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Electrospray Ionization Tandem Mass Spectrometry as a Tool for the Structural Elucidation and Dereplication of Natural Products: An Overview

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1. Introduction

Natural products (NPs) obtained from plants and microorganisms often exhibit biological activities of interest for the discovery of new drugs as well as pharmaceutical and agrochemical products (Fredenhagen et al., 2005; Konishi et al., 2007; Lang et al., 2008). For instance, over 60% of the anticancer drugs have been discovered directly from NPs or are semi-synthetic derivatives of these compounds (Butler, 2008; Costa-Lotufo et al., 2010; Harvey, 2008; Newman & Cragg, 2007). However, the research for new bioactive compounds is usually laborious and slow, once the biological evaluation processes are preceded by isolation, purification, and structural elucidation steps, which are usually expensive and time-consuming (Konishi et al., 2007). This fact, in combination with the large number of known compounds, has led the scientific community to develop new techniques for the direct identification of NPs from extracts and natural broths, thus avoiding reisolation of already known compounds (Bindseil et al., 2001; Konishi et al., 2007; Lee, 2004; Newman et al., 2000; Newman et al., 2003; Shu, 1998).

Dereplication is the process that allows for the rapid identification of bioactive metabolites in crude extracts by distinguishing previously identified compounds from novel ones (Crotti et al., 2006). This technique avoids repetitive work of isolation of already known NPs (Wolf & Siems, 2007), promoting chemical screening or metabolite profiling (Crotti et al., 2006). The dereplication process involves separation of single metabolites by chromatographic methods, identification of these compounds by spectroscopic methods, bioassays for evaluation of the biological activity, and searches in databases for verification of the novelty of these compounds (Konishi et al., 2007; Wolf & Siems, 2007).

Hyphenated techniques have played a key role in the identification of NPs and other organic compounds (Crotti et al., 2006; Prasain et al., 2003). These techniques combine a separation method (i.e., gas chromatography, GC; liquid chromatography, LC) with a structural identification technique (i.e., mass spectrometry, MS; ultraviolet-visible spectroscopy, UV-vis; nuclear magnetic resonance, NMR). Although a number of

approaches dealing with the dereplication of NPs have been based on GC-MS (gas chromatography mass spectrometry), LC-UV (liquid chromatography ultraviolet spectroscopy), and LC-NMR (liquid chromatography nuclear magnetic resonance), LC-MS (liquid chromatography mass spectrometry) has been the most widely employed technique for this purpose (Lang et al., 2008; Lee, 2002; Oliveira & Watson, 2000; Wolfender & Hostettmann, 1996; Wolfender et al., 1994).

In this chapter we present an overview on the use of LC-MS and electrospray ionization tandem mass spectrometry (ESI-MS/MS) techniques for the dereplication of NPs belonging to different classes, such as sesquiterpene lactones, lignans, caffeoyl quinic acid derivatives, flavonoids, and alkaloids.

2. Why use LC-MS?

LC-MS combines the versatility of LC (usually high-pressure liquid chromatography, HPLC), which enables analysis of a wide range of compounds (Degani et al., 1998), with the sensitivity of MS (Niessen, 2006). About 60% to 80% of all the existing compounds are amenable to HPLC analysis, whereas about 15% are analyzable by GC. The use of GC is often limited to thermally stable and volatile compounds, as well as those that can be analyzed after derivatization reactions. On the other hand, HPLC is employed to separate macromolecules, ionic species, polar and high-molecular weight compounds, on condition that they are soluble in the mobile phase. These factors have made HPLC the technique of choice for dereplication and metabolite profiling studies of NPs in complex mixtures (Dong, 2006).

Figure 1 shows a schematic representation of LC-hyphenated techniques for the structural identification of organic compounds. In the HPLC system, the sample is injected into the mobile-phase stream and transported through the column with the stationary-phase where the separation takes place. This separation occurs by selective interaction between the sample and the mobile and stationary phases, and it is monitored with the aid of a flow-through detector, usually UV, NMR, or MS. Although NMR is the most powerful technique for the structural elucidation of organic compounds, some drawbacks of coupling NMR to LC emerges from the high costs of using deuterated solvents as mobile phase in LC and the low sensitivity as compared to MS, thereby limiting its use for the identification of NPs at trace levels. UV-vis does not provide conclusive data for structural elucidation alone, but development of the diode array detector (DAD) has increased its detection power and effectiveness. UV-DAD has been more and more often applied as a first detection method in dereplication studies using LC because it is rapid and inexpensive, as compared to the other techniques (Crotti et al., 2006).

Liquid chromatography mass spectrometry (LC-MS) is a highly sensitive and selective method for identification of NPs in complex mixtures (Fredenhagen et al., 2005; Furtado et al., 2007). Compounds are separated in the column of the LC system and directed to the mass spectrometer by a flow separator, where they are ionized and further separated in the mass analyzer according to their mass-to-charge (m/z) ratio (Niessen, 2006). However, three major difficulties have limited the coupling of LC to MS for many years, namely I) apparent incompatibility of the flow-rate as expressed by the need for introducing a large volume of a liquid effluent from a conventional LC column into the high vacuum of the mass spectrometer; II) incompatibility of the effluent composition as a result of the frequent use of non-volatile mobile phase additives during the LC separation process, and (III) ionization of

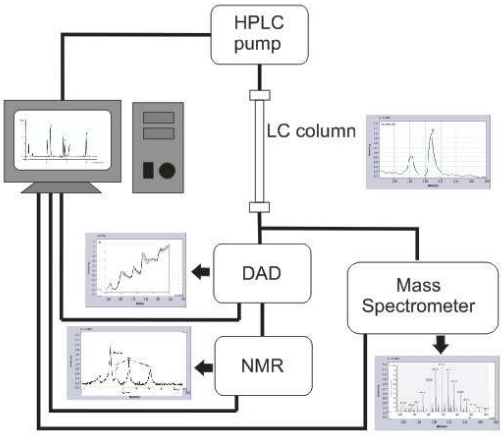


Fig. 1. LC-hyphenated techniques for structural identification.

non-volatile and/or thermally labile analytes (Niessen & Tinke, 1995). These difficulties were overcome with the advent of the atmospheric pressure ionization (API) techniques, more specifically electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) (Tomer, 2001).

In ESI, the sample is dissolved in a polar, volatile solvent and pumped through a narrow stainless steel capillary (75-150 μm i. d.). A sufficiently high voltage, commonly 3-5 kV, is applied at the capillary tip, which is situated within the ionization source of the spectrometer. This high voltage causes charge migration of the species in solution to the metal/solution interface, resulting in an electric double layer. In the capillary tip, electrostatic forces acting on the ion preformed in the double layer counterbalance the surface tension, culminating in a conical surface known as Taylor cone (Vessecchi et al., 2007). The small charged-droplets of the fine spray that emerge from the capillary tip are diminished in size due to a co-axially introduced nebulizing gas (usually nitrogen) flowing around the outside of the capillary, thus leading to solvent evaporation (Herbert & Johnstone, 2003). When the Rayleigh limit is reached, the Coulombic repulsion between charges in the droplet surface overcomes the surface tension, thereby giving rise to the droplet "Coulombic explosion" (Fig. 2). Finally, ions are produced by the droplets and flow into the mass analyzer (Ardrey, 2003; Niessen, 2006; Smeraglia et al., 2002; Vékey, 2001).

