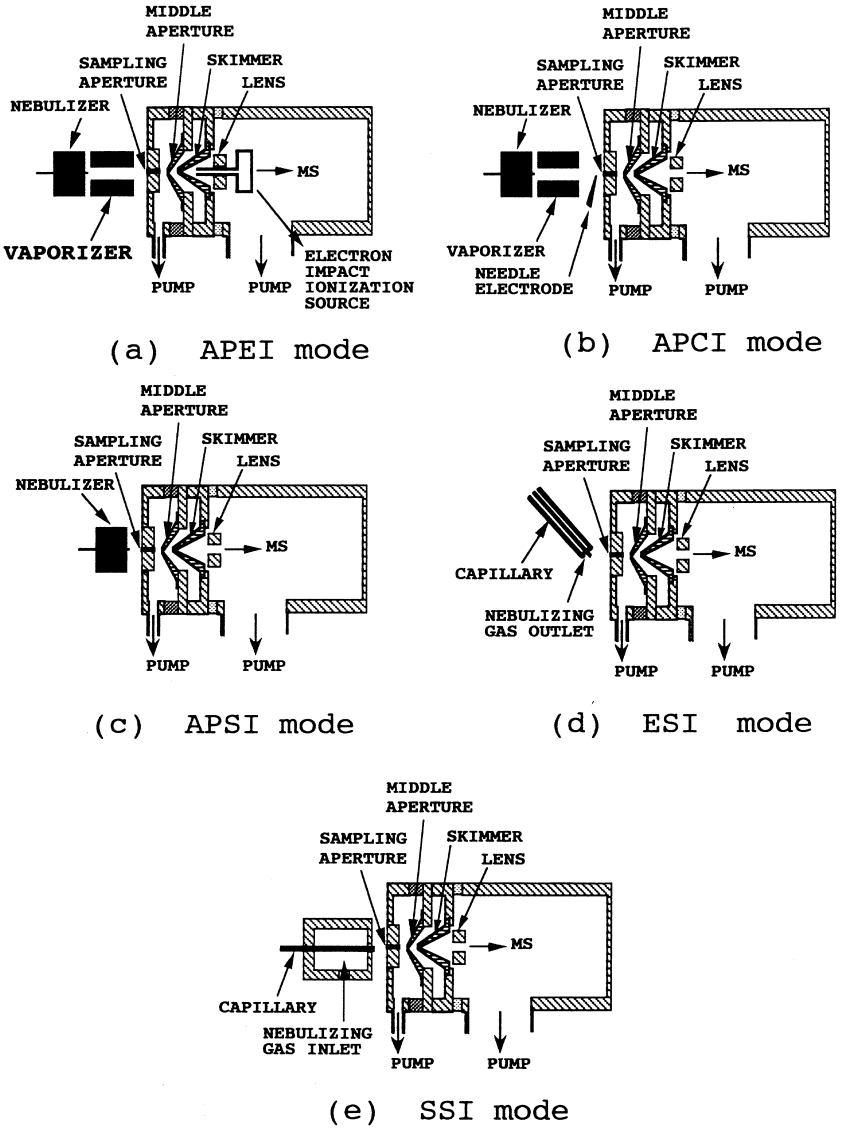


Fig. 1. The schematic diagrams of (a) APEI interface, (b) APCI interface, (c) APSI interface, (d) ESI interface, and (e) SSI interface.

obtain high ion currents of sample molecules. Vaporised sample molecules are introduced into an EI source through a heated guide glass pipe (O.D. 4 mm, I.D. 2 mm, 50 mm long), which efficiently introduces vaporised sample molecules to the EI source. The guide pipe and the EI source were heated to 150 and 250°C in experiments, respectively. The edge of the guide pipe was supported by an einzel lens for focusing ions in the other API modes. The acceleration voltage of the electron beam was set to 70 eV in normal operation. A mass analysing region was evacuated with a turbo-molecular pump to lie in the 10^{-5} Torr range.

3.1.2. Other API modes

In the other API modes (APCI, APSI, ESI and SSI), a focusing lens is set after the skimmer instead of the EI source. These interfaces can be easily replaced by one another.

The APCI technique has a lot of advantages as an LC–MS interface, which include the detection of a wide range of organic compounds and the ability to handle HPLC flows up to 1.5 ml min^{-1}. The schematic diagram of the APCI interface that we have designed is shown in Fig. 1(b) [1]. Briefly, sample solutions are vaporised by the nebuliser consisting of a stainless steel capillary brazed to a stainless steel block and the desolvator consisting of a bored stainless steel block. Vaporised sample and solvent molecules are then ionised by a corona discharge followed by ion–molecule reactions. The ions produced are introduced into a mass analysing region through a differential pumping region via the apertures. The nebuliser and the vaporiser were uniformly heated to 300–350°C by cartridge heaters.

The APSI mode was accidentally discovered during APCI experiments. Very weak ions were observed without a corona discharge in the APCI mode, which led to the discovery of the APSI mode. This mode has proven suitable for neutral saccharides, glycosides and so on, which are hard to detect with good sensitivity using the other API modes, due to the highly sensitive detection of cationised molecules of these compounds. The schematic diagram of the APSI interface is shown in Fig. 1(c) [3]. In essence, fine droplets issued from a stainless steel capillary are directly introduced into a mass analysing region through the differential pumping regions to produce protonated and/or cationised molecules of samples. The stainless steel capillary brazed to a stainless steel block can be indirectly heated by cartridge heaters and temperature controlled. The temperature of this block was set to 420°C so as to obtain high ion currents of samples.

In most reports employing pure electrospray mass spectrometry, a sample solution is simply infused at a low flow-rate of μl range through an electrospray interface. However, the electrospray interface accommodating higher flow-rate is preferable for combining an electrospray mass spectrometer with conventional 4.6 mm or 2.1 mm I.D. HPLC column, because extra column broadening becomes a serious problem in the case of a pure electrospray interface equipped with a post column split. Therefore, we have planned to apply a pneumatically assisted electrospray (or ion spray) developed by Bruins et al. [9] to the multi-API system. At a high flow-rate of over 50 μl min^{-1}, however, the conventional ion spray technique has some limitations of high chemical background in the low mass range, due to ion cluster formation and the accumulation of sample solution at a counter electrode resulting in instability of ion intensity. In order to overcome these difficulties, we redesigned the structure of ESI source [10]. The schematic diagram of this ESI interface is shown in Fig. 1(d). A HPLC column effluent traveled through a connecting Teflon tube connected with a stainless steel capillary (0.1 mm I.D.×0.3 mm O.D.) housed in the interface. High voltage (2.5–4 kV) was applied to the stainless steel 'tee' that houses the two concentric capillaries. Nitrogen nebulising gas was passed through the annular space between the inner and outer capillaries (~1.5 l min^{-1}) to effect pneumatically assisted electrospray ionisation. The capillary tip was positioned approximately 10 mm from a heated sampling aperture as a counter electrode, which consists of a stainless steel capillary (0.3 mm I.D.×1.57 mm O.D.) brazed to a stainless steel cylinder with 30 mm long. This sampling aperture was indirectly heated to 120–130°C by a ceramic heater. The axis of the capillary was set at an angle of 45 degrees against the axis of the sampling aperture, in order that large droplets on the centre of an electrospray jet were vaporised by colliding with a heated flange supporting the sampling aperture. This ESI interface structure can

