Front. Chem. China (2006) 2: 138–144
DOI 10.1007/s 11458-006-0005-1

RESEARCH ARTICLE

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Applications of HPLC-MS in compound Ilex pubescens extract study

Abstract In this paper, high performance liquid chromatography (HPLC) along with mass spectrometry (MS) and HPLC along with a diode array detector (DAD) was used to study the compound *Ilex pubescens* extract. Two ionization techniques: electro spray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were used in this work. The liquid chromatograms obtained by DAD, total ion chromatograms (TIC) from positive- and negative-ion ESI-MS and the positive- and negative-ion APCI-MS were compared. The liquid chromatograms obtained by TIC from ESI-MS provided more information on chromatographic peaks than those obtained by DAD or TIC from APCI-MS. It is suggested that the fingerprints of the compound *Ilex pubescens* extract should be provided by the liquid chromatograms obtained by DAD together with TIC from the negative-ion ESI-MS. The molecular weights of the nine main components in an HPLC-DAD chromatogram were determined by the corresponding positive- and negative-ion ESI and the positive- and negative-ion APCI mass spectra information. In the liquid chromatogram obtained by TIC from the negative-ion ESI-MS, the molecular weights of 23 main components were determined based on the corresponding positive- and negative-ion ESI mass spectra information.

Keywords high performance liquid chromatography - mass spectrometry (HPLC-MS), compound *Ilex pubescens*, Chinese medicine herb, fingerprint, active components, electro spray ionization (ESI), atmospheric pressure chemical ionization (APCI)

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1 Introduction

Chinese medicinal herbs are a rich natural resource and have been used for thousands of years in China [1], and are increasing in popularity worldwide as an alternative to the development of pharmaceuticals in therapeutic applications [2]. Studies of Chinese medicinal herbs by modern analytical methods are increasing; e.g. fingerprint studies and active components identification have been popular topics of research for many years. Further, fingerprint analysis has been introduced and accepted by the World Health Organization (WHO) as a strategy for the assessment of Chinese medicinal herbs [3,4].

Compound *Ilex pubescens* extract, a Chinese medicinal herb extract produced by the Guangzhou Children's Hospital, is formulated from *Ilex pubescens, Radix Isatidis, Follum Isatidis and Houttuynia cordata Thunb*[5]. It has been proved by clinical trials that a compound *Ilex pubescens* injection has an anti-inflammatory and detoxification effect, and has been used to cure tonsillitis, lymphadenitis, parotitis and pneumonia [5]. Compound *Ilex pubescens* extract is the most important material in a compound *Ilex pubescens* injection. Its fingerprint study and active components identification provide essential scientific data and information for compound *Ilex pubescens* injection manufacture, quality control and pharmacological studies.

HPLC-UV or DAD has been the most commonly used method for the fingerprint study of chinese medicinal herbs for many years thanks to its high separation efficiency, rapid analysis, good reproducibility and the popularization of this method [6,7]. In recent years, applications of HPLC-MS in the study of chinese medicinal herbs have been growing rapidly owing to rapid technical advances and the increasing availability of instrumentation [2]. The fingerprint study of compound *Ilex pubescens* injection by HPLC-DAD has been done earlier by Huang *et al* [8]. DAD is a selective detector, in which wavelengths from 220 to 350 nm are commonly used for fingerprint study of chinese medicinal herbs [6-8]. However, there are many active components from chinese medicinal herbs which do not

have ultraviolet absorption in that wavelength range. In such cases, an HPLC-UV or DAD fingerprint is insufficient. Atmospheric pressure ionization (API)-mass spectrometry, which commonly applies ESI, APCI and atmospheric pressure photoionization (APPI) techniques[9], can successful analyze more than 90 percent of organic compounds[10]. Further, API is the first technique to directly interface the solution phase with a mass analyzer [11], and it is very suitable to couple HPLC with MS. A comparative study of the HPLC-DAD and HPLC-MS fingerprints of compound *Ilex pubescens* extract provides much useful information and takes the most advantages of both techniques. *The technical requirements of Chinese medicine herbs injection fingerprint study*, a document made available by the Chinese State Food and Drug Administration in 2000, has a clear prescription: To establish a Chinese medicinal herb fingerprint a feasible method should be selected based on the physicochemical properties of its chemical components, and the application of a chromatography method is recommended. For the more complex of these medicinal herbs, more than one fingerprint by different analytical methods should be carried out if necessary [12]. The identification of the main active components is an important part in the fingerprint study of Chinese medicinal herbs. Due to the lack of reference substances for many natural compounds, HPLC-MS is a very feasible and useful method.

