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An integrated high resolution mass spectrometric and informatics approach for the rapid identification of phenolics in plant extract

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ABSTRACT

An integrated approach based on high resolution MS analysis (orbitrap), database (db) searching and MS/MS fragmentation prediction for the rapid identification of plant phenols is reported. The approach was firstly validated by using a mixture of phenolic standards (phenolic acids, flavones, flavonols, flavanones, flavanols, isoflavones). In particular, the integrated approach consists of the following steps: (1) LC–ESI-MS/MS analysis in data dependent scan mode using an orbitrap mass analyzer (resolution 60,000; positive ion-mode, ESI source); (2) searching the experimental monoisotopic masses (tolerance 1 ppm) in plant phenols databases; (3) filtering the entries on the basis of the phenol class to which the unknown belongs, as determined on the basis of the UV spectrum. Final identification is achieved by matching the isotopic pattern and by MS/MS fragmentation studies. In particular, experimental MS/MS fragments are matched with those predicted by a commercially available software. The method was then successfully applied for the rapid identification of phenolics contained in an EtOH extract of *Angelica keiskei*.

1. Introduction

Phenolic compounds such as phenolic acids, tannins and flavonoids are broadly distributed in plants and are contained as natural ingredients of several beverages and manufactured foods. Although traditionally their interest has mainly been related to their organoleptic properties, such as color, astringency, bitterness or taste [1], in recent decades they are increasingly being recognized for their nutritional value for reducing the risk of chronic diseases and promoting optimal health [2–5]. Due to their biological value, the identification and characterization of phenolic compounds in plants and manufactured foods represent an important analytical issue, which has blossomed during the last decades. High performance liquid chromatography (HPLC) coupled with electrochemical, UV or fluorescent detectors was the analytical technique of choice for the separation and characterization of plant phenolic compounds. The weakness of these analytical approaches resides in the detection methods, in particular, lack of structural information and specificity leading to the possibility of sample matrix interference and misinterpretation of unknown compounds. Moreover, these approaches require a high efficiency of separation since co-eluting compounds can be missed. In addition, an external standard is required for the structure confirmation [6].

Currently, HPLC coupled with mass spectrometry by atmospheric pressure chemical ionisation (APCI) or electrospray ionisation (ESI) source represents the most selective analytical technique for the identification and quantification of phenol compounds from plants and food. Identification of phenol compounds by mass spectrometry (MS) can be carried out in different ways and the most used are full scan and tandem MS which can be run at the same time (data dependent scan mode) [7–9]. The full scan analysis permits to reveal the molecular weight (MW) of the unknown compounds, while tandem MS, the fragmentation pathway. Ion-trap mass spectrometers can carry out sequential fragmentations of the parent molecular ion followed by the *n* daughter ions. Hence, by using this approach, unknown compounds are identified on the basis of UV spectra, nominal MW and MS/MS fragmentation. The LC–ESI-MS approach leads to several other advantages such as the HPLC separation is less crucial than before since co-eluting compounds are scanned separately and identified [6].

Although LC–ESI-MS approach is a powerful technique, phenolics identification is very often time consuming and it requires a deep knowledge in MS/MS fragmentation and plant phenols chemistry.

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Therefore, the aim of the current study is to set-up, validate and apply a novel approach able to greatly facilitate and speed-up the identification of plant phenols. The approach is based on high resolution MS analysis (orbitrap), database searching and MS/MS fragmentation prediction using a commercially available software.

2. Experimental

2.1. Chemicals and materials

Standard flavonoids and phenolic acid (apigenin, daidzein, quercetin, naringenin, catechin, chlorogenic acid) were purchased from Sigma–Aldrich Chemical Co. (Saint Louis, MO, USA). HPLC-grade and analytical grade organic solvents were purchased from the same supplier. HPLC-grade water was prepared using a Milli-Q (Millipore, Billerica, MA, USA) water purification system.