provide; (A) low chemical noise due to the introduction of small droplets only from the centre of an electrospray jet to the sampling aperture and vaporisation of the droplets by heating in the sampling aperture and collision induced dissociation in a differential pumping region [1], and (B) stable ion currents due to avoidance of the accumulation of droplets at the sampling aperture. As a result of applying this electrospray interface, we have succeeded in performing a long stable operation at a high flow-rate of over 200 μl min^{-1}. In addition, it is worthwhile to describe that operation at a low flow-rate of μl range can be performed by setting the capillary on the axis of the sampling aperture. In this case, no nebulising gas is needed.

In the SSI mode, organic compounds are ionised by nebulising sample solutions using a high speed gas flow only, at room temperature. Fig. 1(e) shows a cross-sectional view of the SSI source. For measurement, the solution is pumped through the fused-silica capillary (0.1 mm I.D., 0.2 mm O.D.) at a flow-rate up to 200 μl min^{-1}. The centre axes of the fused-silica capillary and the orifice are met aligned. Nitrogen gas flows through the orifice into the atmosphere. The distance between the fused-silica capillary tip of the ion source and the sampling aperture (0.3 mm I.D., 30 mm long) is 3 mm. The sampling aperture is heated with a ceramic heater (50 W) to 120–130°C and covered with a stainless steel plate with a 2 mm aperture to avoid cooling of the sampling aperture due to gas flow and droplet evaporation.

3.2. Highly sensitive API interface

3.2.1. Advanced collision induced dissociation in differential pumping region (advanced CID^2)

Since ions are produced at atmospheric pressure in the API method, resultant ions must be introduced into a mass analysing region under low pressure to detect them through a differential pumping region. Charged droplets appear to be formed by clustering following adiabatic expansion in the differential pumping region. These are a main source of noise and reduce the sensitivity of the mass spectrometer. For this purpose, we have developed a collision induced dissociation in a differential pumping region (CID^2) [11,1,3] in which declustering is promoted by collision with neutral molecules which exist in the differential pumping region. This technique has also been applied to an electrospray mass spectrometry by Smith et al. to show great capability of declustering and fragmentation [12]. This CID technique is the most important key technology for the API method.

For more efficient declustering and fragmentation, we have developed a new CID method in a differential pumping region. We call this advanced CID^2. In the original CID^2 method, a drift voltage is applied between the sampling aperture and the skimmer for declustering and fragmentation, as shown in Fig. 2(a). In this method, however, a relatively high drift voltage is necessary due to cooling effect in a supersonic region produced after the sampling aperture. In addition, two differential pumping regions are at least needed before a mass analysing region to maintain the pressure of a mass analysing region below 5×10^{-5} Torr if we use turbo-molecular

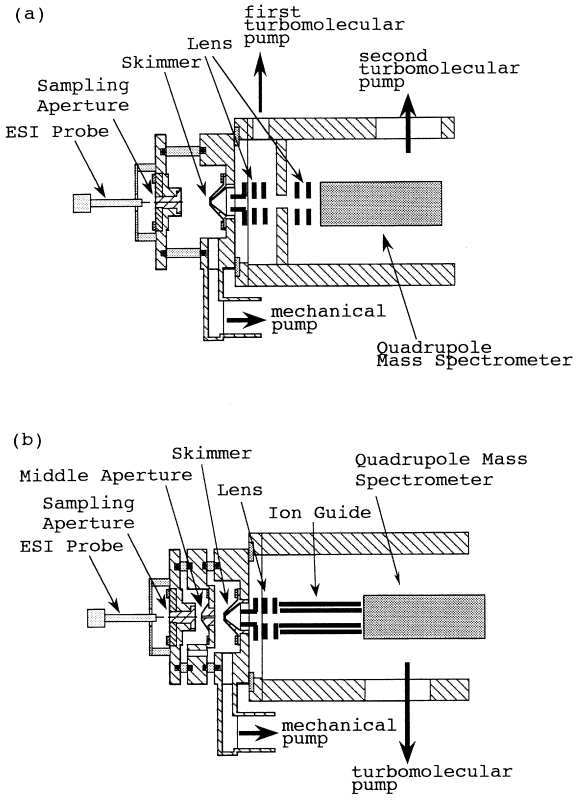


Fig. 2. The difference of ion source structure between (a) CID^2 and (b) advanced CID^2.

pumps with a pumping speed of 200–250 l s^{-1}. This results in a complicated vacuum system. In order to overcome these difficulties, we have applied a middle aperture (0.3 mm I.D.) between the sampling aperture and the skimmer for suppressing a supersonic region after the sampling aperture. Fig. 2(a) and Fig. 2(b) show the difference of ion source structure between CID^2 and advanced CID^2. This middle aperture is very effective for lowering a drift voltage or an electric field strength, for increasing a sensitivity and simplifying a vacuum system. This is confirmed by the result shown in Fig. 3 and a simplified vacuum system shown in Fig. 2(b) of one differential pumping region before the mass analysing region. Fig. 3 reveals the intensity variations of $(M+H)^+$ of tetrabutyl ammonium ($m/z=242$) obtained by the ESI mode with an electric field strength between the sampling aperture and the skimmer for CID^2 and with an electric field between the middle aperture and the skimmer for advanced CID^2. In this case, the potentials of the sampling aperture and the middle aperture were same. It is worthwhile pointing out that the intensity in advanced CID^2 is about three times higher than that in CID^2 although the intensity comes to a maximum at a lower electric field strength in advanced CID^2 than in CID^2.

3.2.2. Electrostatic ion guide

To improve the sensitivity of API–MS, it is important to eliminate the charged droplets. To accomplish this, the mass spectrometer is located far from the ion sampling aperture that introduces the ions and droplets into the vacuum region, and an ion transport system is constructed between the sampling aperture and the mass spectrometer. Recently, rf-only quadrupoles or octapoles have bben used for ion transport. However, a precise configuration of the many electrodes and an rf generator are needed for these ion transport systems.