The traditional methods for identification of active components in Chinese medicinal herbs are time-consuming and expensive, because the manual operations of extraction, isolation, purification and the instrumental analysis of elementary analysis, infrared spectrometry, nuclear magnetic resonance (NMR)，MS are necessary[13]. It is difficult to isolate and purify the active components from these herbs. Sometimes, it is easy to miss the low-content active components [1]. Sometimes, the active compounds may decompose or degrade if they occur after complex chemical processes. Despite this, it was the most common method available to analyze and identify active components in Chinese medicinal herbs before HPLC-MS and HPLC-NMR techniques became widely used. The development of modern analytical sciences provides many more simple, accurate and reliable methods to analyze and identify active components in Chinese medicinal herbs. The discovery of ESI made it possible to couple MS with HPLC and to analyze complicated mixtures by the HPLC-MS system [14,15]. It is possible to determine information on chemical structure using tandem mass spectrometry techniques such as the triple quadrupole and the quadrupole ion trap [9], and it is easy to determine accurate molecular weight and molecular formula using high-resolution mass spectrometry such as time-of-flight (TOF) and the Fourier transform ion cyclotron resonance (FT-ICR)[9]. The increasing development of mass spectrometry instruments makes the HPLC-MS system more suitable and useful for the identification of active components in Chinese medicinal herbs.

2 Experimental

2.1 Materials

Compound *Ilex pubescens* extract was provided by the Guangzhou Children's Hospital (Guangzhou, China). HPLC grade methanol was purchased from Burdick and Jackson (Muskegon, USA). The water was treated with Milli-Q water purification system (Millipore, France). Other chemicals were of analytical grade.

2.2 Instrumentation and analytical methods

All analytical procedures were performed on an Agilent 1100 HPLC/MSD Trap XCT system (Palo Alto, CA, USA) equipped with a G1312A binary pump, a G1379A vacuum degasser unit, a G1313A auto-sampler, a G1315B diode array detector and a G2446A quadrupole ion trap mass spectrometry. The mass spectrometry system was equipped with ESI and APCI. A reversed phased column (Agilent, Eclipse XDB-C18, 4.6 mm×100 mm, 5 μm) was used for separation. The mobile phase consisted of two eluents. Eluent A was water and eluent B was methanol, both contained 0.5 percent formic acid. The gradient program is presented in Table 1 with a flow rate of 0.8 mL/min. The injection volume was 10 μL. DAD, it preceded the MS interface and its detecting wavelength was set at 262 nm with a reference wavelength of 360 nm.

Table 1 Composition of mobile phase with gradient elution program

Time (min)	Solvent A (%)	Solvent B (%)
0	95	5
10	95	5
20	80	20
40	70	30
50	40	60
70	30	70
75	0	100
80	0	100

Solvent A: water contains 0.5 percent formic acid; Solvent B: methanol contains 0.5 percent formic acid.
While using ESI, two thirds effluent from the HPLC column was diverted to waste and the remainder diverted to the ion source.,The ESI operation conditions were: nebulizer pressure 35 psi, drying N_2 gas flow 8 L/min, drying N_2 gas temperature 350°C. While using APCI, all the effluent from the HPLC column was diverted to the ion source. The APCI operation conditions were: nebulizer pressure 60 psi, drying N_2 gas flow 7 L/min, drying N_2 gas temperature 350°C, vaporizer temperature 400°C, corona current 4 μA in the positive-ion mode, whereas 20 μA was in the negative-ion mode. Other mass spectrometry operation conditions were as follows: capillary voltage 3500 V, MS scan ranges from 50 to 1200 m/z, trap target mass 350 m/z,

trap drive level 100 %, trap maximum accumulation time 100 ms. An auto-MS^n mode was used to acquire MS/MS daughter ions of the three most abundant mother ions with a resonance excitation voltage ranging from 0.3 V to 2.0 V.

