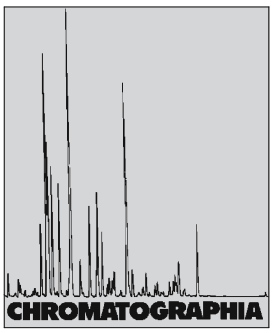
Mass Spectrometric Identification of Multihydroxy Phenolic Compounds in Tibetan Herbal Medicines



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Abstract

A highly selective and accurate method based on derivatization with dansyl chloride coupled with liquid chromatography–mass spectrometry has been developed for identification of natural pharmacologically active phenolic compounds in extracts of *Lomatogonium rotatum* plants (Tibetan herbal medicine) obtained by solid-phase extraction. The number of hydroxyl groups on the dansylated phenols was estimated by LC–MS–MS analysis in positive-ion mode. Dansyl derivatization of the compounds introduced basic secondary nitrogen into the phenolic core structures and this was readily ionized when acidic HPLC mobile phases were used. MS fragmentation of the derivatives generated intense protonated molecular ions of m/z $[MH]^+$ (phenol aglycones were transformed into the corresponding free phenols by cleavage of an aglycone bond). Collision-induced dissociation of the protonated molecule generated characteristic product ions of m/z 234 and 171 corresponding to the protonated 5-(dimethylamino)naphthalene sulfoxide and 5-(dimethylamino)naphthalene moieties, respectively. Selected reaction monitoring based on the m/z $[MH]^+$ to 234 and 171 transitions was highly specific for these phenolic compounds. Characteristic ions with m/z values of $[MH - 234]^+$, $[MH - 2 \times 234]^+$, and $[MH - 3 \times 234]^+$ were of great importance for estimation of the presence of multihydroxyl groups on the phenolic backbone.

Keywords

Column liquid chromatography–mass spectrometry
Tibetan herbal medicines
Phenolic compounds
Dansyl derivatization

Introduction

In the pharmaceutical industry, samples often start as complex matrices containing many compounds, and powerful analytical tools are required for rapid separation and characterization of the components. Liquid chromatography–tandem mass spectrometry (LC–MS–MS) has proved to be a powerful tool for separation and identification of the many components of herbal medicines and has been widely accepted as a useful method for identification of compounds in extracts obtained from plants [1, 2].

Lomatogonium rotatum (*L. rotatum*), a member of the *Lomatogonium* family of the Gentianaceae which grows on the Qinghai–Tibetan Plateau, is used to treat liver, gall, and bladder diseases [3]. Several other biological properties have also been reported, including anti-allergenic, anti-depressant, anti-inflammatory, anti-virus, and hepatoprotective activity [4]. Previous studies of *L. rotatum* have shown that the major pharmaceutically active components of the plant are multihydroxy phenols, for example xanthones and flavones [5–7]. The roots, leaves, and flowers of the plants are known to contain several multihydroxy phenolic compounds (sometimes called flavonoids). A large amount of phenolic compounds is essential for the development and normal functioning of a living organism, because of the anti-oxidant properties of the compounds.

Traditionally, reversed-phase liquid chromatography and capillary electrophoresis have been used for effective separation of many components for drug discovery [8]. Over the past decade applications of high-performance liquid chromatography (HPLC) have dramatically expanded into almost every area of chemical and biochemical research. Separation and determination of multi-

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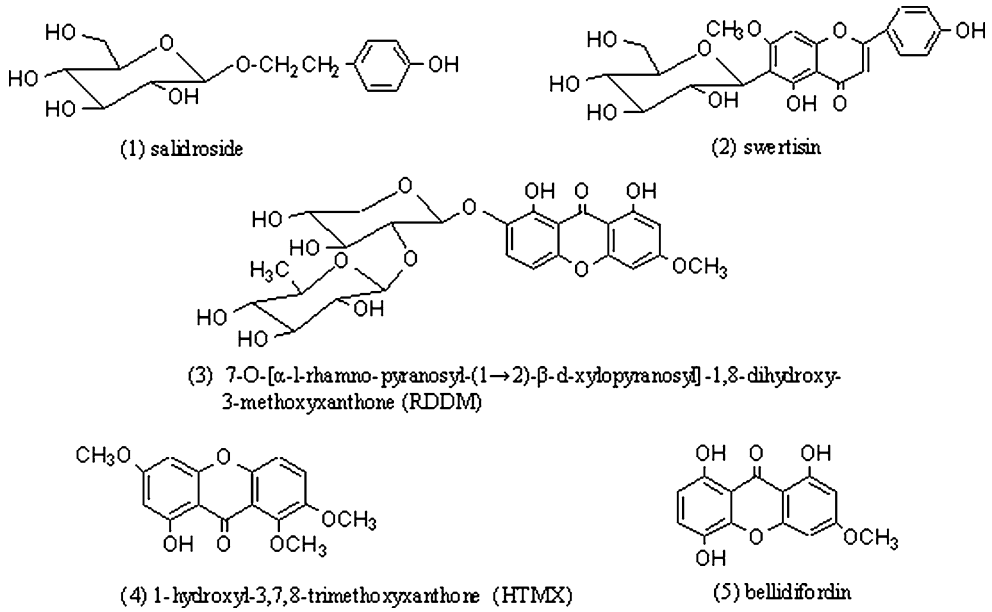

Fig. 1. The structures of the compounds investigated

phenolic compounds, components extracted from *L. rotatum* samples, have been reported [9, 10]. Many papers have reported methods for identification of phenolic compounds by tandem mass spectrometry [11–18]. Liquid chromatography coupled with electrospray ionization (ESI) has recently been used for identification of phenolic compounds in strawberries [19]. Shortcomings of these methods have also been reported, however. One possible drawback of LC–MS–MS analysis of free phenolic compounds in plant extracts is the low ionization efficiency of the compounds in ESI or APCI sources. Analysis of free phenolics by LC–MS–MS also usually gives information about molecular ions, with few fragment ions, so accurate estimation of the number of hydroxyl groups on the phenolic core structure is usually difficult.