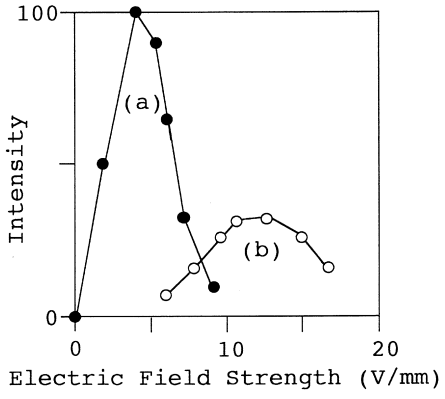


Fig. 3. The intensity variations of $(M+H)^+$ of tetrabutyl ammonium obtained by the ESI mode with (a) an electric field strength between the sampling aperture and the skimmer for CID^2 and (b) with an electric field between the middle aperture and the skimmer for advanced CID^2.

To provide easy transport of ions to a mass spectrometer, we have developed a novel electrostatic ion lens which consists of two cylindrical electrodes positioned coaxially with each other. This is called an electrostatic ion guide (EIG) [13]. The EIG consists of an inner electrode (15 mm I.D., 17 mm O.D., 211 mm long) and an outer electrode (21 mm I.D., 25 mm O.D., 211 mm long) positioned coaxially with each other, as shown in Fig. 4. Square openings (10×10 mm) are formed in the inner electrode, and their angular position alternates by 90° with respect to the preceding opening in the axial direction. The outer electrode has rectangular openings (14×4 mm). They are used to evacuate the interior of the inner electrode. This EIG is set between an einzel lens and a mass analysing region, as shown in Fig. 2(b). When a potential difference is applied between the inner and outer electrodes of the EIG, an electric field penetrates into the interior of the inner electrode through the openings. Ions introduced into the EIG can be transported and focused by the periodic electric field along the axis of the cylindrical electrodes. Several focus points exist on the centre axis of the EIG and these focus points move along the centre axis as the electrostatic potential created by the outer electrode varies. In this case, ions were transported and focused by applying −300 V to the inner electrode and 500 V to the outer.

The S/N ratios with and without the EIG is compared. Fig. 5 shows the mass chromatograms of 5 pg tetrabutyl ammonium ($m/z=242$) obtained by (a) our conventional instrument, and (b) using the EIG, respectively. Of course, the advanced CID^2 method was also used. The ESI mode was used for ionisation at a flow-rate of 200 μl min^{-1}. The S/N ratio is improved by a factor of about 4 due to noise level reduction by using the EIG although signal

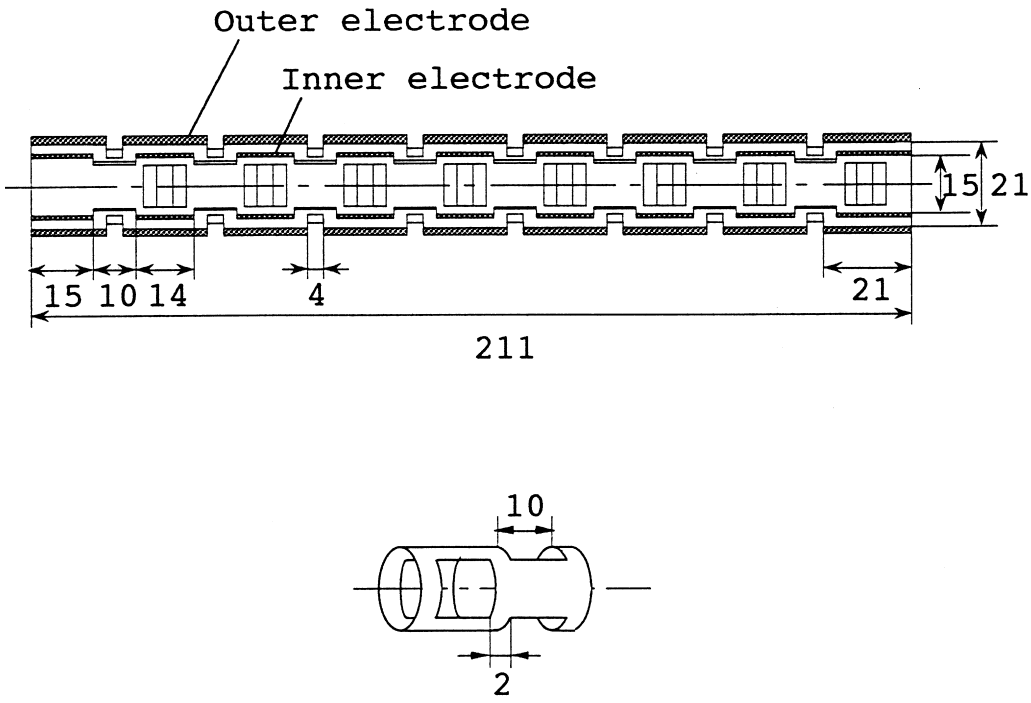


Fig. 4. The double cylindrical electrode electrostatic ion guide.

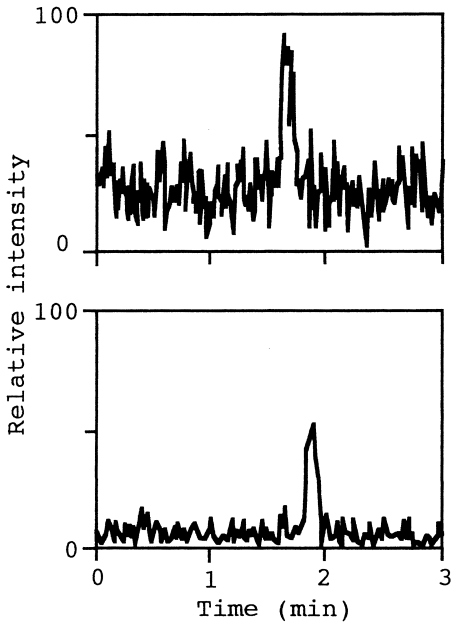


Fig. 5. Mass chromatograms of 5 pg tetrabutyl ammonium obtained by (a) our conventional instrument and (b) using the EIG.

level is not changed. These results show that the ions can be efficiently transported to the mass spectrometer by the EIG, but the charged droplets are eliminated by diffusion.

These experiments were carried out at a unit mass resolution of the quadrupole mass spectrometer. With lower resolution mode, 1 pg detection of tetrabutyl ammonium could be performed.