As a result of ESI, protonated ($[M+H]^+$)/deprotonated ($[M-H]^-$) molecules, and cationized (usually $[M+Na]^+$ or $[M+K]^+$)/anionized molecules (i.e, $[M+Cl]^-$) can be produced, depending on the molecular structure of the analyte (Herbert & Johnstone, 2003; Todd, 1995). Natural products exhibiting acidic groups (i.e., flavonoids, and caffeoyl quinic acid derivatives) are easily deprotonated, which enables their analysis in the negative ion mode, whereas for NPs with basic groups (i.e., alkaloids), which can be easily protonated, the positive ion mode is more adequate (Ardrey, 2003). The major advantage of interfacing ESI with LC is related to the ion formation, which occurs at atmospheric pressure in the condensed phase outside the high vacuum of the mass spectrometer, thereby eliminating one of the greatest difficulties inherent to the analysis: the negative influence of the vacuum

system (Ackermann et al., 1996). Furthermore, some other characteristics make the ESI an important ionization process: elimination of solvent ejected from droplets by the electrical field and difference in pressure; generation of multiply charged ions, which is important for molecules of high weight because the effective mass range of the mass spectrometer is increased; detection of molecular weight; and low solvent flow, which is required for operation at optimum sensitivity, thereby reducing sample consumption (Crotti et al., 2006).

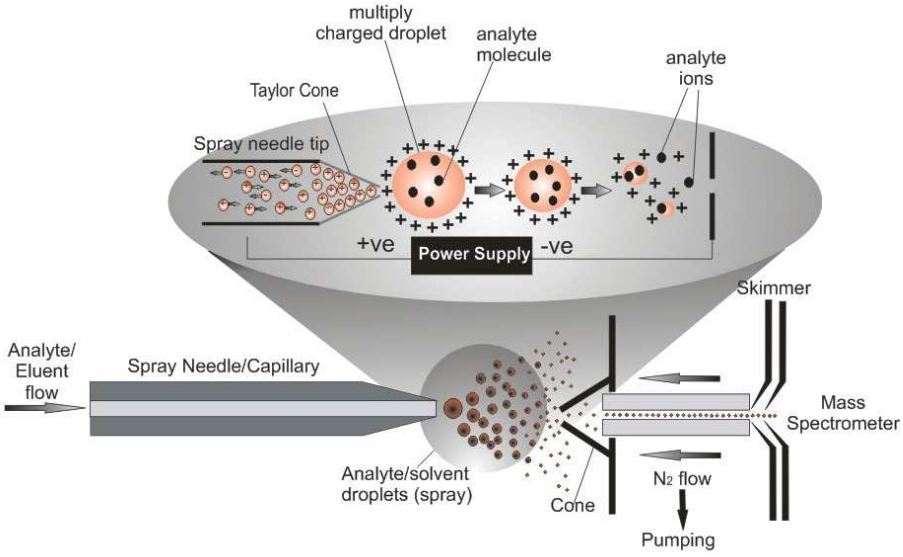


Fig. 2. Representation of an electrospray ion source and the electrospray process (Crotti et al., 2006).

3. How to use LC-MS and ESI-MS/MS for the dereplication of NPs?

ESI is considered to be a "soft ionization" process, since the internal energy of the formed ions is low, and little or no fragmentation takes place (Ardrey, 2003; Núñez et al., 2005; Vékey, 2001). This feature is very interesting for identification of the molecular weight or molecular formula. However, it represents an inconvenience for structural elucidation studies, which are based on fragment ions (Herbert & Johnstone, 2003; Wolfender et al., 2000). In tandem mass spectrometry (MS/MS), an ion (called precursor ion) from the first stage of MS is selected and activated, to produce fragment ions, which are then analyzed in the second stage of MS. The most widely employed ion activation method is the collision-induced dissociation (CID), which consists in promoting the energy-controlled collision of a chemically inert gas, (e.g. Ar, He, N_2, or CO_2) with the precursor ion (Fig. 3). The collision energy may be chosen, in order to optimize the MS/MS spectrum. Low collision energy values promote soft fragmentation and produce few fragments, whereas high collision energy values prompt extensive fragmentation, so the produced fragment ions can be used to obtain structural information. On the other hand, important structural information can be obtained and eventual comparison with spectral libraries may be made when moderate

collision energy values are utilized (Gates et al., 2006). MS spectrometers that are used in MS/MS experiments can be of two main types: i) instruments that are able to store the ions, thus allowing for selection of the target ions by injection with authentic patterns, followed by fragmentation, hence generating the mass spectra (e.g. ion cyclotron resonance, ICR; and quadrupole ion trap, IT); and ii) instruments that use a sequence of mass spectrometers in space consisting of quadrupole mass analyzers in sequence (e.g, triple quadrupole, QqQ), or a hybrid conformation with quadrupole in sequence (e.g., quadrupole-time-of-flight, Q-TOF) (De Hoffmann & Stroobant, 2007).

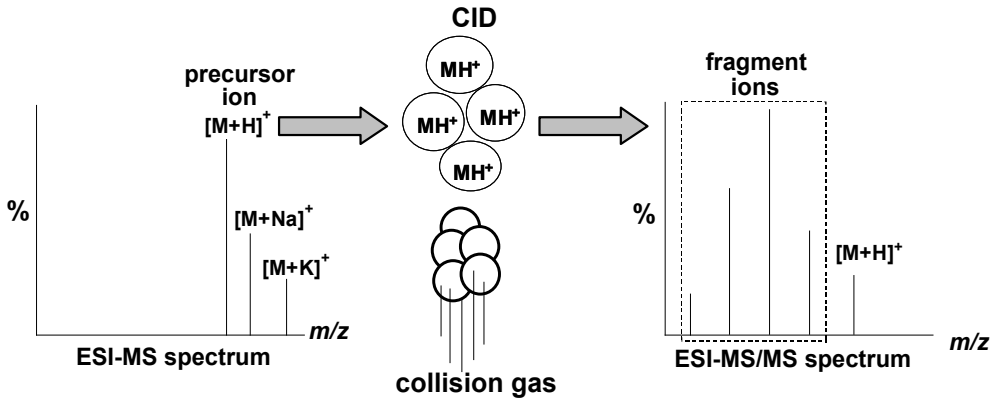


Fig. 3. Representation of the CID process in tandem mass spectrometry (MS/MS).

Dereplication of NPs by means of LC-MS and ESI-MS/MS has often been accomplished on the basis of retention times, UV-DAD spectra, and MS/MS data obtained for each peak of the chromatogram of the crude extract as compared to those of previously analyzed authentic standards. MS/MS data of the authentic standards are obtained using the same collision energy value, which is also employed in LC-MS/MS experiments involving the crude extract (Gobbo-Neto & Lopes, 2008). In the case of standards belonging to the same class of NPs and with similar structures, the acquisition of the MS/MS spectra using the same collision energy value ensures that differences in the fragmentation are the sole result of differences in their structures, so that structure-fragmentation relationships can be established (Crotti et al., 2005). The presence of structure diagnostic fragment ions (DFI) is very important for distinction between structural features (Aguiar et al., 2010) or maybe for characterization of a class of NPs. This methodology enables direct online identification of already known compounds from crude extracts.

Besides the importance of ESI-MS/MS for the online identification of NPs using LC-MS, this technique can also be used for offline identification. In this case, authentic standards are also previously investigated by MS/MS, and diagnostic fragment ions are identified. Accurate mass data, which allows for identification of the molecular formulas of the compounds, are especially important for this purpose. After that, the crude extracts are analyzed directly by MS without undergoing previous chromatographic separation in the LC system. The MS spectrum is analyzed, and those ions with the same *m/z* as the authentic standards are selected as precursor ions for the MS/MS experiments. Finally, the MS/MS spectra of the

standards and those obtained from the selected precursor ions are compared. The disadvantage of this offline identification as compared to online identification by LC-MS is that data from the LC system, such as retention time and UV-DAD spectra, are lost. A good example of offline dereplication of NPs is the identification of plumeran alkaloids in the crude methanol extract of stem bark from *Aspidosperma spruceanum* (Aguiar et al., 2010). In this study, Aguiar and co-workers employed ESI-MS/MS and accurate-mass data of six authentic standards (**1-6**) to identify important diagnostic fragment ions, which were then used to distinguish aspidoscarpine (**1**) from aspidolimidine (**2**) in the crude methanol extract (Fig. 4).