The purpose of this work was to develop a simple aqueous-phase derivatization procedure for labeling of the hydroxyl functional groups of phenolic compounds. Fundamental research on multihydroxy phenolic compounds in extracts of *L. rotatum* provides basic information for quality assurance. For analysis of the phenolic compounds and determination of the number of hydroxyl groups in the molecules by MS it was necessary to introduce a highly ionizable group into the compounds. We therefore introduced the highly ionizable 5-(dimethylamino)naphthalene moiety into the phenolic core structure, because this group is readily ionized in the acidic mobile phases commonly used for HPLC. The spectra of the derivatives contained an intense molecular ion signal at m/z $[MH]^+$ when APCI–MS was performed in positive-ion mode. Selected reaction monitoring based on the transitions m/z $[MH]^+$ to m/z 234 and m/z 171 was highly specific for phenolic compounds. The abundant characteristic ions were valuable information for elucidation of the number of hydroxyl functional groups in the phenolic core structure.

Experimental

Chemicals and Reagents

Sodium hydroxide was purchased from Jining Chemical Reagent (Shandong, China). HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Formic acid, sodium biphosphate, and sodium bicarbonate (used to prepare buffers) were of analytical grade and purchased from Jinning Chemical Reagent. Dansyl chloride [5-(dimethylamino)-1-naphthalenesulfonyl chloride] of HPLC purity (>99.0%) was obtained from Sigma–Aldrich (St Louis, MO, USA). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Other reagents used in this study were of analytical grade unless otherwise stated.

Standards of salidroside (SA, **1**), swertisin (SW, **2**), 7-*O*-[α-L-rhamnopyranosyl-(1 → 2)-*β*-D-xylopyranosyl]-1,8-dihydroxy-3-methoxyxanthone (RX, **3**), 1-hydroxyl-3,7,8-trimethoxyxanthone (HT, **4**), and bellidifolin (BE, **5**) were kind gifts from Mr Li (Northwest Plateau Institute of Biology, Chinese Academy of Sciences, PR China). The purity of the standards was >99.0% [checked by liquid chromatography with diode–array detection (DAD) at 234 nm]. The molecular structures of the compounds are shown in Fig. 1.

All solvents were of analytical-grade from Shanghai Chemical Reagent (Shanghai, China). Before use all solutions were filtered through a 0.2-μm Nylon membrane (Alltech, Deerfield, IL, USA) and degassed.

Preparation of Standard Solutions

The derivatization solution, 1.0×10^{-3} mol L^{-1}, was prepared by dissolving 5.4 mg dansyl chloride in 20 mL anhydrous acetonitrile prepared by distillation of HPLC-grade acetonitrile dried over P_2O_5.

Individual stock solutions (1.0×10^{-3} mol L^{-1}) of the phenolic compounds were prepared in 50% aqueous acetonitrile; if necessary, 0.1 mol L^{-1} sodium bicarbonate (pH 10.5) was added until the compound dissolved. Individual

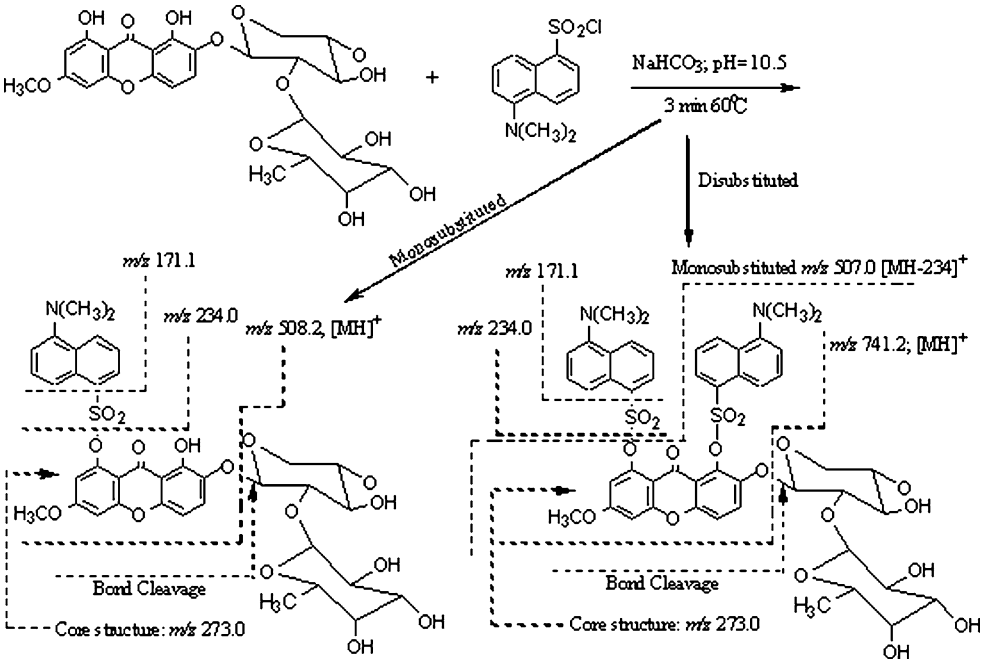

Fig. 2. Schematic diagram of the derivatization procedure and of MS cleavage of a typical dansylated derivative, that of 7-*O*-[α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranosyl]-1,8-dihydroxy-3-methoxyxanthone (RX)

standard solutions (5.0×10^{-5} mol L^{-1}) of the phenolic compounds for HPLC analysis were prepared by diluting the corresponding stock solutions with 50% aqueous acetonitrile. Test mixtures were prepared by mixing the individual standard solutions with 50% acetonitrile solution. When not in use all standards were stored at 4 °C.

Sodium bicarbonate buffer for the derivatization reaction was prepared from 0.2 mol L^{-1} sodium bicarbonate solution adjusted to pH 10.5 with 4 mol L^{-1} sodium hydroxide solution prepared from sodium hydroxide pellets. Formic acid–ammonia buffer was prepared from 2.0 mol L^{-1} formic acid adjusted to pH 3.5 with aqueous ammonia solution (17%, *w*/*w*). Formic acid–ammonia buffer solutions at five low concentrations (5.0, 10.0, 20.0, 25.0, and 30 mmol L^{-1}), used to test the retention of the dansyl derivatives of the phenolic compounds, were obtained by dilution the corresponding stock solution with water.