3.3. Applications

3.3.1. LC–MS using APEI mode

Figs. 6 and 7 shows the LC–MS total ion chromatogram (TIC) and mass chromatograms of the analysis of a mixture of testosterone (M_r 288) and progesterone (M_r 314) and the mass spectra of these compounds, respectively. The molecular ion species of these compounds can be observed with good signal to noise ratios and without chromatogram tailing. In addition, the obtained EI mass spectra are similar to those obtained by direct probe sampling. In this experiment, a sample amount of 30 ng was consumed for obtaining mass spectra by a scan

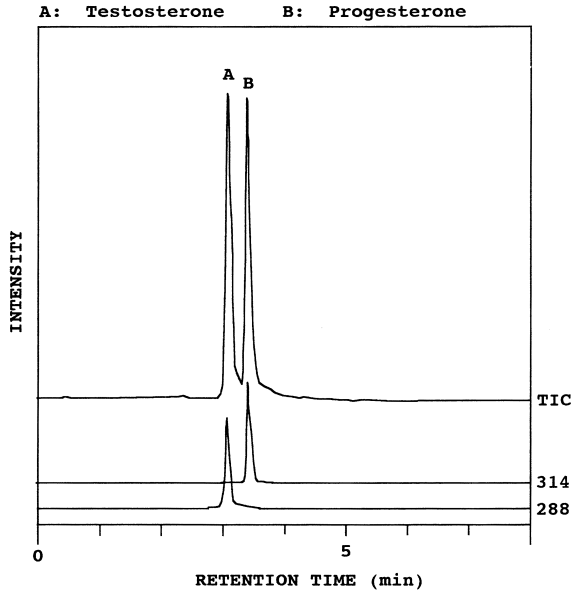


Fig. 6. The total ion chromatogram of a mixture of testosterone and progesterone and the extracted ion chromatograms at m/z 288 and 314 by the APEI mode.

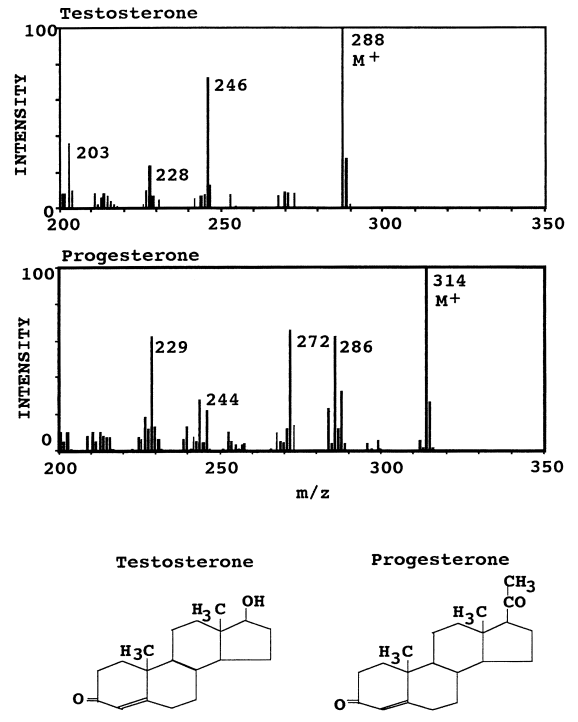


Fig. 7. The mass spectra of testosterone and progesterone by the APEI mode.

mode. However, 0.1 ng was enough for detecting by a selected ion monitoring (SIM) mode. This technique may be very useful for the analyses of hydrocarbons, aromatic compounds and other compounds with relatively high volatility and thermal stability.

3.3.2. LC–MS using APCI mode

The APCI mode is usually best suited for those applications where conventional HPLC flow-rates are preferred, because total HPLC effluent flows ranging from 0.5 to 1.5 ml min^{-1} can be directed through the APCI interface.

The TIC obtained by the analysis of a mixture of oxytetracycline (M_r 460), tetracycline (M_r 444), chloro-tetracycline (M_r 478) and deoxycycline (M_r 444) and the mass spectrum of oxytetracycline are shown in Fig. 8. In our system, sample solutions are nebulised not pneumatically but by heating to high temperatures [1]. We have succeeded in nebulising

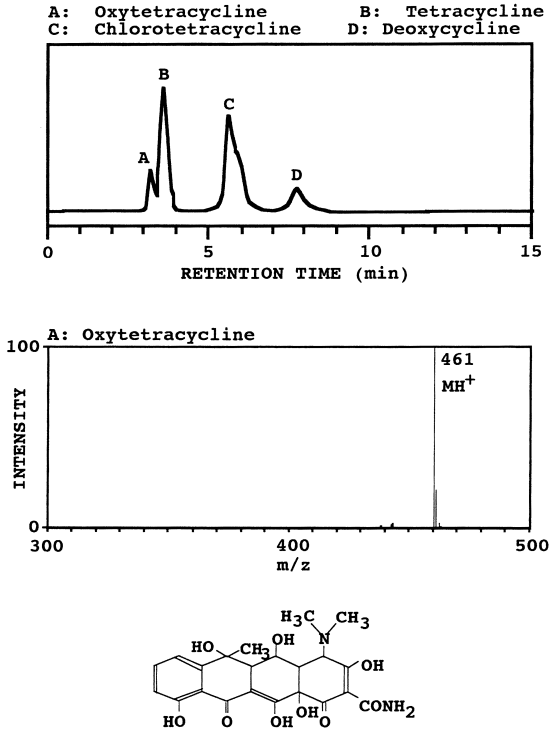


Fig. 8. (a) The total ion chromatogram of a mixture of oxytetracycline, tetracycline, chlorotetracycline and deoxycycline and (b) the mass spectrum of oxytetracycline by the APCI mode.

and analysing protonated molecules of a variety thermolabile compounds such as amino acids, alkaloids, antibiotics, steroids and small peptides which cannot be directly measured by GC–MS [1]. These experimental results have proven that the APCI mode has great ability of ionising nonvolatile and thermolabile compounds than expected.

Recently, we have found that several kinds of ion species can be observed in the APCI mode. Fig. 9 shows three mass spectra of spiramycin, glycocholic acid and salinomycin using 0.1 M ammonium acetate/acetonitrile (50/50) as a mobile phase. In spite of the same mobile phase, spiramycin, glycocholic acid and salinomycin produce different major ion species, $(M+H)^+$, $(M+NH_4)^+$ and $(M+Na)^+$, respectively. Especially, salinomycin is the first case of detecting cationised molecules by the APCI mode. Affinity between salinomycin molecules, which is a kind of ionophore, and sodium ions may be very large. These experimental results reveal that the APCI mode remains a challenging target as an LC–MS interface.