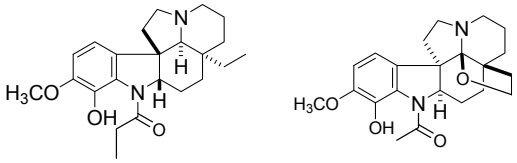


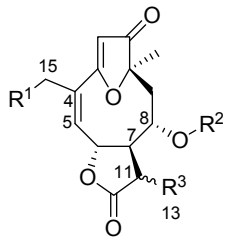
Fig. 4. Structures of aspidoscarpine (**1**) and aspidolimidine (**2**).

4. Applications of MS/MS in the dereplication of NPs

4.1 Sesquiterpene lactones (STL)

Sesquiterpene lactones (STL) are an important class of natural products that occur in several plant families, mainly in Asteraceae (Crotti et al., 2005). These compounds are interesting due to their several biological activities (Picman, 1986) and relevance as chemotaxonomic markers (Crotti et al., 2005). The biosynthesis of STLs is initiated by condensation of three isoprene molecules followed by oxidation, and their chemical structures are divided into groups according to their carbocyclic skeleton (e.g., germacranolides, eudesmanolides, guaianolides, glaucolides, cadinanolides, hirsutinolides, and furanoheliangolides) (Crotti et al., 2005; Dewick, 2004).

The fragmentation pattern of the protonated STL belonging to the goyazensolide type of furanoheliangolides has been investigated by Crotti and co-workers using ESI-MS/MS (Crotti et al., 2005). Firstly, the authors selected ten STL standards exhibiting the same structural core, but differing in terms of the presence/absence of a hydroxyl group at C-15, a single/double bond between C-4 and C-5, and the presence of an acyloxy group or hydroxyl group at C-8 (Fig. 5). All the MS/MS spectra were obtained at 10 eV, as optimized by varying the collision energies between 5 and 50 eV. The authors reported that the fragment ion $[M+H- R_2CO_2H]^+$ is diagnostic for compounds that exhibit an acyloxy group at C-8 (**3-7**, **9**, **11** and **12**), whereas the fragment ion $[M+H-CO_2]^+$ indicates the presence of a hydroxyl group at C-8. The acylium ion R_2^+, which is formed for compounds **3-7**, **9**, **11**, and **12**, was useful for identification of the ester bound at C-8. Moreover, the relative configuration of C-8 of centratherin (**7**) and budlein A (**9**) could also be identified on the basis of the relative intensity of the fragment ion $[M+H-R_2CO_2H]^+$. These data were further used by Gobbo-Neto & Lopes, in combination with retention times and UV-DAD spectra, for the online identification of 36 compounds in the methanol extract of leaves from *Lychnophora ericoides* (Asteraceae), including STLs **6**, **7**, and **12** (Gobbo-Neto & Lopes, 2008).



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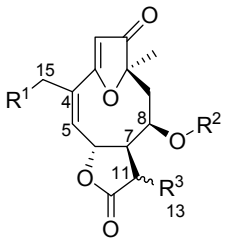

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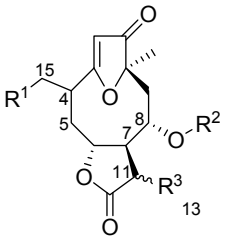


11-12

	3	4	5	6	7	8	9	10	11	12
R^1	H	H	OH	H	OH	H	OH	H	H	H
R^2						H		H		
R^3	CH_2	CH_2	CH_2	CH_2	CH_2	α-CH_3	CH_2	CH_2	CH_2	CH_2
MW	344	346	360	358	374	278	374	276	346	360

Fig. 5. Sesquiterpene lactones of the goyazensolide type (Crotti et al., 2005).

4.2 Phenylpropanoids

Phenylpropanoids is one of the largest and most important classes of NPs. It includes a vast range of phenolic compounds, from small and simple forms to complex molecular structures. Moreover, from a biosynthetic viewpoint phenylpropanoids are the precursors of important secondary metabolites, such as aromatic acids, benzoic acids, cinnamic acids, lignins, lignans, phenylpropenes, coumarins, styrylpyrones, flavonoids, stilbenes, flavolignans, isoflavonoids, terpenoid quinones, and tannins, among others.

4.2.1 Aromatic acids

Gómes-Romero and co-workers have identified several phenol derivatives in foods used in diets (e.g., propolis, lemon, borage, cabbage-broccoli, garlic, onion, etc) by using a MS/MS library previously established from commercially available standard phenolic compounds (Gómez-Romero et al., 2011). The authors reported that the negative ion mode electrospray ionization was more adequate than the positive ion mode for this purpose, although the latter was also utilized when necessary. They described that loss of CO_2 (44 Da) or H_2O (18 Da) from the $[M-H]^-$ ion, which was used as precursor ion in MS/MS experiments, are the major fragmentation routes for the selected compounds. However, they did not result in diagnostic fragment ions (DFI). Thus, in order to adjust the intensity of some specific and diagnostic fragment ions, the collision energy values were varied.

Samples of these foods were freeze-dried, powdered, and extracted with methanol 80% in ultrasonic bath, which was followed by centrifugation, filtration, and dilution with water/acetonitrile 1:1 (v/v). After that, samples were analyzed by LC coupled with a diode array detector (DAD) set at 254 nm, and a quadrupole orthogonal acceleration time-of-flight mass spectrometer (micrOTOF-Q™) equipped with an electrospray (ESI) ion source. The authors compared the MS/MS data of each peak of the chromatogram with those of the

previously established library in both the positive and negative ion modes, and they identified nine hydroxybenzoic acid derivatives, nine cinnamic acid derivative, and six simple phenolic compounds, apart from eleven flavonoids. Vanillic (**13**) and syringic (**14**) acids were identified on the basis of their DI *m/z* 152 and 182, respectively, as well as on the DI *m/z* 92, which is common for both compounds in the negative ion mode (Fig. 6). Moreover, syringic acid (**14**) produced the ions *m/z* 166 and 123 as DI, via direct loss of two $CH_3^{\bullet}$ from the deprotonated molecule, and loss of $CH_3^{\bullet}$ elimination followed by CO_2 elimination, respectively. *Trans*-cinnamic (**15**), caffeic (**16**), and three coumaric acid isomers (**17-19**) were also shown to eliminate CO_2 from the deprotonated molecule (Gómez-Romero et al., 2011).

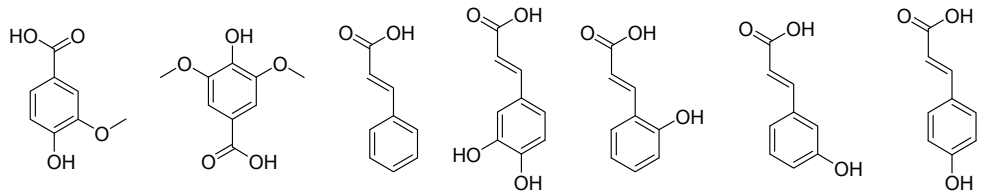


Fig. 6. Structures of some hydroxybenzoic acids (**13** and **14**) and cinnamic acid derivatives (**15-19**).

4.2.2 Caffeoylquinic acid (CQA) and feruloyl quinic (FQA) derivatives

Miketova and co-workers have published a study on the fragmentation of protonated and deprotonated 3,5- and 4,5-dicaffeoylquinic acid (di-CQA) derivatives using electrospray ionization tandem mass spectrometry (Miketova et al., 1999). They demonstrated that both the positive and negative ion modes of analysis allow for identification of the ester groups bound at the quinic acid moiety, although the negative ion mode is the most informative method of analysis for the free compounds. The presence of a phenolic hydroxyl at the *ortho* or *para* position was proposed to be important for the formation of some diagnostic ions in the negative ion mode, as shown in Figure 7.