Extraction of Multihydroxy Phenolic Compounds

Ultrasound-assisted Extraction

Pulverized *L. rotatum* (0.5 g) and aqueous ethanol (80%, 10.0 mL) were placed in a 25-mL round-bottomed flask and the flask was sonicated in a water bath for 30 min. The *L. rotatum* sample was extracted three times with the same solvent mixture and the extracts were combined and evaporated to dryness by rotary vacuum evaporation at 60 °C. The residue was re-dissolved in 4.0 mL 80% aqueous ethanol and filtered through a 0.2-μm Nylon membrane filter. This solution (2 mL) was then purified by solid-phase extraction on a reversed-phase ODS (C_{18}) cartridge column (5 mL, 400 mg; Agilent Technologies, Stockport, Cheshire, UK) previously conditioned with 2 mL methanol and 2 mL water. The desired multi-phenolic compounds were eluted with 5 mL acetonitrile and the eluent was evaporated to dryness under a stream of nitrogen. The resulting residue was redissolved in 80% (*v*/*v*) aqueous ethanol to a total volume of 2.0 mL and stored at 4 °C until derivatization and LC–MS analysis.

Shaking Extraction

Pulverized *L. rotatum* (0.5 g) was extracted with aqueous ethanol (80%, 3 × 10.0 mL; 30 min for each extraction) in a mechanical shaker and the extracts were combined and purified as described above.

Extraction under Reflux

Pulverized *L. rotatum* (0.5 g) and aqueous ethanol (80%, 10.0 mL) were placed in a 25-mL round-bottomed flask and the contents were rapidly heated to reflux for 30 min with vigorous stirring. After cooling the aqueous ethanol was transferred to a 50-mL of round-bottom flask. The sample was extracted three times with the same solvent mixture and the extracts were combined and purified as described above.

Derivatization Procedure

The phenolic compounds were derivatized in aqueous acetonitrile in the presence of a basic catalyst. The extract of the phenolic compounds obtained by SPE (40–50 μL) was placed in a vial and 100 μL sodium bicarbonate solution (pH 10.5) and 100 μL dansyl chloride solution were added in succession. The mixture was shaken for 30 s, then heated at 60 °C for 3 min, then cooled to room temperature. After addition of an appropriate volume of water the solution was injected directly on to the column. The derivatization procedure and MS cleavage are shown in Fig. 2.

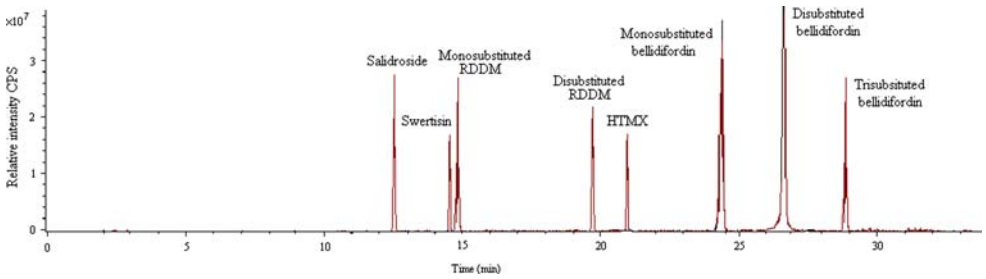

Fig. 3. Total-ion-current chromatogram obtained by HPLC–APCI–MS analysis in positive-ion mode, of the dansyl derivatives of the standards of the phenolic compounds

High-Performance Liquid Chromatography–Mass Spectrometry

Analysis was performed with an 1100 Series LC–MSD-Trap-SL liquid chromatograph–mass spectrometer.

The HPLC system comprised a model G1322A vacuum degasser, a model G1311A quaternary pump, a model G1329A autosampler, a model G1316A thermostatted column compartment, and a model G1315B DAD detector, all from Hewlett–Packard (HP). The HPLC system was controlled by HP Chemstation software. The dansyl-phenol derivatives were separated on a 200 mm × 4.6 mm i.d., 5-μm particle, Hypersil BDS C_{18} column (Yilite, Dalian, China). The injection volume was 10 μL, after which the injection syringe was automatically washed with 100% acetonitrile by use of the autosampler. The mobile phase was a linear gradient prepared from 25% aqueous acetonitrile containing 30 mmol L^{-1} formic acid–ammonia buffer (pH 3.5) (component A) and acetonitrile containing 30 mmol L^{-1} formic acid–ammonia buffer (pH 3.5) (component B). Before use the mobile phase components were filtered through a 0.2-μm Nylon membrane filter (Alltech, Deerfield, IL, USA). During conditioning of the column and before injection the system was equilibrated with 100% A. After injection the composition of the mobile phase was changed to 100% B in 30 min and maintained at this composition for 5 min. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 30 °C. After passage through the flow cell of the DAD the column eluate was directed to the APCI source of the mass spectrometer, without splitting.

The mass spectrometer, from Bruker Daltonik (Bremen, Germany), was equipped with an APCI source which was operated in positive-ion mode. The mass spectrometer was controlled by Esquire-LC NT software, version 4.1. The nebulizer pressure was 60 psi, the dry gas temperature 350 °C, the dry gas flow 5.0 L min^{-1}, the APCI Vap temperature 450 °C, the corona current 4,000 nA (pos), and the capillary potential 3,500 V.

A typical MS ion–current chromatogram obtained from a standard solution is shown in Fig. 3.