3 Results and discussion

3.1 Comparison of the liquid chromatograms obtained by DAD, TIC from ESI-MS and APCI-MS

The comparison of the liquid chromatograms of compound *Ilex pubescens* extract obtained by DAD, TIC from positive-and negative-ion ESI-MS,positive-and negative-ion APCI-MS are shown in Fig. 1. As can be seen, there are 13 obvious chromatographic peaks in the liquid chromatogram obtained by DAD. They are important characteristic peaks of the HPLC-DAD fingerprint of the compound *Ilex pubescens* extract. The chromatographic peaks whose retention times are less than the third minute have serious interferences and are eluted together, that is proved by the corresponding ESI and APCI mass spectra information, and they are too insignificant for our study. The profile of the liquid chromatogram obtained by DAD is different with that obtained by TIC from positive- or negative-ion ESI-MS and positive-or negative-ion APCI-MS.The liquid chromatograms obtained by TIC from ESI-MS provide more chromatographic peaks information both in the positive- and negative-ion modes. The retention times of the main chromatographic peaks obtained by DAD are different from the TIC from ESI-MS and APCI-MS. The corresponding ESI-MS and APCI-MS response results of chromatographic peaks No.1 to No.13 obtained by DAD are shown in Table 2. As can be seen, peaks No.4, No.5 and No.13 showed a good response by DAD but no response by either ESI-MS or APCI-MS, peaks No.6 and No.7 have responded to positive- and negative-ion ESI-MS, while there was no response by positive- and negative-ion APCI-MS, peak No. 3 has a response by negative-ion ESI- and APCI-MS while there was no response by positive-ion ESI- and APCI-MS. There are no obvious chromatographic peaks in the liquid chromatogram obtained by DAD after peak No.13, while there are still obvious chromatographic peaks in the corresponding chromatograms obtained by TIC from ESI-MS, both in the positive- and negative-ion modes.