Gobbo-Neto & Lopes have reported on a sensitive analytical method for the dereplication of various classes of secondary metabolites found in the *L. ericoides* leaf extracts, including caffeoylquinic acid (CQA) and feruloyl quinic (FQA) derivatives (Gobbo-Neto & Lopes, 2008). The authors employed a methodology based on HPLC coupled with a diode array detector (HPLC-DAD) and HPLC coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS) using collision-energy values ranging between 10 and 25 eV, in combination with co-injection of authentic standards and accurate-mass measurements. The chemical structures of the identified compounds are depicted in Fig. 8.

For chlorogenic acids (**20**, **22** and **23**), the authors utilized the ion with *m/z* 353 as precursor ion and reported that the fragment ion at *m/z* 173, which is a result of water elimination from the quinic acid moiety, is diagnostic for CQA derivatives esterified at position 4. The CQA derivatives substituted at position 5 and di-CQA isomers (3,5- and 4,5-substituted) were confirmed by co-elution with authentic standards. HPLC-DAD analysis was used in combination accurate mass measurements for identification of feruloylquinic acids (FQA) and feruloyl-caffeoylquinic acids (FCQA). The ion at *m/z* 367 was used as the precursor ion, yielding the fragment ion with *m/z* 173, which is diagnostic for 4-FQA derivatives. 3-FQA and 5-FQA were identified on the basis of the ions *m/z* 193 and 191, respectively, as well as

by comparison with previously reported studies (Clifford et al., 2003; Clifford et al., 2005). On the other hand, the FCQA isomers could not be identified, even when DAD, ESI-MS/MS in the positive and negative ion modes, and accurate mass data were employed.

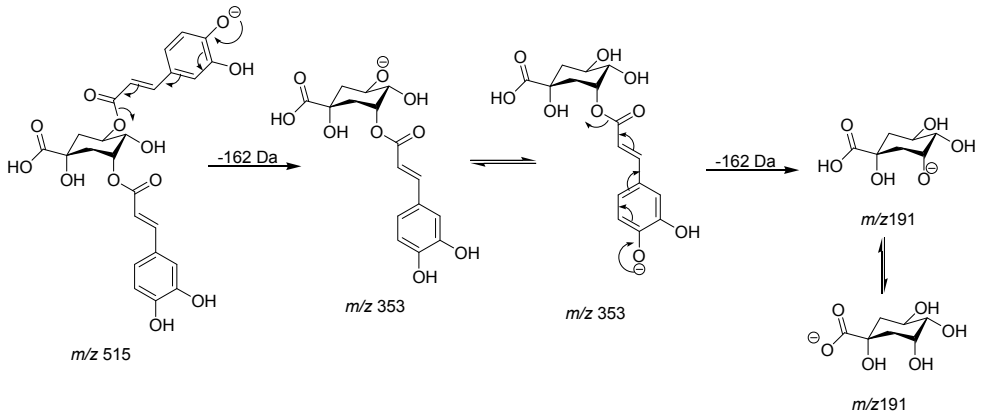
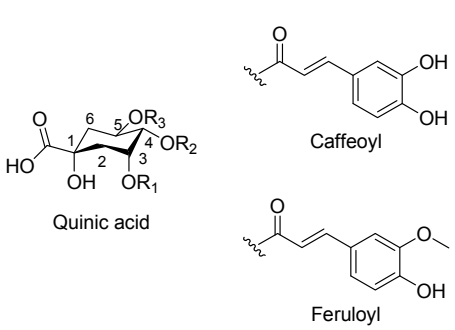


Fig. 7. Fragmentation of 3,5-dicaffeoylquinic acid (Miketova et al., 1999).



	R_1	R_2	R_3
20	caffeoyl	H	H
21	feruloyl	H	H
22	H	caffeoyl	H
23	H	H	caffeoyl
24	H	feruloyl	H
25	H	H	feruloyl
26	caffeoyl	caffeoyl	H
27	caffeoyl	H	caffeoyl
28	H	caffeoyl	caffeoyl
29	feruloyl	feruloyl	H
30	caffeoyl	caffeoyl	caffeoyl

Fig. 8. Structures of the caffeoylquinic and feruloylquinic acid derivatives identified in the *L. ericoides* leaf extract.

4.2.3 Lignans

Lignans are a group of secondary metabolites consisting of compounds containing two phenylpropanoid units linked by oxidative coupling between C-8 and C-8′ in their structures (Willfor et al., 2006). Several biological activities, such as anti-tumoral, fungicide, and anti-viral actions, have been attributed to the presence of these compounds, (Ayres & Loike, 1990; Botta et al., 2001; Pan et al., 2009).

Shengmai San (SMS), a prescription comprised of *Panax ginseng*, *Schisandra chinensis*, and *Ophiopogon japonicus*, has long been used in traditional Chinese medicine. Wang and co-workers have employed HPLC-DAD-MS/MS for identification of the multiple SMS constituents (Wang et al., 2011). These authors identified 53 compounds, including 21 lignans, 6 steroidal glycosides, and 12 homoisoflavonoids. Twenty-seven compounds were

identified by comparison of their retention times, MS data, and UV spectra with those of authentic compounds, and the other twenty-six were tentatively identified by comparing their UV spectra, molecular weights, and structural information from MS/MS spectra with those previously published in the literature. Studies on the dereplication of lignans in the ethanol extract from the fruits of *Schisandra chinensis* have also been conducted by He and co-workers using high-performance liquid chromatography coupled with a photodiode-array detector and an electrospray ionization ion source. The authors identified seventeen dibenzylcyclooctadiene lignans on the basis of the relative intensities of protonated and cationized molecules, their UV spectra, and the respective retention times (He et al., 1997).

Zheng and co-workers have utilized HPLC coupled with an ESI ion source and a hybrid ion IT-TOF mass analyzer for dereplication of lignans in *Panax ginseng*, *Radix ophiopogonis*, and *Schisandra chinensis baill* extracts, which are used in traditional Chinese medicine for treatment of tumoral diseases, coronary atherosclerosis, and some other cardiopathies (You et al., 2006; Yu et al., 2007). They reported that elimination of $CH_3^{\bullet}$ or $^{\bullet}OCH_3$ from the protonated molecule is diagnostic of a methoxyl group at rings A or B of lignans, and that there is not an oxygen atom on ring C, as in the case of schizandrin A (**31,** Fig. 9). Elimination of C_5H_{10} by cleavage of the eight-membered ring to produce a five-membered ring was also reported. On the other hand, elimination of a water molecule (loss of 18 Da) produces the diagnostic product ion (DPI) for lignans that have a hydroxyl group at C-6 or C-7 of ring C, such as shizandrol A (**32**).

Lignans that have a hydroxyl group at C-7 of ring C and an ester function at rings A or B, such as angeloyl gomisin H (**33**) , have been reported to fragment by elimination of H_2O and an olefinic ketene (C_5H_6O or C_7H_4O), the latter being associated with the presence of the ester at rings A or B. Elimination of the phenol ester as an olefinic ketene from lignans using electron ionization (EI) has been previously reported by Zhai & Cong (Zhai & Cong, 1990). In addition, the authors described that the formation of the sodiated molecule ($[M+Na]^+$) is a more favored ionization process, as compared to protonation, for lignans that have a hydroxyl group at C-7 and an ester bound at C-6 of ring C, such as schisantherin A (**34**). In the case of compound **34**, elimination of the corresponding carboxylic acid (e.g., benzoic acid, $C_7H_6O_2$) is the major fragmentation process (Zheng et al., 2009).