Results and Discussion

Chromatographic Conditions

Separation of these natural products was investigated by performing reversed-phase chromatography on a variety of hydrophobic stationary phases. The standards used for the study included SA, SW, RX, HT, and BE. Several gradient programs were investigated to ensure satisfactory HPLC separation in the shortest time. With methanol as mobile phase separation times were too long and resolution was poor. Acetonitrile was therefore selected as the best choice of organic mobile phase component. Unbuffered aqueous acetonitrile mobile phases have traditionally been used with great success for HPLC separation of phenolic compounds [20]. Initial assessment of the use of RPLC for the phenolic compounds mentioned above was performed without buffer solution. The results indicated that in the absence of buffer these compounds were eluted from the C_{18} column with severe tailing if traditional RPLC was used. The RPLC retention of the standards was investigated for three different stationary phases and a 30-min 25–100% aqueous acetonitrile gradient. Simultaneous separation of the phenolic derivatives was initially studied on Zorbax SB-C_{18} and ODS-C_{18} columns, but severe peak tailing was encountered for several double and trisubstituted phenolic derivatives and good baseline resolution could not be achieved. The best choice was the Hypersil BDS C_{18} column with formic acid-ammonia (pH 3.5) buffered mobile phase containing acetonitrile, which enabled outstanding separation of a mixture of phenolic derivatives.

Optimization of the Extraction Conditions

Yields from extraction of the multi-phenolic compounds followed the pattern:

extraction under reflux > ultrasound extraction > shaking extraction.

Maximum extraction efficiency was obtained by use of 80% aqueous ethanol and extraction under reflux, probably because the multi-phenolic compounds are more soluble in heated aqueous ethanol solution than at room temperature. Relatively low extraction efficiency was usually obtained when shaking extraction was used. The total amount of multi-phenolic compounds obtained by use of aqueous ethanol as extraction solvent and extraction under reflux was 1.42 times greater than that obtained by shaking extraction. All extractions were subsequently performed by extraction under reflux with 80% aqueous ethanol as extraction solvent.

MS–MS Analysis of the Phenolic Compounds

LC–MS identification of phenolic compounds has relied mainly on electron-impact (ESI) or chemical ionization (CI) to give information about the corresponding molecular ions $[MH]^+$, molecular fragments, and molecular ion

adducts [21]. Although a wide range of mass spectrometric ionization techniques is available, few are suitable for use in LC–MS. Atmospheric pressure chemical ionization (APCI) is a relatively recently introduces soft ionization technique with much potential in LC–MS analysis. Direct LC–APCI–MS analysis of some natural multihydroxy phenolic compounds extracted from plants has, however, resulted in low detection limits, mostly because the low efficiency of ionization of underivatized phenolic compounds in the APCI source. The best detection limit (signal-to-noise ratio >5) achieved with APCI in positive-ion mode for these compounds in our laboratory is $>3.0 \times 10^{-5}$ mol L^{-1} (10 µL injection) (No signal is obtained for most free phenolic compounds in negative-ion mode.) This value is higher than the concentrations of the phenolic compound in plants (0.05–10 ng mL^{-1}) [22].

APCI–MS coupled with selective dansyl derivatization for phenolic compounds is relatively simple and typically results in an intense signal for the protonated molecule and some valuable fragment ions which enable estimation of the number of hydroxyl groups on the phenolic core structure. In our studies it was found that collision-induced dissociation of the underivatized standard phenolic compounds did not generate an intense stable pseudomolecular ion signal and corresponding specific fragment ion signals that could be used for specific identification when using APCI–MS detection in positive-ion mode. Because of these shortcomings derivatization of the phenolic compounds with dansyl chloride was used to enhance APCI–MS detection.

To prove this high ionization efficiency the relative intensities of the molecular ions of α-naphthalenesulfonyl chloride (NS) and 5-dimethylamino-1-naphthalenesulfonyl chloride (Dans) were determined for APCI–MS in positive-ion mode under otherwise identical conditions. The core structure of NS does not contain a dimethylamino functional group, unlike Dans. Only a weak pseudomolecular ion signal was obtained for NS under APCI–MS conditions in positive-ion mode. Dans, in contrast, generated intense ion current signals. The ratio $I_{dans}/I_{NS} = 25.2/1$ (where, I_{dans} and I_{NS} are, respectively, the intensity of the ion current signals from the Dans and NS derivatives obtained under the same APCI–MS conditions). This difference can be attributed to the secondary basic nitrogen in the Dans core structure, which results in high ionization efficiency. Using HT as an example, the relative MS intensities of free HT and its derivative were investigated by APCI in positive-ion mode. The relative ion current intensities were, respectively, $I_{HTMX} = 0.74 \times 10^7$ and $I'_{HTMX} = 1.01 \times 10^5$, and the ratio $I_{HTMX}/I'_{HTMX} = 73.3{:}1$ (here I_{HTMX} and I'_{HTMX} are the relative intensity of the signals from derivatized and underivatized HT, respectively).

As expected, acidic mobile phases (for example, mobile phases containing 30 mmol L^{-1} formic acid–ammonia buffer, pH 3.5) promoted ionization of the dansyl derivatives. The dansylated phenolic derivatives contain basic secondary nitrogen atoms (predicted pK_a 3.3 ± 0.4) which can easily combine with a hydrogen ion to form the corresponding conjugated acid, resulting in high ionization efficiency. The relative intensities of the molecular ion signals in the presence and absence of 30 mmol L^{-1} formic acid were evaluated. The I_{ion}/I'_{ion} ratios observed were 2.5:1 for SA, 3.2:1 for SW, and 2.6:1 for HT (where I_{ion} and I'_{ion} are, respectively, the relative intensities of molecular ion signals in the presence and absence of 30 mmol L^{-1} formic acid).

In this study we found that the APCI–MS spectra of aglycone derivatives of phenolic compounds in which the aglycone was directly attached to the phenolic core structure by a phenolic hydroxyl functional group did not generate intense molecular ion signals at m/z $[MH]^+$. Using RX as an example, the RX molecule contained two free hydroxyl groups in the 1 and 8 positions in the phenolic core structure (Fig. 2). Theoretically, LC–MS–MS spectra should contain peaks for two major pseudomolecular ions at m/z [508.3 + disaccharide]$^+$ (corresponding to the monosubstituted form) and m/z [741.2 + disaccharide]$^+$ (corresponding to the disubstituted form), and APCI–MS spectra did contain peaks of major pseudomolecular ions at m/z 508.3, and m/z 741.2. They were, respectively, mono and disubstituted species resulting from loss of saccharide moieties by cleavage of the aglycone bond attached to the phenolic core structure. This was probably because the labeling procedure results in degradation by cleavage of the aglycone bond, because the derivatization procedure used to label the phenolic hydroxyl groups was usually performed in solution buffered at high pH (pH > 10.5).