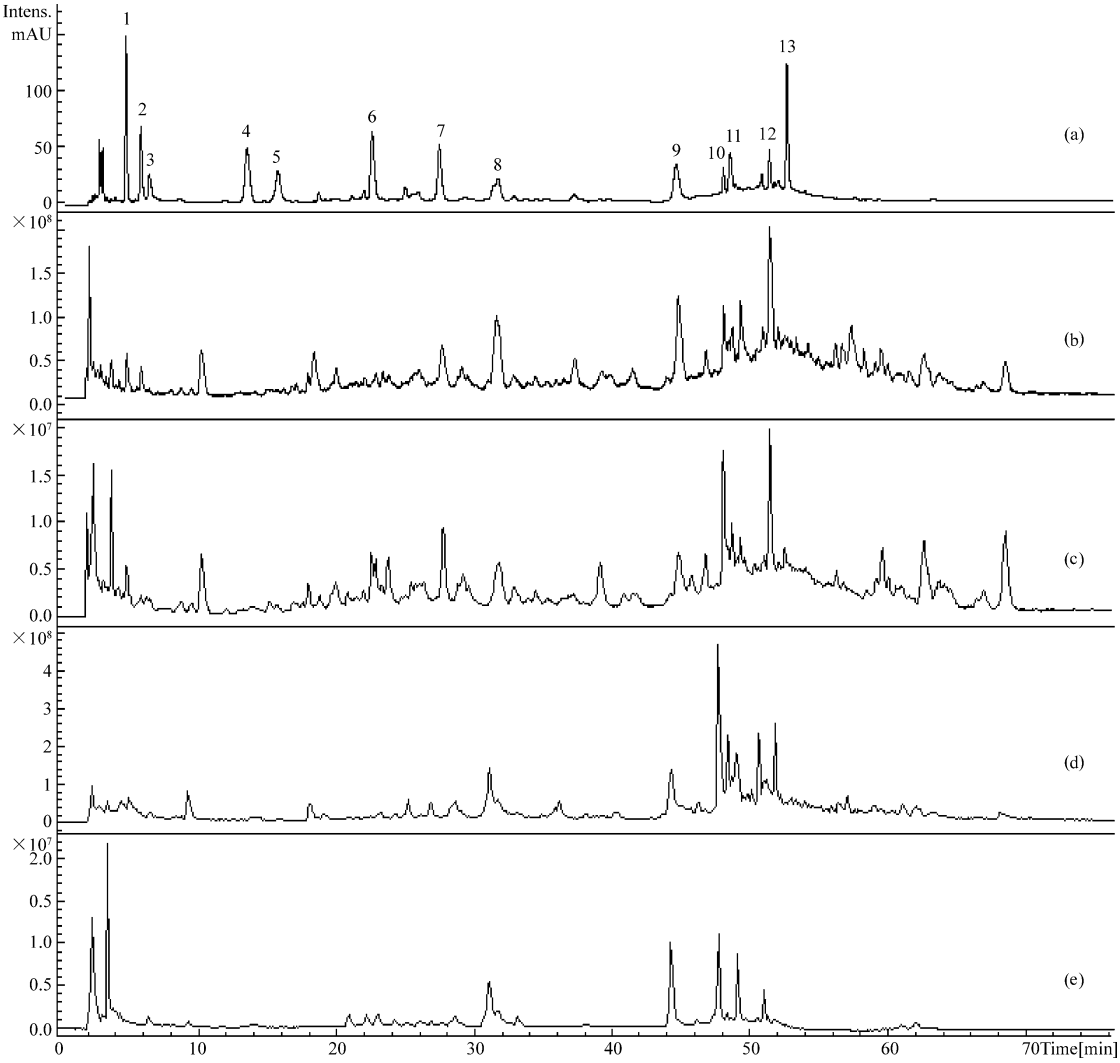

Fig. 1 The liquid chromatograms obtained by DAD (a), TIC from positive- ion ESI-MS (b), negative-ion ESI-MS (c),positive-ion APCI-MS (d) and negative-ion APCI-MS (e) of compound *Ilex pubescens* extract

Table 2 The ESI-MS and APCI-MS response results and molecular weights of chromatographic peaks No.1 to No.13 in the liquid chromatogram obtained by DAD of compound *Ilex pubescens* extract

HPLC-DAD chromatographic peaks	Positive-ion ESI	Negative-ion ESI	Positive-ion APCI	Negative-ion APCI	Component molecular weight
1	+	++	+	+	244
2	++	+	+	−	267
3	−	+	−	++	200
4	−	−	−	−	/
5	−	−	−	−	/
6	+	++	−	−	462
7	++	+	−	−	372
8	+++	++	+++	+++	339
9	+++	++	+++	+++	452
10	++	+++	+++	+++	580
11	++	++	++	+	580
12	+++	+++	+	++	194
13	−	−	−	−	/

+: weak response; ++: normal response; +++: strong response; −: no response; /: cannot be calculated

The fingerprint study provided essential scientific data and information for the modernization of Chinese medicinal herbs. An HPLC-DAD or HPLC-UV method is necessary in these fingerprint studies because of its stability and reproducibility which cannot be attained by an HPLC-ESI-MS or an HPLC-APCI-MS method. It is well known that there are many natural organic acids, alkaloids and glycosides in Chinese medicinal herbs. These compounds respond well to detection by ESI- or APCI-MS, but may not respond to DAD. In this work, an HPLC-ESI-MS method is more suitable than an HPLC-APCI-MS method because it provides more componential information. A negative-ion HPLC-ESI-MS method is more suited to a fingerprint study because its matrix interferences are less than in a positive-ion method. It is suggested that the fingerprints of compound *Ilex pubescens* extract should be provided by the liquid chromatograms obtained by DAD together with TIC from negative-ion ESI-MS.

3.2 Determination of the molecular weights of main components

3.2.1 Determination of the molecular weights of main chromatographic peaks in the liquid chromatogram obtained by DAD

In this work, the molecular weights of nine main components in the liquid chromatogram obtained by DAD were determined by corresponding positive- and negative-ion ESI, positive- and negative-ion APCI mass spectra information (Table 2.).