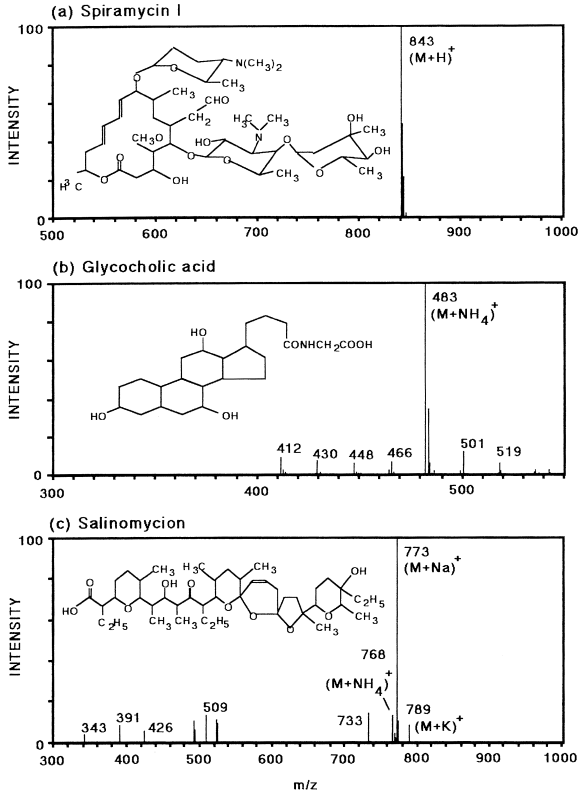


Fig. 9. The mass spectra of spiramycin I, glycocholic acid and salinomycin by the APCI mode.

3.3.3. LC–MS using APSI mode

Although the APSI interface is almost the same as a thermospray interface [14], obtained mass spectra are very different each other [3]. Many experimental results have proven that the APSI mode is very suitable for the compounds with high cation affinity such as saccharides and glycosides, which are difficult to perform highly sensitive detection by the other API modes. The APSI mode is one of the most sensitive methods for these compounds, judging from the fact that a sample amount of pmol level is sufficient for obtaining the stachyose mass spectrum by using the APSI mode. This compares with nmol levels which are needed for obtaining stachyose mass spectra using the ESI or SSI methods, and the APCI mode is not available for detecting protonated or cationised molecules of stachyose.

Fig. 10 shows the TIC and the mass spectrum of sucrose in the analysis of a mixture of glucose (M_r

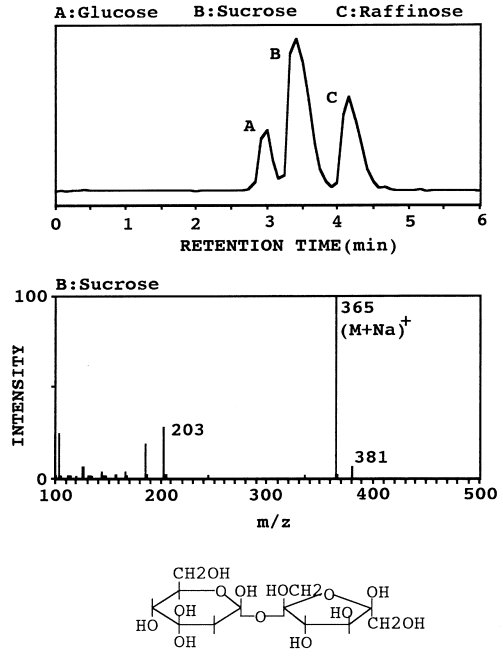


Fig. 10. (a) The total ion chromatogram of glucose, sucrose and raffinose and (b) the mass spectrum of sucrose by the APSI mode.

180), sucrose (M_r 342) and raffinose (M_r 504). It is worthwhile to describe that we can observe the fragment ions due to glycosidic bond cleavages, which are very useful for estimating saccharide sequences, in addition to cationised molecules. In the case of sucrose, the fragment ions corresponding to mono-saccharide structure (m/z=203) are obtained with the cationised molecules at m/z 365. From these ions, we can easily estimate that a sucrose molecule have the structure of hexose-hexose.

In the same way, the APSI mode can distinguish saccharides with same molecular weights. The APSI mass spectra of raffinose and maltotriose which have the same molecular weight (M_r 504) are shown in Fig. 11. Only maltotriose produces several intense fragment ions above m/z 365 such as the ions at m/z 407 although the observed fragment ions below m/z 365 are almost the same both in raffinose and maltotriose. Therefore, raffinose is distinguishable from maltotriose by the fragment ions above m/z 365. It is anticipated that the ions at m/z 407 are produced by the ring rupture of maltose molecules accompanied by sodiation ($(C_{18}H_{32}O_{16}-C_4H_8O_4+Na)^+$).

In addition, the APSI mode can be used as a rapid determination of the selectivity between ionophores and alkali metal ions [15]. Although a valinomycin mass spectrum without any alkali metal chlorides mainly shows the protonated molecule $(M+H)^+$ at m/z 1111, this ion vanishes by doping alkali metal chlorides of LiCl, NaCl, KCl, RbCl or CsCl and cationised molecules are produced at m/z 1117 ($(M+Li)^+$), m/z 1133 ($(M+Na)^+$), m/z 1149 ($(M+K)^+$), m/z 1195 ($(M+Rb)^+$) or m/z 1243 ($(M+Cs)^+$), respectively. In these mass spectra, only cationised molecules are observed and it is easy to identify ion species. Fig. 12 shows the comparison of ion intensities in valinomycin–alkali metal ion complexes. The order of the ion intensities is $(M+Rb)^+>(M+K)^+>(M+Cs)^+>>(M+Na)^+>(M+Li)^+$. Although there is a small gap between $(M+K)^+$ and $(M+Cs)^+$ intensities, this result is correlated well with the result that the stability constant of $(M+Rb)^+$ is the highest and the constants of $(M+Na)^+$ and $(M+Li)^+$ are very small compared to those of $(M+K)^+$, $(M+Rb)^+$ and $(M+Cs)^+$ [15]. For reference, the stability constants between valinomycin and alkali metals (Li^+, Na^+, K^+, Rb^+ and Cs^+) in methanol are <5, 4.7, 80 000, 180 000, and 25 000, respectively [16]. This reveals that the APSI