The LC–MS–MS data collected for these compounds contained only pseudomolecular ion of the corresponding parent phenolic backbone. When, however, the aglycone group was attached indirectly to the phenolic core structure by a carbon atom linkage in the forms RCH_2CH_2–O–aglycone or RCH_2–O–aglycone (where R is the phenolic core structure) intense pseudomolecular ion signals at m/z $[MH]^+$ and the corresponding characteristic fragment ion formed by loss of one glucose moiety were observed.

Using salidroside (SA) as an example (Fig. 4), the ions formed during APCI–MS can be interpreted as follows: m/z 534.1 $[MH]^+$; m/z 372.0 [MH - one aglycone moiety]$^+$; m/z 234.0 [5-(dimethylamino) naphthalene sulfoxide moiety]; m/z 170.0 [5-(dimethylamino)naphthalene moiety]. Loss of the aglycone moiety in the salidroside molecule resulted in a very intense specific fragment ion signal at m/z 372.0.

Using HT as an example, the APCI–MS–MS spectra obtained from a monohydroxyl phenolic compound are shown in Fig. 5. The pseudomolecular ion at m/z $[MH]^+$ 536.2, and specific fragment ions at 302.1 [MH - 234]$^+$, m/z 234 [5-(dimethylamino)naphthalene sulfoxide moiety], and m/z 170.1 [5-(dimethylamino)naphthalene moiety] are usually generated. The specific ion at m/z 234 is from cleavage of the O–S bond and the characteristic fragment ion at m/z 170.1 is from cleavage of the C–S bond; both are followed by rearrangement of one hydrogen atom toward the 5-dimethylamino naphthalene sulfoxide and naphthalene nucleus moieties, respectively.

Major fragment ions at m/z $[MH]^+$, m/z [MH - 234]$^+$, [MH - 2 × 234]$^+$, m/z 234, and m/z 171.1 are formed from the dihydroxy phenolic derivatives. Selected reaction monitoring of the transitions from m/z $[MH]^+$ to these ions is specific for these derivatives. In the positive-ion mass spectra for dihydroxy phenolic derivatives, the corresponding collision-induced dissociation of the pseudomolecular ion is clearly distinguishable.

Figure 6 shows the APCI–MS–MS spectra of a dihydroxy phenolic derivative, using RX as an example which generates mono and disubstituted forms. The spectrum of the monosubstituted form contains ions at m/z 508.3 $[MH]^+$, m/z 273.0, m/z 236.1 [5-(dimethylamino)naphthalene

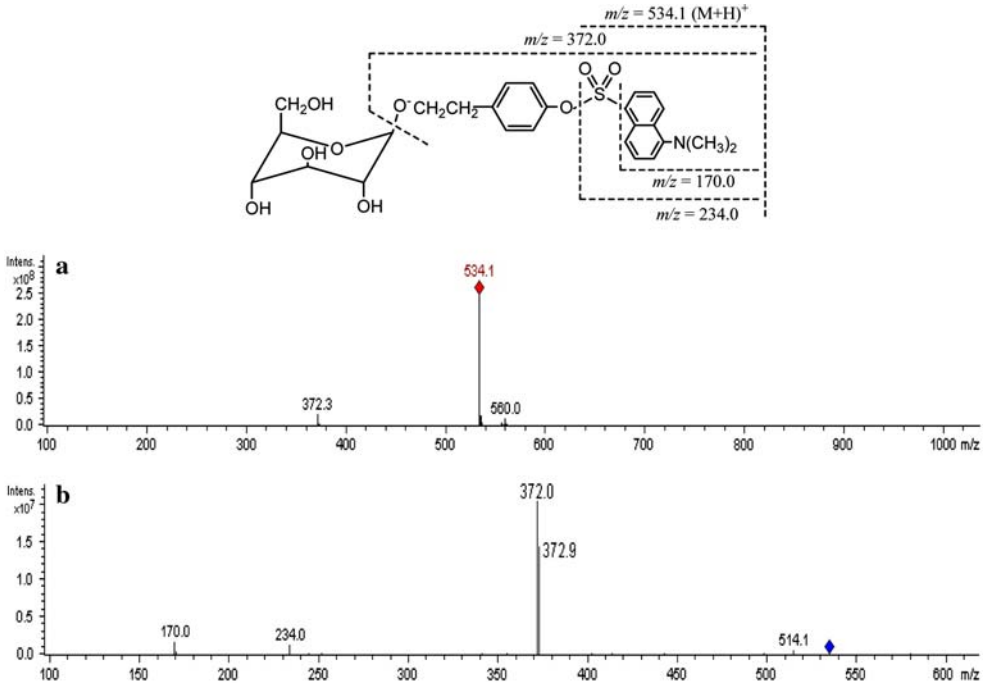

Fig. 4. Typical mass spectra between 100 and 1,000 amu, obtained by APCI–MS in positive-ion mode, of the dansylated derivative of salidroside: **a** molecular ion; **b** MS–MS

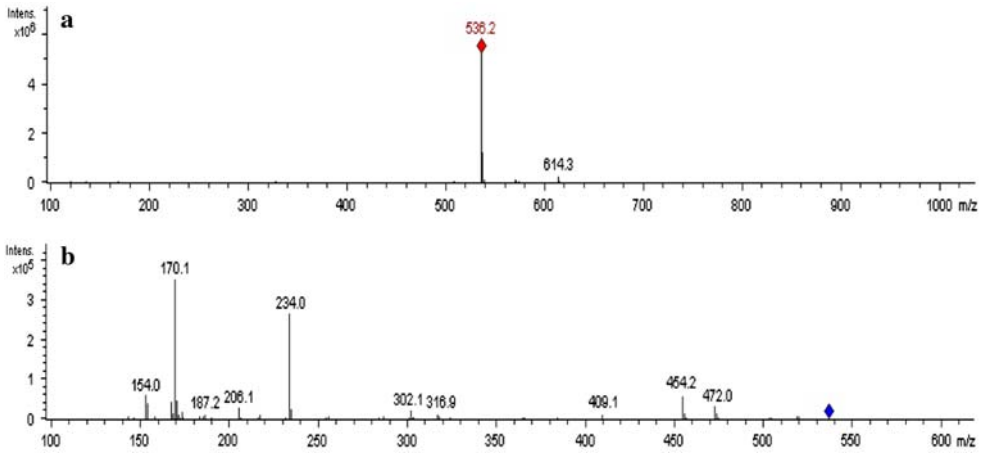

Fig. 5. Typical mass spectra between 100 and 1,000 amu, obtained by APCI–MS in positive-ion mode, of the dansylated derivative of HTMX: **a** molecular ion; **b** MS–MS