As an example, Fig. 2 shows the corresponding mass spectra information of peak No. 9 in the liquid chromatogram obtained by DAD. In the ESI mass spectra, $[M + H]^+$ and $[M + Na]^+$ ions are observed in the positive-ion mode, while $[M + Cl]^-$, $[M + HCOO]^-$and $[M + H_2O+HCOO]^-$ ions are observed in the negative-ion mode. In the APCI mass spectra, only the $[M + H]^+$ ion is observed in the positive-ion mode, while $[M + HCOO]^-$ is observed in the negative-ion mode. The molecular weight of the component of peak No. 9 is calculated to be 452.

The identification of the main components is an important part in the fingerprint study of Chinese medicinal herbs. Determining molecular weights is the first step. The next step is to infer chemical structures by the mass spectra information of MS/MS or MS^n and confirm this by reference substances. This part of the work, which is complicated, is in process.

3.2.2 Determination of the molecular weights of main chromatographic peaks in the liquid chromatogram obtained by TIC from negative-ion ESI-MS

As discussed in 3.1, a negative-ion HPLC-ESI-MS method is suitable for a fingerprint study of compound *Ilex pubescens* extract. It is important to identify the components of the main chromatographic peaks in the chromatogram obtained by TIC from negative-ion ESI-MS.

As can be seen from Fig. 3a, there are 23 obvious chromatographic peaks in the liquid chromatogram obtained by TIC from negative-ion ESI-MS. Their corresponding positive-ion chromatographic peaks are shown in Fig. 3b. The corresponding positive- and negative-ion ESI mass spectra information together with the molecular weights of the 23 obvious chromatographic peaks in the liquid chromatogram obtained by TIC from negative-ion ESI-MS are shown in Table 3. The positive-ion ESI mass spectra information commonly includes $[M + H]^+$, $[M + NH_4]^+$, [M

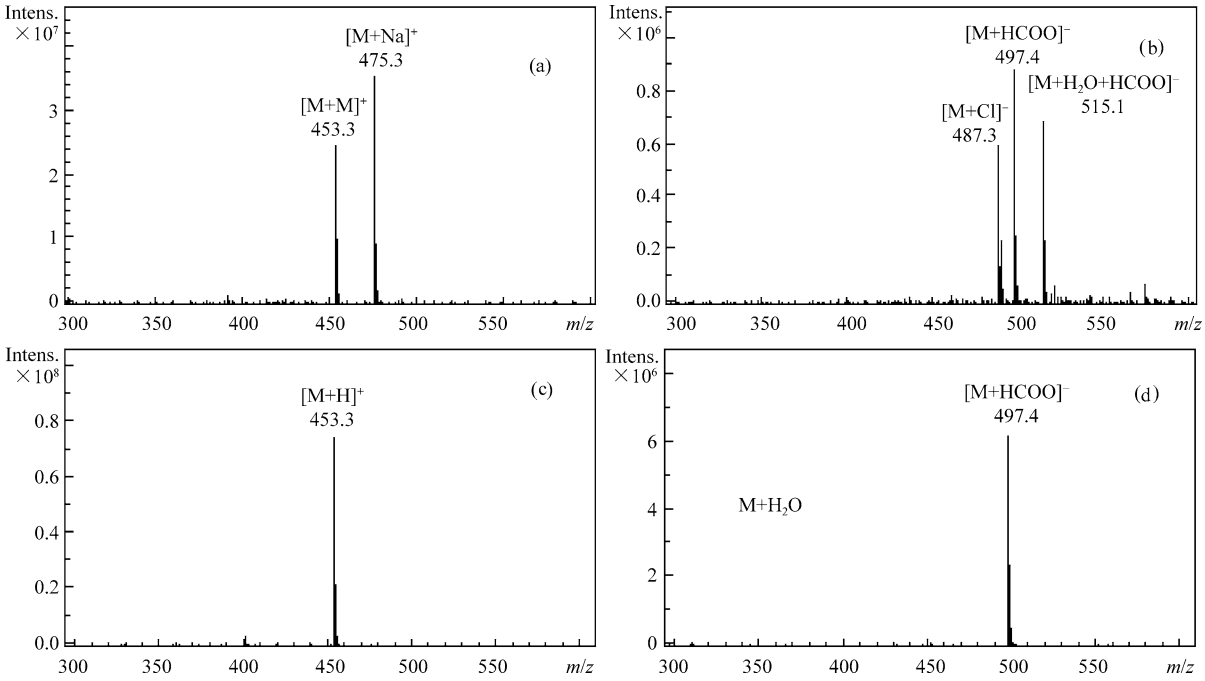

Fig. 2 The positive-ion ESI (a), negative-ion ESI (b), positive-ion APCI (c) and negative-ion APCI (d) mass spectra of peak No. 9 in the liquid chromatogram obtained by DAD of compound *Ilex pubescens* extract