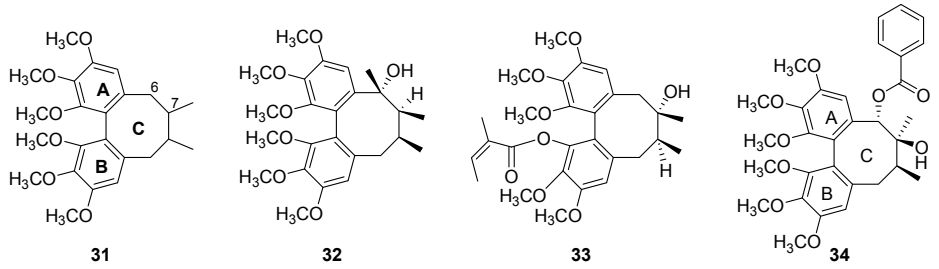


Fig. 9. Structures of dibenzylcyclooctadiene lignans schizandrin A (**31**), schizandrol A (**32**), angeloyl gomisin H (**33**), and schisantherin A (**34**).

4.2.4 Flavonoids

Flavonoids are an important class of secondary metabolites that are biosynthesized by plants. They are related to protection against predators and UV-Vis light, attraction of

pollinators, and antioxidant and hormonal control, among other functions (Dewick, 2004). They also have economic importance because they can be used as pigments, tanning substances, nutritional complements, and food flavors. Moreover, they display pharmacological properties, such as anticarcinogenic, anti-inflammatory, allergen, antiviral, and anti-ulcerogenic actions, among others (Simões et al., 2004).

The fragmentation of aglicone and glycoside flavonoids by MS/MS has been extensively investigated. The major fragment ions result from different retrocyclization cleavages (e.g., retro-Diels-Alder reactions, RDA), as shown in Fig. 10. The nomenclature adopted for the RDA cleavages was firstly proposed by Ma and co-workers (Ma et al., 1997; Ma et al., 1999). The superscripts on the left of rings A or B indicate the bonds that have been broken.

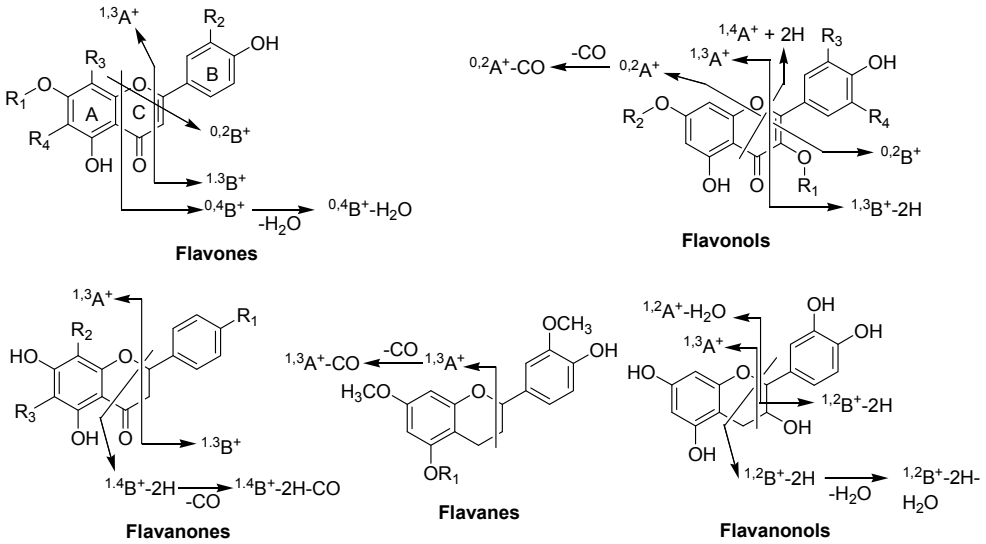


Fig. 10. Main retrocyclization cleavages of some classes of flavonoids.

Wolfender and co-workers have proposed an LC-MS/MS methodology for the online characterization and dereplication of selected commercial and isolated flavonoids (Wolfender et al., 2000). ESI-MS/MS experiments were carried out in hybrid quadrupole time-of-flight (Q-TOF) and ion trap (IT) mass spectrometers. In the IT mass analyzer, the precursor ions are trapped in a short space for varied periods of time, which allows for an increased number of collisions to take place between the collision gas and the precursor ions, thus raising the internal energy of these ions. On the other hand, in the q-TOF mass analyzer the interactions between the precursor ion and the collision gas occur during short periods of time, so that a spatial separation between the ionization and the collision induced dissociation (CID) process comes into effect, thereby diminishing the collision energy. Due to these differences, the MS/MS spectra obtained using IT and q-TOF mass analyzers are different from each other. In order to improve the efficiency of the transmission energy, the precursor ions are accelerated in a linear beam instrument, promoting formation of different fragment ions in both cases. The authors reported that the higher energy in Q-TOF causes hard fragmentation of flavonoids, so that some fragment ions may not be observed using IT, which compromises the feasibility of using

both techniques for dereplication. Considering the flavone apigenin (**35,** Fig. 11) described in that work, the authors firstly approached the generation of ring cleavages in the positive ion mode. In the positive IT-MS mode at 35% energy, only the ion with *m/z* 153 was detected, while the same fragments produced during Q-TOF were achieved when the energy was amplified up to 50%(Wolfender et al., 2000).

Rak and co-workers have put forward a strategy for the dereplication of flavonoids in a sample of commercial black currant juice without any preliminary study about its components. This strategy was based on the fact that most of the flavonoid derivatives have an aglycone part. Firstly, the authors developed a procedure for detection of aglycone flavonoids in the juice chemical constituents using multiple reaction monitoring (MRM) in the negative ion mode. In this type of scan, both the precursor and the product ion are specified for the detection of only one pair at the detector (Sleno & Volmer, 2004). Considering the great number of possible aglycone derivatives, the authors chose apigenin (**35**), luteolin (**36**), quercetin (**37**), myricetin (**38),** and naringenin (**39**), which are amongst the commonest aglycone flavonoids (Fig. 11). In addition, the authors performed the experiment using a high declustering potential (DP) with some standard solutions of aglycone analogues, aiming to minimize formation of cluster ions from solvents. The authors showed that the major compounds in the black currant juice can be identified by selecting the characteristic *m/z* values, in combination with their retention times. They also conducted full scan MS experiments, which evidenced that odd-electron fragment ions resulting from homolytic cleavages were more abundant than even-electron ion fragment ions when the highest negative DP values were employed. The tendency toward radical fragmentation reactions was confirmed by MRM experiments, which were accomplished in parallel with the chromatographic run (Rak et al., 2010). A third chromatographic run was carried out on a quadrupole linear ion trap apparatus, in order to confirm the structure of the precursor ions on the basis of diagnostic fragment ions. Twelve flavonoid derivatives were identified in the sample of black currant juice, demonstrating the great versatility of these MS techniques for the dereplication of flavonoids (Rak et al., 2010). However, the authors reported some difficulties in distinguishing between isomer flavonoids using this methodology.

	CID	Precursor ion	Fragment ions					
			$^{1,3}A^+$	$^{1,3}B^+$	$^{0,2}B^+$	$^{0,4}B^+$	$^{0,4}B^+$-H_2O	Main ion
q-TOF MS-MS (POS)	25	271	153 (25)	119 (5)	121 (2)	163 (2)	145 (2)	271 (100)
	30		153 (85)	119 (20)	121 (10)	163 (10)	145 (10)	271 (100)
	35		153 (100)	119 (35)	121 (20)	163 (10)	145 (15)	153 (100)
IT POS MS	35	271	153 (4)	-----	-----	-----	-----	271 (100)
	40		153 (35)	119 (5)	121 (3)	-----	145 (5)	217 (100)
	50		153 (100)	119 (15)	121 (10)	163 (5)	145 (15)	153 (100)
	60		153 (100)	119 (20)	121 (10)	163 (3)	-----	153 (100)
IT NEG MS	50	269	151 (20)	117 (10)	-----	-----	-----	225 (100)

Table 1. MS/MS data of protonated and deprotonated apigenin (**35**).