sulfoxide moiety], and *m/z* 171.1 [5-(dimethylamino)naphthalene moiety]. The mode of cleavage expected should lead to an intense characteristic fragment ion signal at *m/z* 274 rather than the characteristic fragment ion signal at *m/z* 273. This ion is only one unit-mass different from the *m/z* 274 $[MH - 234]^+$ ion expected and may be an isotopomeric ion peak of the *m/z* 274 $[MH - 234]^+$ ion. A peak can, in fact, be observed at *m/z* 274, but its intensity is quite weak compared with that at *m/z* 273. Disubstituted RX derivatives form the ions *m/z* 741.2 $[MH]^+$, *m/z* 507.0 $[MH - 234]^+$, *m/z* 273.0 $[MH - 2 \times 234]^+$, *m/z* 234 [5-(dimethylamino)naphthalene sulfoxide moiety], and *m/z* 171.1 [5-(dimethylamino) naphthalene moiety]. Note that disubstituted RX derivatives generate the characteristic fragment ion signal at *m/z* 507.0 $[MH - 234]^+$; this ion is only one mass unit different from the pseudomolecular ion of the corresponding monosubstituted derivative (at *m/z* 508.3, $[MH]^+$) and can be explained by loss of one 5-(dimethylamino)naphthalene sulfoxide moiety. The 5-(dimethylamino)naphthalene sulfoxide (dansyl) moiety is usually detected as the *m/z* 234 ion, and is apparent in this spectrum; sometimes this species is also detected as the *m/z* 236 ion, probably because of combination of the secondary nitrogen and sulfonyl functions of the cleaved dansyl moiety with two hydrogen ions. A similar phenomenon explains the fragment ions at *m/z* 171.1 and *m/z* 170.0.

APCI–MS spectra obtained from bellidifordin, an example of a trihydroxy phenolic derivative, are shown in Fig. 7 and can be interpreted as follows. The spectrum of the monosubstituted form contains ions at *m/z* 508.2 $[MH]^+$, *m/z* 273.0 $[MH - 234]^+$, *m/z* 236 [5-(dimethylamino)naphthalene sulfoxide moiety],

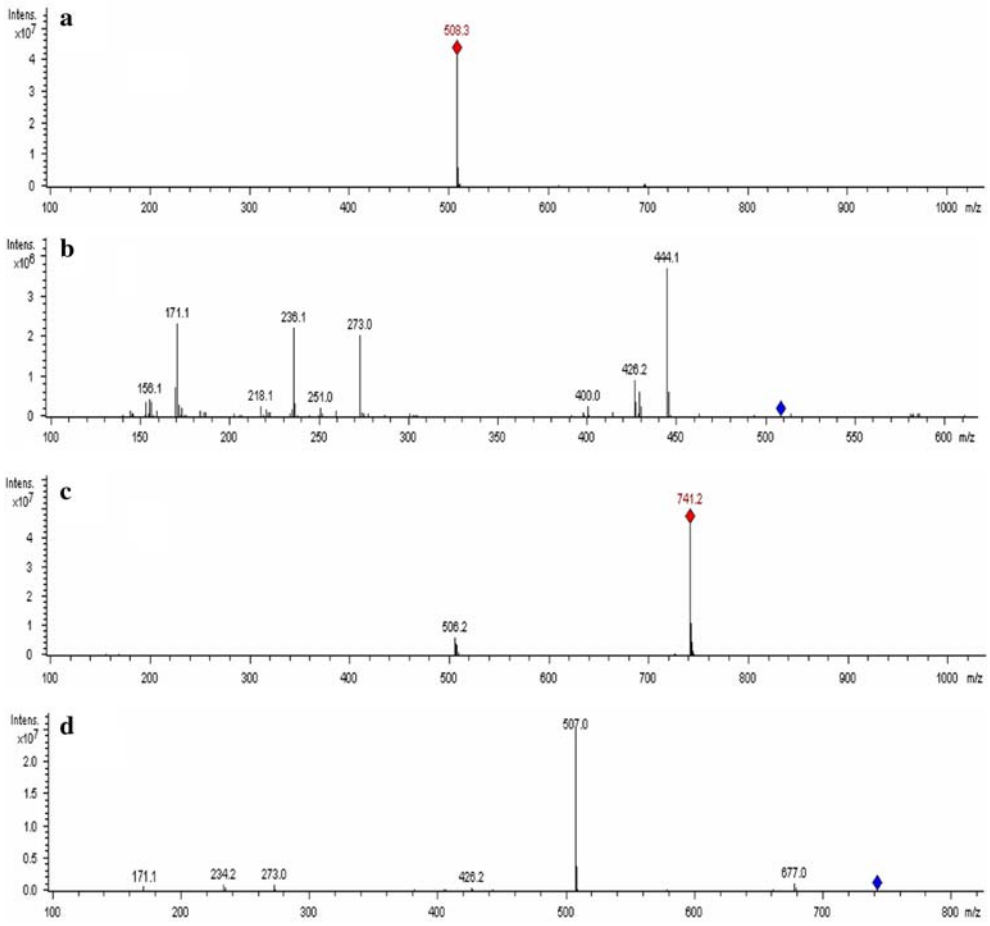

Fig. 6. Typical mass spectra between 100 and 1,000 amu, obtained by APCI–MS in positive-ion mode, of the dansylated derivatives of RDDM: **a** and **b** are, respectively, results from MS and MS–MS of the monosubstituted derivatives, and **c** and **d** are, respectively, results from MS and MS–MS of the disubstituted derivatives