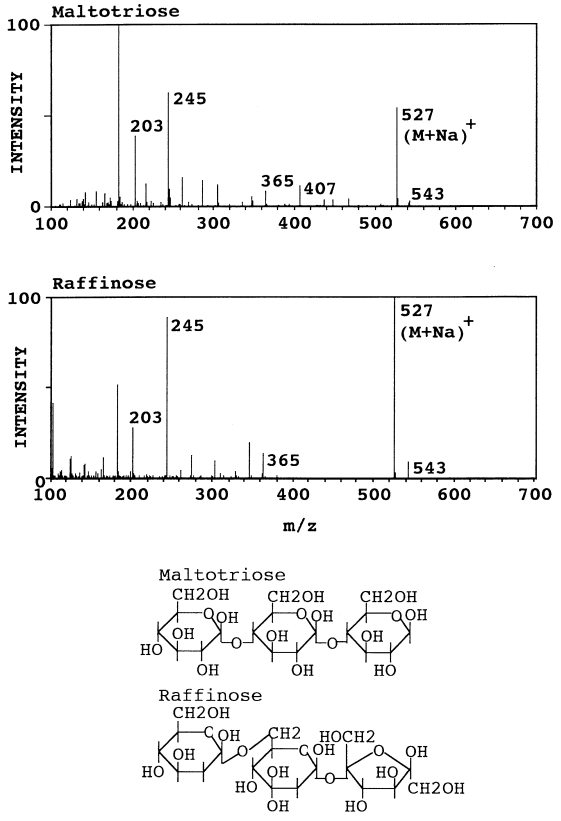


Fig. 11. The mass spectra of (a) maltotriose and (b) raffinose.

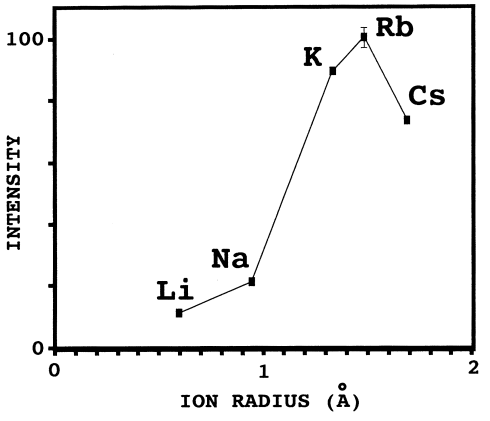


Fig. 12. Comparison of ion intensities of valinomycin–alkali metal ion complexes.

method is very useful for rapid determination of selectivity relating to ionophores.

3.3.4. LC–MS using ESI mode

As several groups have already reported, the ESI method is very powerful for analytes which exist as ions in solution or are associated with ions in solution [4,5]. Peptides and proteins especially can be measured with high sensitivity by detecting multiply charged ions of these samples. In a traditional ESI mode, optimum sensitivity can be achieved using micro HPLC flows [8], but its most analytically important use is with larger bore columns. The ESI mode proposed here can provide operations with a wide range of flow-rate from 1 $\mu l\,min^{-1}$ to 1 $ml\,min^{-1}$. Fig. 13 shows the mass spectra of cytochrome c at flow-rates of 1 and 200 $\mu l\,min^{-1}$ obtained by the newly designed interface. This result proves that electrospray mass spectra do not strongly depend on the flow-rate of solution in this interface. Therefore, both electrospray mass spectrometric experiments infusing sample solutions at a low flow-rate and LC–MS experiments using conventional 4.6 mm I.D. or 2.1 mm HPLC columns can be performed in this system.

Recently, peptide mapping using electrospray mass spectrometry has become an established and powerful tool for the structural or mutation analysis of proteins. This peptide mapping method is divided into two types; direct peptide mapping [17] and LC–MS peptide mapping [18]. Since the ESI mode described here can provide operation at low and high flow-rates, both direct peptide mapping and LC–MS peptide mapping can be easily utilised in this system. Fig. 14 shows the electrospray mass spectrum of the tryptic digests of human haemoglobin A_0. This

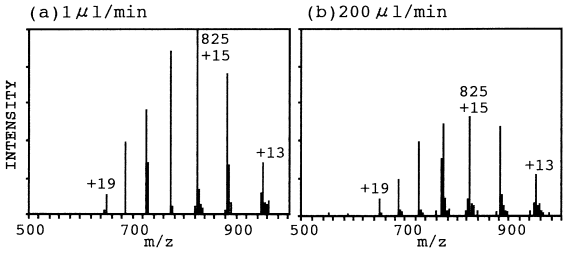


Fig. 13. Comparison of mass spectra of cytochrome c at (a) 1 and (b) 200 $\mu l\,min^{-1}$ flow-rates in the ESI mode.

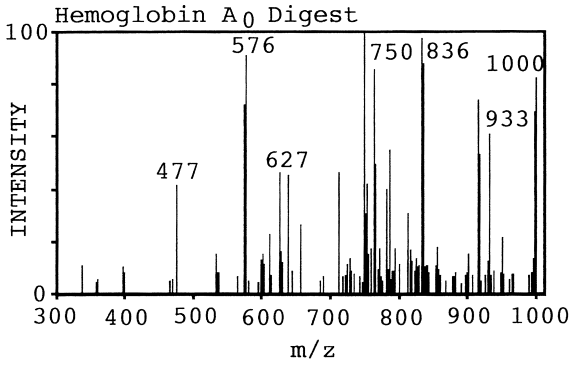


Fig. 14. Direct peptide mapping result of human haemoglobin A_0 by the ESI mode.

spectrum, shown between m/z 300 and 1000, is an average of five scans. The solution of the peptide mixture was electrosprayed at a flow-rate of 1.0 $\mu l\,min^{-1}$. The spectrum shows the presence of a large number of ion peaks, each corresponding to a tryptic digest. Ions resulting from all the tryptic fragments of human haemoglobin A_0 are observed with the exception of mono and di-peptides. However, multiply charged ions resulting from incomplete tryptic cleavage confirms the presence of these small peptides, as shown in Table 2.

We also carried out an LC–MS analysis of human haemoglobins by directly combining a semi-micro column (2.1 mm I.D. column with 250 mm long) with the mass spectrometer equipped with the ESI interface. Fig. 15 shows the mapping results to show the point mutation of sickle-cell haemoglobin (Hb-S). The disagreement between the tryptic digests of normal haemoglobin (Hb-A_0) and sickle-cell haemoglobin (Hb-S) is only one in fragment. This fragment consists of residues 1 to 8 of the beta chain; the sequence Val–His–Leu–Thr–Pro–Glu–Glu–Lys in Hb-A_0, but Val–His–Leu–Thr–Pro–Val–Glu–Lys in Hb-S. The observed molecular weight difference between the fragments of Hb-A_0 and Hb-S clearly corresponds to the mutation from glutamic acid to valine in Hb-S. These results show that peptide mapping using LC–MS is a very powerful technique for analysing mutant proteins.