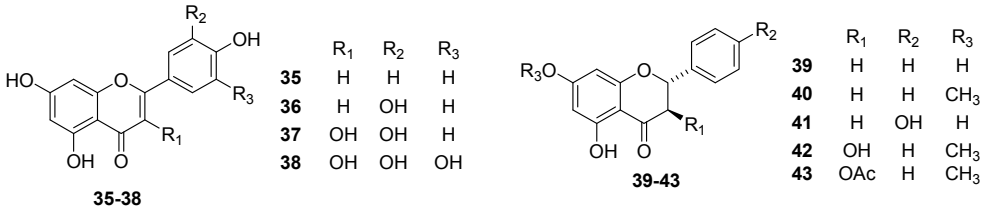


Fig. 11. Structure of some aglycone flavonoids.

For the online identification of aglycon flavonoids present in the hydroalcoholic extract of *L. ericoides* leaves, Gobbo & Lopes have compared the retention time, the UV spectra and the accurate mass of each peak of the chromatogram with authentic standards, besides comparing the MS data with those previously published in the literature (Gobbo-Neto & Lopes, 2008). A peak exhibiting absorbance maximum above 340 nm, which is characteristic of flavanones previously identified in *L. ericoides* (e.g, pinocembrin, **40;** and pinostrombin, **41**) was identified in the MS/MS spectrum of the deprotonated molecule, as well as by comparison with literature results (Cuyckens & Claeys, 2004; Fabre et al., 2001; Ma et al., 1997; Zhang & Brodbelt, 2003). MS/MS spectrum of the corresponding protonated molecule displayed the base peak at *m/z* 153, which indicates a dihydroxyl substituent at ring A of pinocembrin (**40**). On the other hand, the presence of a fragment ion at *m/z* 167, together with the absence of diagnostic ion at *m/z* 153, indicates a hydroxyl and a methoxyl substituent at ring A, indicative of the presence of the flavanone pinostrombin (**41**) (Gobbo-Neto & Lopes, 2008). Pinobanksin (**42**) was identified on the basis of its UV spectra, comparison with retention time of previously isolated standards, and accurate mass data. 3-*O*-acetylpinobaskin (**43**) was elucidated by comparison with pinobanksin ($[M+H]^+$ at *m/z* 313), being the mass difference due to the methyl group atom bound at the phenol oxygen. Clearly, the substance was assigned as 3-*O*-acetylpinobaskin not only because of the difference of 14 mass units at PI, but also because of the appearance of the same product ions in 3-*O*-acetylpinobaskin, which differs by 14 mass units from the pinobaskin product ions (*e.g.*, the diagnostic product ion of pinobaskin, *m/z* 153, presented a difference of 14 mass units from 3-*O*-acetylpinobaskin, *m/z* 167, thus confirming the presence of an acetyl group instead of a hydroxyl group in ring A).

Waridel and co-workers have proposed an LC-MS/MS methodology for the differentiation between C-6 and C-8 glycoside flavonoids (Waridel et al., 2001). Firstly, the authors analyzed standards of selected flavonoids (Fig. 12) in a reverse-phase C_{18} column using isocratic elution with acetonitrile/water 4:1 containing 0.5% acetic acid. Low energy CID experiments were performed on ion-trap (IT) and hybrid quadrupole time-of-flight (Q-TOF) instruments. ESI and APCI were used in both the positive and negative ion modes of analysis. The optimal CID collision energy was chosen so that the same MS/MS spectral profile would be generated and the relative intensities and *m/z* values of the fragment ions could be compared as a result of the structural differences. The authors postulated the distinction between C-8 and C-6 glycoside flavonoids on the basis of the relative intensity of some peaks of the MS/MS spectra. Taking into account the stabilities of the fragment ions, which were considered to be associated with their respective relative intensities, the authors reported that loss of H_2O and formation of *m/z* 379 are processes that are more favored for C-6 isomers (e.g., isovitexin, **44**; and isoorientin, **45**) than for C-8 isomers (e.g., vitexin, **46**; and orientin, **47**). Another proposition to distinguish between C-6 and C-8 glicoside

flavonoid isomers was based on the intensity of the product ion $[M+H-120]^+$, which had low intensity when the skimmer voltage (to produce *in source* dissociation) was not employed, but was the base peak when the skimmer voltage was used. The authors selected the ion $[M+H-120]^+$ as the precursor ion in Q-TOF and used the multiple-stage mass spectrometry (MS^n) in IT. The results revealed a large difference between the isomers for the collision energy at 30 eV or 50%, which enabled distinction by hard fragmentation and some elimination reactions, specific for each isomer (Fig. 12). In the case of Q-TOF, the product ion *m/z* 283, which results from CH_2O loss from $[M+H-120]^+$, was the base peak in the MS/MS spectra of both C-6 and C-8 isomers. On the other hand, when IT was employed, the product ion at m/z 283 was the base peak for C-8 isomers, whereas the product ion at *m/z* 295 $[M+H-120-H_2O]^+$ was the base peak in the MS/MS spectrum of C-6 isomers. The authors also reported that differentiation between C-6 and C-8 glycoside flavonoid isomers in the negative ion mode was not possible when the MS/MS experiments were performed in the ion trap equipment, once CO elimination from $[M-H-120]^-$ is the only fragmentation process for both isomers. The fragmentation map proposed by the authors is illustrated in Fig. 13 (Waridel et al., 2001). Nevertheless, the main difficulty reported by the authors was method standardization, because screening and online identification of these compounds requires previous optimization of the parameters of both IT and q-TOF apparatus.

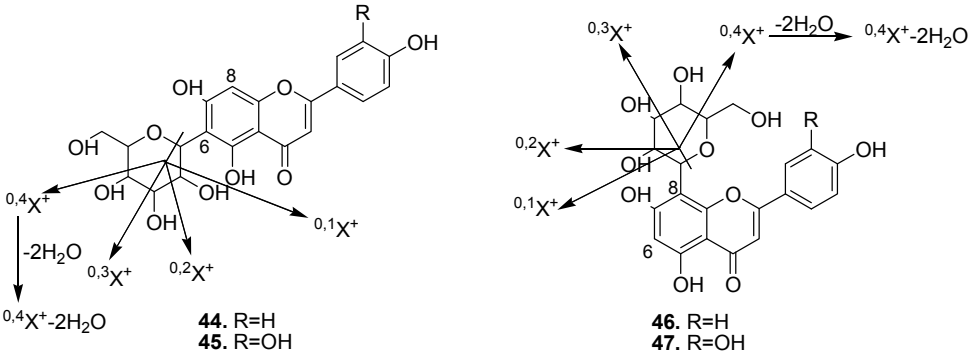


Fig. 12. Structure of C-6 (**44** and **45**) and C-8 (**46** and **47**) glicoside flavonoid isomers.