and *m/z* 171.1 [5-(dimethylamino)naphthalene moiety], that of the disubstituted form contains ions at *m/z* 741.2 $[MH]^+$, *m/z* 507.0 $[MH - 234]^+$, *m/z* 273.0 $[MH - 2 \times 234]^+$, *m/z* 234.1 [5-(dimethylamino)naphthalene sulfoxide moiety], and *m/z* 171.1 [5-(dimethylamino)naphthalene moiety], and that of the trisubstituted form contains ions at *m/z* 974.0 $[MH]^+$, *m/z* 740.1 $[MH - 234]^+$, *m/z* 506.2 $[MH - 2 \times 234]^+$, *m/z* 273.1 $[MH - 3 \times 234]^+$, *m/z* 234 [5-(dimethylamino)naphthalene sulfoxide moiety], and *m/z* 170.1 [5-(dimethylamino)naphthalene moiety]. As found in earlier studies, retention of multihydroxy phenolic derivatives increases with increasing number of hydroxyl groups on the phenolic backbone—the monosubstituted derivatives were eluted before the di and trisubstituted derivatives, the trisubstituted derivatives being eluted last. This is because the hydrophobic properties of the compounds increase with increasing dansyl substitution, resulting in stronger retention.

The merit of on-line LC–MS–MS has been demonstrated by analysis of dansyl derivatives of phenolic compounds. Interpretation of MS–MS fragmentation is very important for determination of the number of hydroxyl groups on the phenolic backbone of multihydroxy phenolic compounds. Characteristic ions indicative of loss of one or two dansyl moieties provide valuable information about the number of hydroxyl groups present. Although other endogenous hydroxyl compounds present in plants samples are, presumably, coextracted and derivatized by dansyl chloride, no inferences were observed, because of the highly specific parent mass-to-charge ratio and the corresponding characteristic fragment ions. Solid-phase extraction in conjunction with HPLC with gradient elution is also an efficient means of reducing interference to a minimum in the separation and simultaneous identification of the derivatized phenolic compounds. APCI–MS–MS analysis coupled with selective dansyl derivatization enabled the development of a highly selective and accurate method for analysis of the phenolic compounds.

Separation and Identification of Phenolic Compounds in a Sample of *L. rotatum*

Phenolic compounds extracted from samples of *L. rotatum* were analyzed by the method described above. As expected, multihydroxy phenolic compounds (flavonoids) were the main constituents of the aerial parts of *L. rotatum*. The ion-current chromatogram obtained by APCI–MS in positive-ion mode is shown in Fig. 8. The peaks were identified by use of the characteristic fragment ion peaks