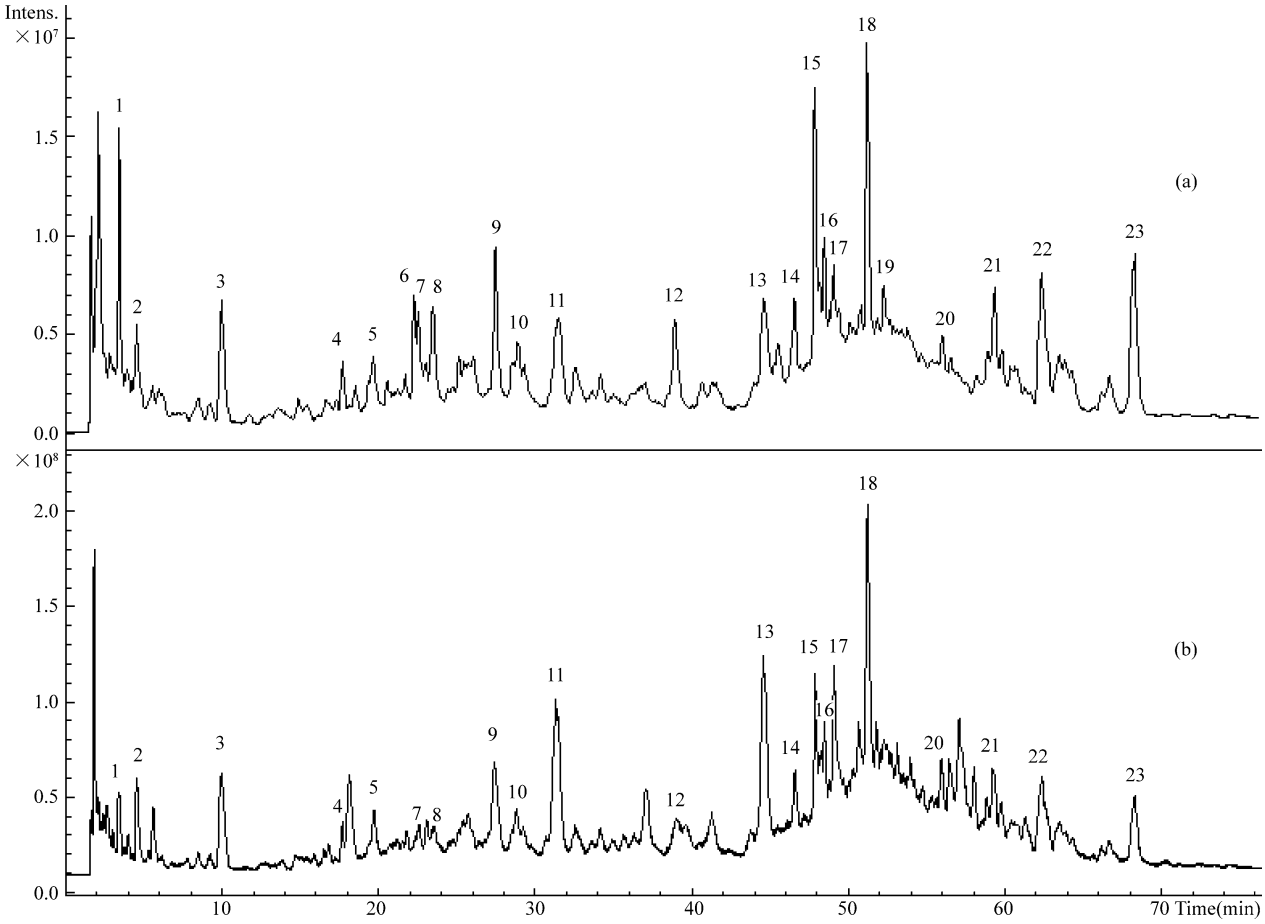

Fig. 3 The liquid chromatograms obtained by TIC from negative-ion ESI-MS (**a**) and positive-ion ESI-MS (**b**) of compound *Ilex pubescens* extract