3.3.5. LC–MS using SSI mode

Fig. 16 shows the analysis result from a mixture of pesticides using semi-micro LC–MS [19].

Table 2
Expected tryptic digests of haemoglobin A_0 and observed ions

Fragment	Sequence	Calculated molecular weight	Observed m/z values					
			$(M+H)^+$	$(M+2H)^{2+}$	$(M+3H)^{3+}$	$(M+4H)^{4+}$	$(M+5H)^{5+}$	$(M+6H)^{6+}$
A_1 (1–7)	VLSPADK	729						
A_2 (8–11)	TNVK	461						
A_3 (12–16)	AAWGK	532						
A_4 (17–31)	VGAHAGEYGAEALER	1530		766	511	383		
A_5 (32–40)	MFLSFPTTK	1071		537				
A_6 (41–56)	TYFPHFDLSHGSAQVK	1834		918	612	460		
A_7 (57–60)	GHGK	397		398				
A_8 (61)	K	146						
A_9 (62–90)	VADALTNAVAHVDDMPNALSALSDLHAHK	2997				750		
A_{10} (91–92)	LR	287						
A_{11} (93–99)	VDPVNFK	818	819	410				
A_{12} (100–127)	LLSHCLLVTLAAHLPAEFTPAVHASLDK	2969						
A_{13} (129–139)	FLASVSTVLTSK	1252		627				
A_{14} (140–141)	YR	337						
$B_{1\text{-}A}$ (1–8)	VHLTPEEK	952		477				
B_2 (9–17)	SAVTALWGK	932	933	467				
B_3 (18–30)	VNVDEVGGEALGR	1314		658				
B_4 (31–40)	LLVVYPWTQR	1275		638				
B_5 (41–59)	FFESFGDLSTPDAVMGNPK	2059						
B_6 (60–61)	VK	245						
B_7 (62–65)	AHGK	411						
B_8 (66)	K	146						
B_9 (67–82)	VLGAFSDGLAHLDNKLK	1670		836	558			
B_{10} (83–95)	GTFATLSELHCDK	1422						
B_{11} (96–104)	LHVDPENFR	1126						
B_{12} (105–120)	LLGNVLVCKVLAHHFGK	1720						
B_{13} (121–132)	EFTPVQAAYQK	1379		690				
B_{14} (133–144)	VVAGVANALAHK	1149		576	384			
B_{15} (145–146)	YH	318						
A_1+A_2		1171		587	391			
A_2+A_3		974		488				
A_8+A_9		3126				782	626	
$A_{10}+A_{11}$		1087		545				
$A_{12}+A_{13}$		4203						
$A_{13}+A_{14}$		1572			525			
$B_5+B_6+B_7+B_8$		2808				703	563	469
$B_6+B_7+B_8$		767	768	384				
B_8+B_9		1798			600	451		
B_9+B_{10}		3073				770	616	
$B_{10}+B_{11}$		2530				634		
$B_{11}+B_{12}$		2828					567	
$B_{14}+B_{15}$		1450	726	484	363			

Simazine, thiuram, and thiobencarb were used in this measurement and each injected component was 50 pmol. The flow-rate of the mobile phase was set at 200 $\mu l\,min^{-1}$. Since intense protonated molecules can be obtained for these compounds, we monitored these ions at m/z values of 202, 241, and 258 for simazine, thiuram, and thiobencarb, respectively. Peaks at each m/z value are clearly observed at retention times of 15, 14, and 37 min, respectively. A smaller peak corresponding to protonated molecules of thiuram combined with water molecules is also observed at 14 min.

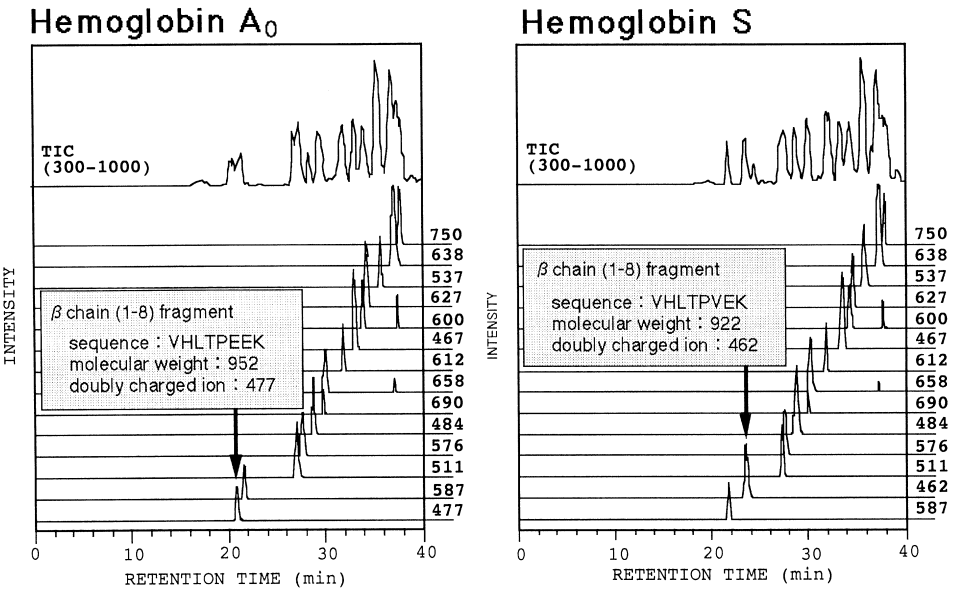


Fig. 15. The total ion chromatograms by the analysis of human haemoglobins A_0 and S digests and several extracted ion chromatograms.

Recently, it has been found that large peptides and proteins can be also measured with this method [20].

3.3.6. Fragmentation by Advanced CID²

The advanced CID^2 is very useful for fragmentation of organic compounds as well as CID^2. One example is a peptide called tuftsin. The mass spectrum at 30 V only shows singly and doubly charged ions at m/z 501 and 251, respectively. However, several fragment ions are observed if 70V is applied as a drift voltage, as shown in Fig. 17. The ions at m/z 74, 129, 164 and 272 correspond to A_1 fragment, Y_1 fragment+H–COOH, (B_3 fragment+H)/2 and Y_2 fragment+2H, respectively.