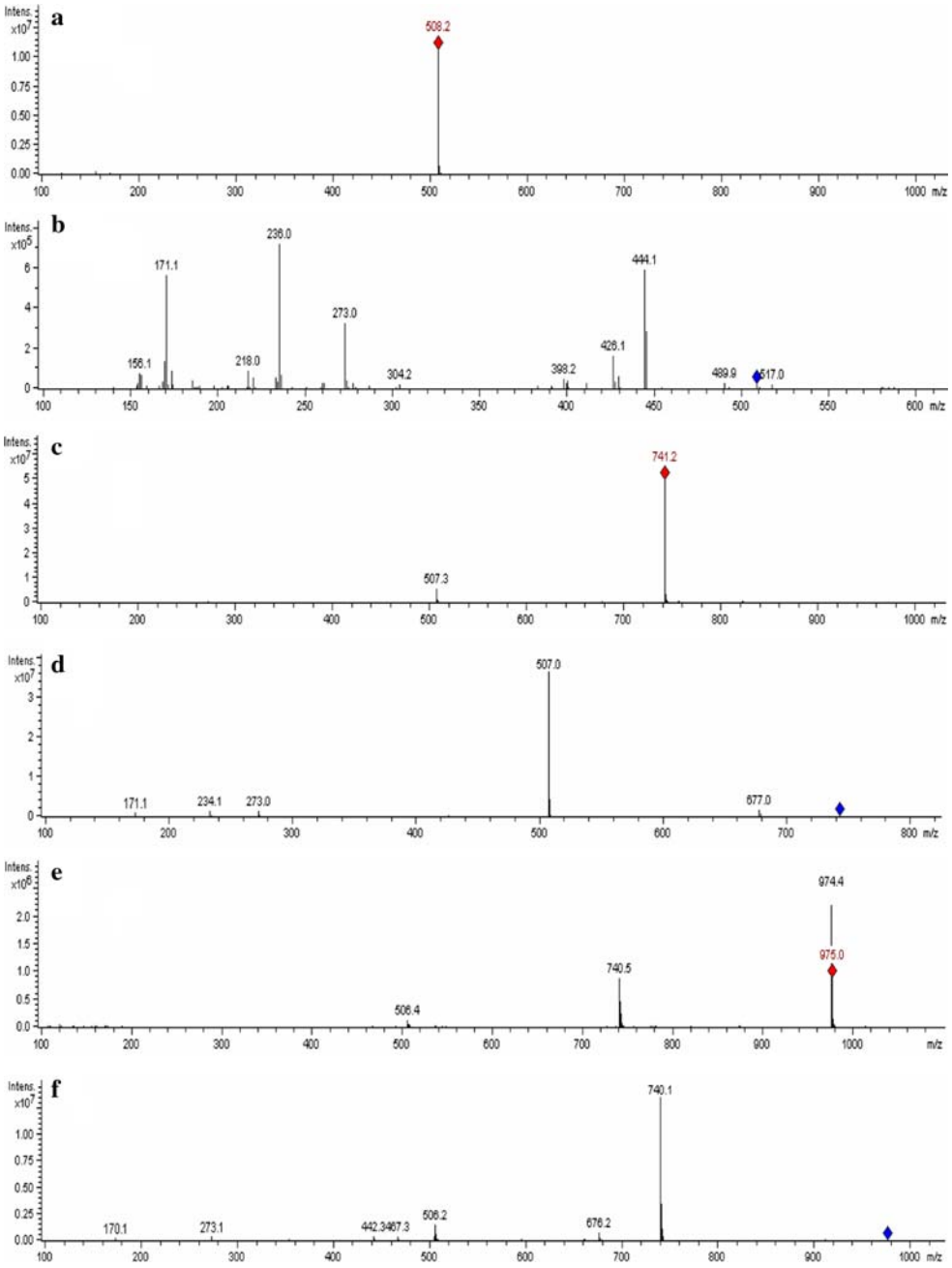

Fig. 7. Typical mass spectra between 100 and 1,000 amu, obtained by APCI–MS in positive-ion mode, of the dansylated derivatives of bellidifordin: **a** and **b** are, respectively, results from MS and MS–MS of the monosubstituted derivatives, **c** and **d** are, respectively, results from MS and MS–MS of the disubstituted derivatives, and **e** and **f** are, respectively, results from MS and MS–MS of the trisubstituted derivatives

from APCI–MS data and by spiking with standards of the compounds. Although the chromatogram is more complex than that obtained from the standards (Fig. 3), it is apparent that most of the dansyl derivatives of the phenolic compounds were resolved with good baseline resolution. The compounds identified in extracts obtained from samples of *L. rotatum* were SW, BE, RX, HT, and 1,8-dihydroxy-3,5-dimethoxyxanthone (DD). APCI–MS and APCI–MS–MS data for the derivatives are listed in Table 1.

Conclusions

This paper describes the performance of a method for identification of multihydroxy phenolic compounds in extracts of *L. rotatum*. The selectivity for estimation of the number of hydroxyl groups on the phenolic core structure by LC–MS–MS analysis is effectively improved by formation of the dansyl derivatives. Dansyl derivatization introduces weakly basic secondary nitrogen into the phenolic core structures and this is readily ionized after chromatography by use of the acidic mobile phases commonly used in HPLC. The protonated molecular ions generate cleavage products by loss of one or several 5-(dimethylamino)naphthalene sulfoxide moieties, giving the characteristic fragment ions $[MH - 234]^+$, $[MH - 2 \times 234]^+$, and $[MH - 3 \times 234]^+$. The major drawback is that aglycones of the phenolic compounds give only the pseudomolecular ion of the parent phenolic compound by cleavage of the aglycone attached to the phenolic core structure, so

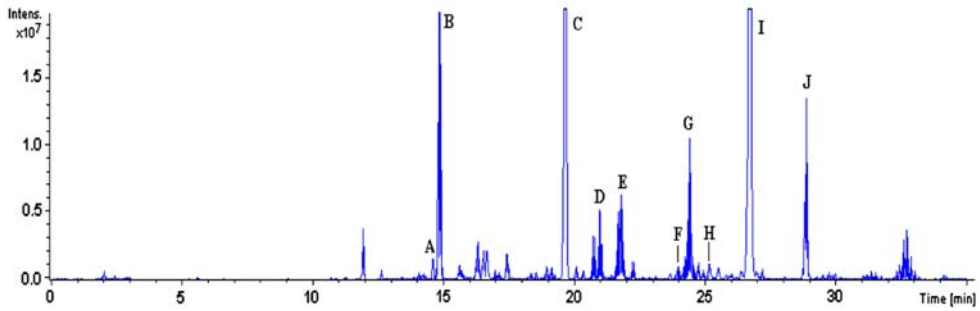

Fig. 8. Total-ion current chromatogram obtained by HPLC–APCI–MS analysis in positive-ion mode, of the dansyl derivatives of the phenolic components extracted from a sample of *L. rotatum*. **A** swertisin; **B** monosubstituted RDDM; **C** disubstituted RDDM; **D** HTMX; **E** monosubstituted 1,8-dihydroxy-3,5-dimethoxyxanthone (monosubstituted DHDMX); **F** not identified; **G** monosubstituted bellidifordin; **H** disubstituted 1,8-dihydroxy-3,5-dimethoxyxanthone (disubstituted DHDMX); **I** disubstituted bellidifordin; **J** trisubstituted bellidifordin

Table 1. Retention times (RT) and LC–MS–MS data

Compound	RT (min)	$[MH]^+$	APCI–MS–MS peaks in positive-ion mode	Identity
A	14.6	680.2	236.3, 446.3 $[MH - 234]^+$	Monosubstituted SW
B	14.8	508.3	171.1, 236.1, 273.0 $[MH - 234]^+$ (isotopomeric ion peak), 400.0, 426.2, 444.1	Monosubstituted RX
C	19.6	741.2	171.1, 234.2, 273.0 $[MH - 2 \times 234]^+$, 507.0 $[MH - 234]^+$	Disubstituted RX
D	21.0	536.2	170.1, 234.0, 302.1 $[MH - 234]^+$, 316.9, 409.1, 454.2, 472.0	HT
E	21.8	522.1	170.1, 234.1, 288.1 $[MH - 234]^+$, 458.4	Monosubstituted DD
F	24.0	522.1	Not identified	Not identified
G	24.4	508.2	171.1, 236.0, 273.0 $[MH - 234]^+$ (isotopomeric ion peak), 304.2, 398.2, 426.1, 444.1	Monosubstituted BE
H	25.2	755.1	271.2, 288.3 $[MH - 2 \times 234]^+$ (isotopomeric ion peak), 521.1 $[MH - 234]^+$	Disubstituted DD
I	26.7	741.2	171.1, 234.1, 273.0 $[MH - 2 \times 234]^+$, 507.0 $[MH - 234]^+$	Disubstituted BE
J	28.8	974.0	170.1, 273.1 $[MH - 3 \times 234]^+$ (isotopomeric ion peak), 506.2 $[MH - 2 \times 234]^+$, 740.1 $[MH - 234]^+$	Trisubstituted BE

the positions of the hydroxyl groups cannot be confirmed. The method does, however, enable highly selective and accurate estimation of the number of hydroxyl groups on the phenolic core structure, and the efficiency of APCI–MS ionization of the derivatized multihydroxy phenolic compounds in positive-ion detection mode is much greater than that for the underivatized compounds.

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