+ Na]$^+$ and [M + K]$^+$ ions, and cluster ions of [2M + H]$^+$, [2M + NH_4]$^+$, [2M + Na]$^+$ and [2M + K]$^+$ which are observed in some chromatographic peaks. In the negative-ion mode, mass spectra information commonly includes [M - H]$^-$, [M + Cl]$^-$ and [M + CH_3COO]$^-$ ions, and the corresponding cluster ions are also observed in some chromatographic peaks. As examples, the positive- and negative-ion ESI mass spectra of peak No.9 and No.18 are shown in Fig.4.

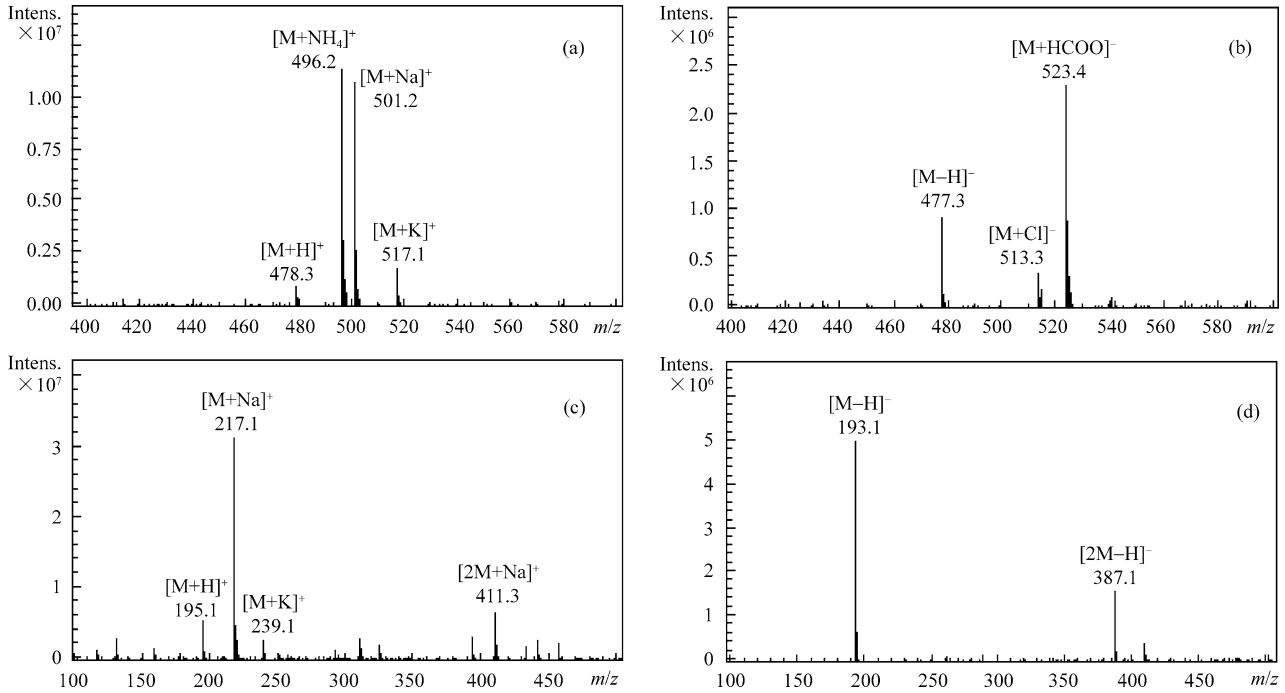

Fig. 4 The positive-(a), negative-ion(b) ESI mass spectra of peak No. 9 and positive-(c), negative-ion(d) ESI mass spectra of peak No. 18 in the liquid chromatogram obtained by TIC from negative-ion ESI-MS of compound *Ilex pubescens* extract