One of the most important steps in dereplication studies of natural products by LC-MS is the chromatographic separation process. Resolution (e.g, the distance between two adjacent peaks in the chromatogram) is essential for the correct interpretation of the subsequent UV-DAD, MS and MS/MS data. The addition of formic or acetic acid to the LC mobile phase (usually methanol/water or acetonitrile/water) has been the most used analytical strategy to improve the chromatographic resolution in HPLC. Shi and co-workers have identified glycoside flavonoid isomers in crude extracts of *Fructus aurantii* and *Fructus aurantii immaturus* using HPLC-UV-MS^n (Shi et al., 2007). The chromatographic separation between the compounds **48-50** on reverse phase C_{18} column was achieved by using a mixture of acetonitrile and water/formic acid (0.1%) and applying a linear gradient from 10% and 95% of water/formic acid (0.1%). The differentiation between the compounds was made on the basis of the comparison of retention times, maximum UV wavelengths, and MS and MS/MS data of each individual peak with those of authentic standards. MS/MS spectrum of deprotonated **49** (*m/z* 593) displayed the fragment ion $[M-H-308]^-$, suggesting elimination of

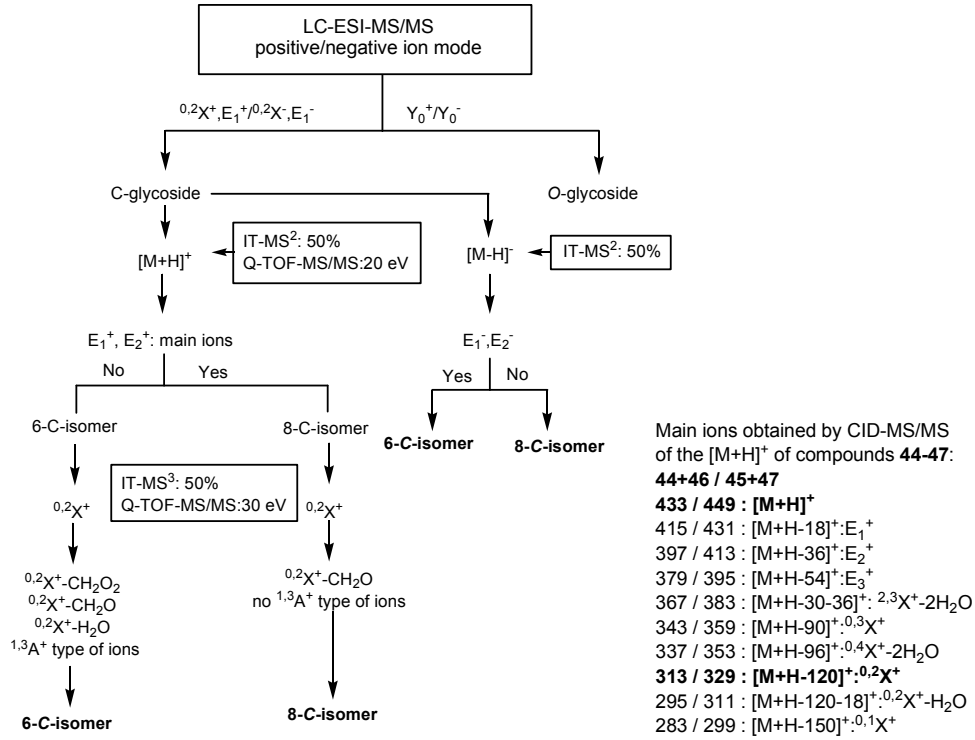


Fig. 13. Fragmentation map proposed for the differentiation between C-6 and C-8 glicoside flavonoid isomers (Waridel et al., 2001).

a rutinose molecule, whereas its MS^3 spectrum showed the same profile as that of the aglycone of poncirin (**50**) (Figure 14). Moreover, the retention time of didymin (**49,** t_R=46,1 min) in the total ion chromatogram was higher than hespiridin (**48**, t_R=32,9 min), thus indicating a structure with fewer oxygen atoms as compared to **48**.

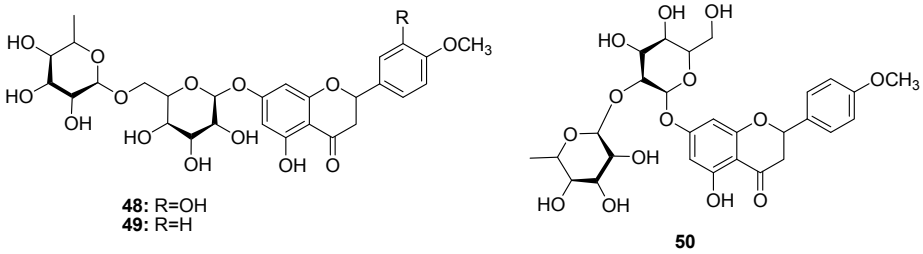


Fig. 14. Chemical structures of hesperidin (**48**), didymin (**49**) and poncirin (**50**).

4.3 Alkaloids

Alkaloids comprise a vast group of secondary metabolites found mainly in higher plants, such as angiosperms (Cordell et al., 2001). Although there is no general definition that

encompasses the great structural diversity of this class of NPs, it is known that these compounds exhibit alkaline properties due to the nitrogen atoms found in their cyclic skeleton. Another chemical property of alkaloids is their solubility in aqueous solutions when salt complexes are formed in the presence of mineral acids (Cordell et al., 2001; Dewick, 2004).

Zhou and co-workers have proposed a methodology involving ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) for the screening of some pyrrolizidine alkaloids exhibiting toxic effects against human beings, such as fetal problems and hepatotoxic and tumorigenic effects (Zhou et al., 2010). The pyrrolizidine alkaloids were extracted directly from the powdered plant using diluted hydrochloric acid, followed by mixed-phase cation exchange (MCX) and solid-phase extraction (SPE) and elution with methanol/ammonia 3:1 (v/v). After solvent removal, the residue was analyzed by UPLC coupled with DAD and triple quadrupole tandem mass spectrometry, equipped with an electrospray ion source operating in the positive ion mode. The authors demonstrated that the fragment ions with *m/z* 150 and *m/z* 168 are diagnostic for pyrrolizidine alkaloids belonging to the Otonecine type group (e. g., **51** and **52**), whereas the fragment ions *m/z* 120 and *m/z* 138 are diagnostic for compounds of the Retronecine type (e.g., **53-57**), as represented in Fig. 15. Some pyrrolizidine alkaloids analogs (e.g., compounds senkirkine, **51;** and clivorine, **52**) that have fragment ions in common could not be distinguished from each other on the basis of their precursor ion spectra only. In this case, dereplication was achieved by combining data from the spectra of the precursor ion with those of multiple reaction monitoring (MRM) experiments.

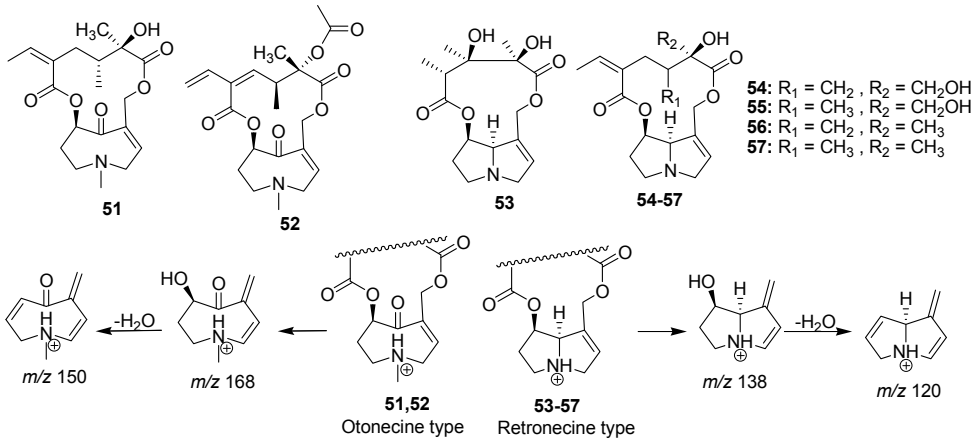


Fig. 15. Structure of pyrrolizidine alkaloids of the Otonecine and Retronecine type, and formation of their diagnostic fragment ions.