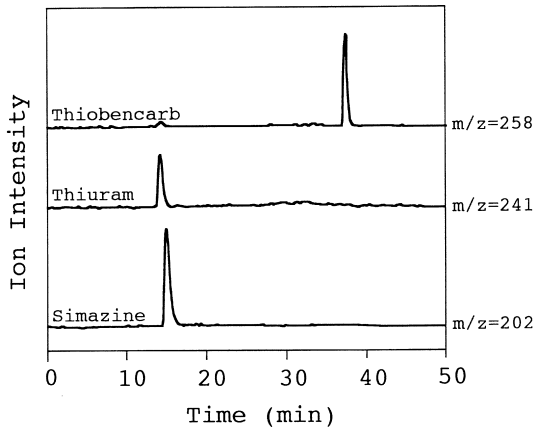
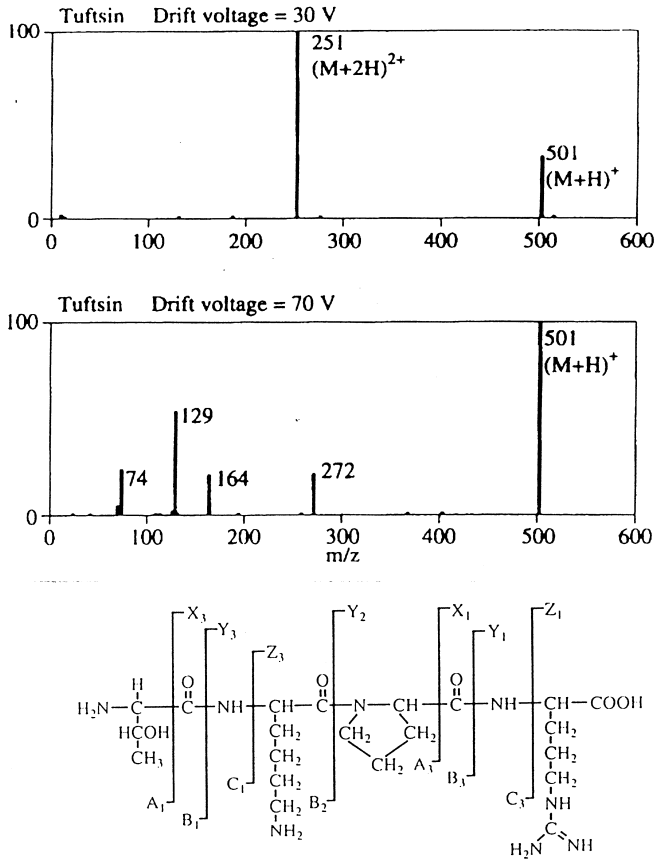


Fig. 16. LC–MS analysis of pesticides by the SSI mode.

Another example is a case of the APSI mode. Fig. 18 shows the APSI mass spectra of digitonin, which is a kind of glycoside, at drift voltages of 100 and 200 V. Although significant fragment ions are not observed in the mass spectrum at 100 V, many fragment ions are observed at 200 V. The ion at $m/z=133$ corresponds to the pentose moiety and that at m/z 163 to the hexose moiety. These ions are useful for showing the existence of pentose and hexose in a measured compound. The ions due to the loss of pentose and hexose, accompanied by protonation, are observed at $m/z=1097$ and 1067, respectively. The presence of a hexose–hexose structure is shown by the ion at m/z 325. The ions corresponding to the digitonin moiety are shown as the ions at $m/z=449$ by protonation and $m/z=471$ by sodiation, while the residual moiety is shown as the ions at $m/z=781$ and $m/z=803$ by sodiation. The ions at $m/z=611$ and 633 correspond to the hexose–digitonin structure accompanied by protonation and sodiation, respectively. As demonstrated here, sig-



Ion at m/z 74 = A1 fragment
Ion at m/z 129 = Y1 fragment + H-COOH
Ion at m/z 164 =(B3 fragment + H)/2
Ion at m/z 272 = Y2 fragment + 2H

Fig. 17. Advanced CID[2] result of tuftsin.

nificant fragment ions, which are very useful for structure elucidation, can be easily obtained by advanced CID[2].

4. Conclusions

The multi-atmospheric pressure ionisation (multi-API) interface for liquid chromatography–mass spectrometry (LC–MS) has been described. Although LC–MS development has continued for more than twenty years and a variety of interfaces have been developed, we are sure that the LC–multi-API–MS system including the APEI, APCI, APSI, ESI and SSI modes is very promising, because the five interfaces, which can be easily replaced by one another, can deal with a wide variety of organic compounds from hydrocarbons with low polarity to proteins with high polarity in a single system. It is very difficult to exactly describe what type of organic compounds can be measured by each mode, but we have an impression; (1) the APEI mode is applied to low polarity organic compounds such as aromatic compounds, (2) the APCI mode is very

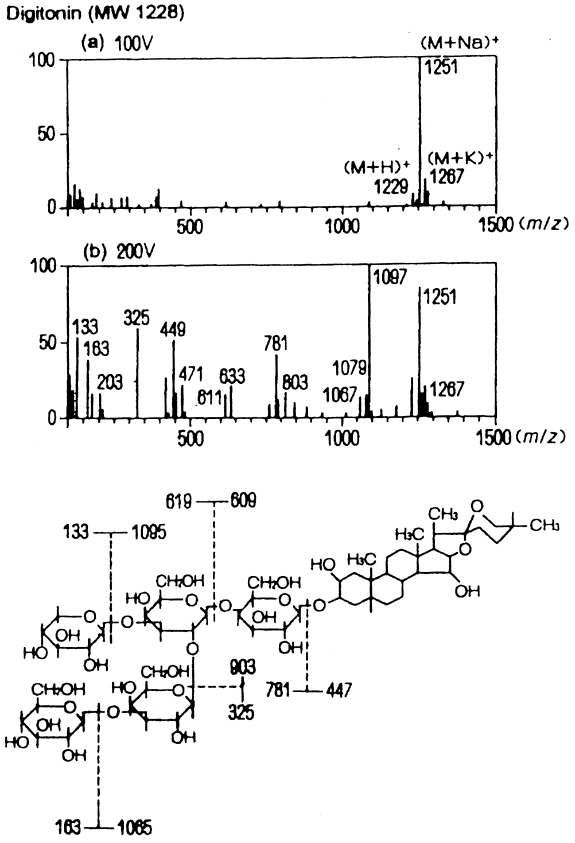


Fig. 18. Advanced CID[2] result of digitonin.

useful for relatively polar organic compounds with nitrogen and/or oxygen atoms which are neutral in solution, (3) the APSI mode is available for organic compounds with high cation affinity such as saccharides, and (4) the ESI or SSI method is very powerful for polar analytes which exist as ions in solution or are associated with ions in solution. If a lot of data relating to these five modes are accumulated, we will clarify this point further.

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