Table 3 The positive- and negative-ion ESI mass spectra information and the molecular weights of 23 chromatographic peaks in the liquid chromatogram obtained by TIC from negative-ion ESI-MS of compound *Ilex pubescens* extract

Peak No.	R. T. (min)	Positive-ion ESI				Negative-ion ESI			Molecular weight
		[M + H]$^+$	[M + NH_4]$^+$	[M + Na]$^+$	[M + K]$^+$	[M – H]$^-$	[M + Cl]$^-$	[M+HCOO]$^-$	
1	3.41	281	—	303*	319	279*	315	325	280
2	4.50	—	424	429*	445	—	441	451*	406
3	9.93	—	282	287*	303	—	299	309*	264
4	17.66	—	414*	419	435	395	431	441*	396
5	19.60	397	414*	419	435	395	431	441*	396
6	22.21	463	480*	485	501	461*	497	—	462
7	22.52	—	466*	471	487	447*	483	—	448
8	23.43	—	464*	469	485	445*	481	—	446
9	27.42	479	496*	501	517	477	513	523*	478
10	28.81	493	510*	515	531	—	527	537*	492
11	31.45	403	420*	425	441	401*	437	447	402
12	38.87	—	600	605*	621	581*	617	—	582
13	44.52	453	—	475*	—	—	487	497*	452
14	46.50	—	614	619*	435	595*	—	—	596
15	47.80	—	598	603*	619	579*	—	—	580
16	48.41	—	598	603*	619	579	—	625*	580
17	49.00	566	—	588*	—	—	600	610*	565
18	51.13	195	—	217*	239	193*	—	—	194
19	52.22	—	428	433*	—	409	—	455*	410
20	55.92	—	—	441*	—	—	453	463*	418
21	59.26	—	—	351*	—	327*	—	—	328
22	62.30	—	348	353*	—	329*	—	—	330
23	68.20	—	682	687*	—	663*	—	—	664

The ions with * are in the highest intensity; —: no response or very weak response

In positive-ion ESI mass spectra, a majority of the main chromatographic peaks have a high intensity $[M + Na]^+$ ion while low intensity $[M + H]^+$ ion. In the mass spectra of negative-ion ESI, a majority of main chromatographic peaks have a high intensity $[M - H]^-$ or an $[M + CH_3COO]^-$ ion. It can be inferred that they are mo.st likely glycosides or natural organic acids. In many cases, glycosides have high intensity $[M + Na]^+$ and $[M + K]^+$ ions, while they may display low intensity or even no $[M + H]^+$ ion in the positive-ion ESI-MS mode, and have high intensity $[M - H]^-$ or an $[M + CH_3COO]^-$ ion in the negative-ion ESI-MS mode. If a substance has more glucosidic-linkages, the higher intensity $[M + Na]^+$, $[M + K]^+$ ions and lower intensity $[M + H]^+$ ion are observed in the positive-ion ESI mass spectra.

The active components identification provides an important scientific basis and data for the pharmacologicalal study of Chinese medicine herbs. Glycosides and natural organic acids are the common active components obtained from Chinese medicinal herbs. The next important steps are referencing corresponding study reports, inferring chemical structures by corresponding MS/MS or MS^n mass spectra information and confirming by reference substances.

4 Conclusions

HPLC-DAD, HPLC-ESI-MS and HPLC-APCI-MS methods were developed to study the compound *Ilex pubescens* extract. The HPLC-ESI-MS method provides more chromatographic peaks information than the HPLC-DAD and the HPLC-APCI-MS methods. A negative-ion HPLC-ESI-MS method was suggested to study the fingerprints of compound *Ilex pubescens* extract together with an HPLC-DAD method. It is easy to determine the molecular weights of the main components by corresponding positive- and negative-ion mass spectra information, and it is possible to determine molecular structural information based on the corresponding MS/MS or MS^n mass spectra information. All these provide essential analytical methods for increasing the information regarding Chinese medicinal herbs.

Acknowledgement The compound *Ilex pubescens* extract was provided by the Guangzhou Children's Hospital, Guangzhou, China.

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