Pivatto and co-workers have utilized accurate mass electrospray ionization tandem mass spectrometry for the offline dereplication of selected piperidine alkaloids from flowers and fruit extracts of *Senna spectabilis*, which is commonly used in traditional medicine as anti-inflammatory, analgesic, laxative, antimicrobial, and antiulceral agent in some countries (Samy & Ignacimuthu, 2000; Viegas Jr. et al., 2004). The authors obtained the ethanol extract from powdered dried flowers and green fruits, followed by redissolution with aqueous

H_2SO_4 and washing with *n*-hexane. The acid residue was basified with NH_4OH (pH 9) and then extracted with dichloromethane. The analysis showed that some protonated compounds have common mass losses (e.g., loss of H_2O) and known fragment patterns (e.g., the charge-induced fragmentation that forms the acylium ion in *p*-coumaroyl derivatives), as shown in Fig. 16. Also, the authors reported that the extracts obtained from flowers contained more oxygen atoms than those obtained from fruits. On the other hand, the formation of some ions as evidenced in the spectrum of the precursor ion could not be explained on the basis of their ESI-MS/MS data, but they can also be of great importance when this technique is used in combination with NMR (Pivatto et al., 2005).

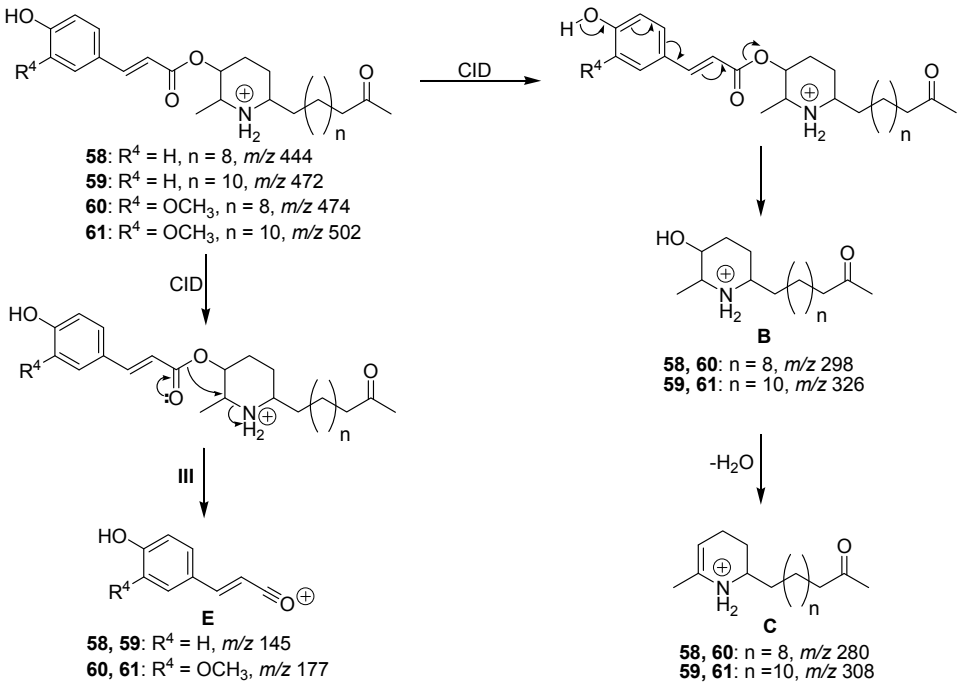


Fig. 16. Fragmentation of piperidine alkaloids identified in *Senna spectabilis* by ESI-MS/MS.

The dereplication of alkaloids has also been achieved by means of UV-Vis spectroscopy in combination with tandem mass spectrometry (MS/MS). Fabre and co-workers have studied a direct and fast characterization of isoquinoline alkaloids from the aerial parts of *Eschscholtzia californica*, which is used in European folk medicine as analgesic, anodyne, diaphoretic, diuretic, soporific, and spasmolytic agent (Fabre et al., 2000). Firstly, the authors analyzed authentic standards of the isoquinoline alkaloids berberine (**62**) and papaverine (**63**) by HPLC and ESI-MS/MS using direct infusion and collision-induced dissociation, aiming to identify possible diagnostic ions for each compound. After that, the powdered aerial parts of *E. californica* were extracted with MeOH for 15 min at 60°C. The residue obtained after solvent elimination was dissolved in aqueous HCl (pH 1), then dodecylsulfate sodium salt was added and further extracted with chloroform (three times). The organic phase was concentrated and dried, then suspended in MeOH and analyzed by high-

performance liquid chromatography coupled with a diode array detector (HPLC-DAD) and HPLC-ESI-MS/MS. The UV and the MS/MS spectra of each peak of the chromatogram were compared with those of the authentic standards. They found that the fragment ions with *m/z* 177, 205, 235, 283, and 293 are diagnostic of compounds **64** and **65**; whereas the fragment ions *m/z* 235 and 263 are diagnostic for compounds **66** and **67** (Fabre et al., 2000). The major compounds identified in the crude extract were **64**, **65**, and **71**. The structures of the identified alkaloids are summarized in Fig. 17.

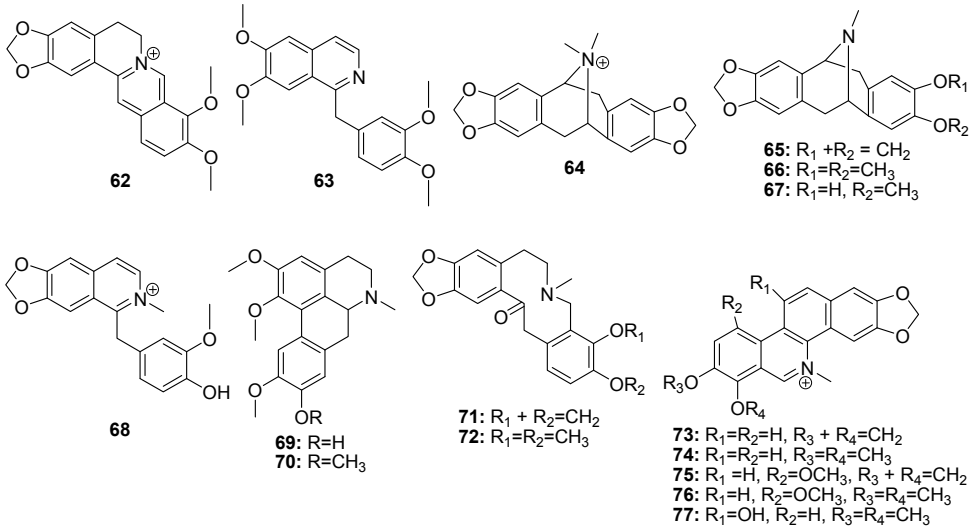


Fig. 17. Structures of the isoquinoline alkaloids identified in the crude methanol extract of aerial parts of *Eschscholtzia californica* (Fabre et al., 2000).

5. Concluding remarks

The use of electrospray ionization tandem mass spectrometry (ESI-MS/MS) for the online and offline dereplication of natural products (NPs) has been discussed herein. Online identification using ESI-MS/MS coupled with liquid chromatography with UV-DAD as first detection has shown to be a more powerful technique as compared to the sole use of ESI-MS/MS. Structural determination is usually achieved on the basis of a combination between retention times, UV and MS/MS spectra and comparison with data of a previously established library of authentic standards. However, this strategy is still limited because previously isolated (or commercially acquired) standards are necessary, not to mention the difficulty in establishing reliable libraries using MS/MS data acquired on different equipments. These are the main challenges to be overcome in coming years in this research field.

6. Acknowledgment

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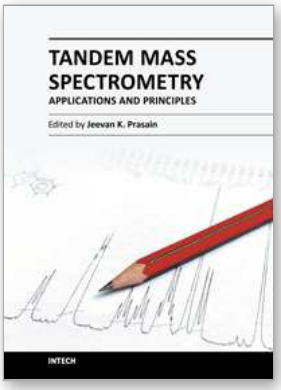

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