2.2. Sample preparation

Angelica keiskei was harvested from its major planted areas in Korea and sample processing was conducted as previously reported [10]. Extraction of free phenols was performed as following: 100 mg of dry powder of *A. keiskei* (prepared using 100 mesh sieve, Hankook Crusher Co. Ltd., HK-100, Haman-Gun, Korea) were extracted with 1 ml of EtOH:H_2O (70:30 v/v) and the supernatant was filtered with a 0.45 μm filter. The extraction procedure was repeated five times and the combined organic layer was evaporated to dryness using a rotary evaporator at room temperature. The residue was dissolved in 500 μl EtOH:H_2O (70:30 by vol), diluted 1:10 with formic acid (0.1%), and then analyzed by LC–ESI-MS.

2.3. LC/UV/ESI-MS analysis

LC/UV/ESI-MS analyses were performed using a Thermo Finnigan Surveyor LC system equipped with a quaternary pump, a Surveyor UV–vis diode-array programmable detector 6000 LP, a Surveyor autosampler, and a vacuum degasser and connected to an LTQ/orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separations were done by reversed-phase elution with a Phenomenex Sinergy MAX-RP column (150 × 2 mm, 4 μm; Torrance, CA, USA) protected by a MAX-RP guard column (4 × 2 mm, 4 μm; Phenomenex, Torrance, CA, USA) kept at 25 °C. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 0.2 ml min^{-1} (injection volume 90 μl). Samples were desalted and concentrated online by a Opti-Lynx trap cartridge (Optimize Technologies, Inc., Oregon City, OR, USA) at 0.2 ml min^{-1} with 100% of phase A for 10 min before the separation on the analytical column. The gradient was linearly from 0% to 40% B in 60 min, to 100% B from 65 to 70 min, and held at 100% B from 70 to 75 min. The composition of the eluent was then restored to 100% A within 2 min, and the system was re-equilibrated for 14 min. The mass spectrometer was equipped with an electrospray interface, which was operated in positive-ion mode and controlled by the Xcalibur software (version 2.0). The parameters of the ESI source were as follows: positive-ion mode spray voltage 4.0 kV, capillary voltage 49 V, capillary temperature 275 °C, tube lens 120 V. During the analysis, the mass spectrometer continuously performed scan cycles in which a high resolution (resolving power r = 60,000 at m/z 400) full scan (130–1500 m/z) in the profile mode was first made by the orbitrap, after which the MS^2 spectra were recorded in the centroid mode for the three most intense ions (isolation width, m/z 3; normalized collision energy, 30 collision-induced dissociation (CID) arbitrary units). For real time internal mass calibration, a list of common contaminants was used according to Keller et al. [11]. Dynamic exclusion was enabled (repeat count, 3; repeat duration, 10 s; exclusion list size, 25; exclusion duration, 120 s; relative exclusion mass width, 5 ppm).

2.4. Informatics

In order to simplify the data mining process of plant phenolics databases, a specific plug-in for the VEGA ZZ package [12] was developed in C++ programming language. The choice was due to the requirement to have a tool able to manage large databases and this software satisfies all needs to do it in fast and easy way. In particular, VEGA ZZ allows to play more than one database at the same time and it provides all tools to expand and update them. The plug-in, called MassTools, is a graphic interface to the SQLite relational database engine (http://sqlite.org) integrated in VEGA ZZ. This interface allows to submit queries including a limited number of search fields (monoisotopic mass and the related isotopic confidence range), that are a subset of all molecular properties stored in the general purpose databases managed by VEGA ZZ. The query results are shown in a report including monoisotopic mass, formula and molecule name and they can be sent automatically to Microsoft Excel (Microsoft Corporation. Redmond, WA, USA), copied to the clipboard and saved to a file (CSV and DIF formats). For each found molecule, it's possible to visualize the 3D structure and to show the isotopic distribution in an interactive chart, that can be compared with the experimental data through a matching algorithm.

The VEGA ZZ and the MassTools plug-in, including the flavonoid database used in this study, are free for academic non-profit use and they can be downloaded in two separated packages at http://www.vegazz.net.

2.4.1. Database

Two different flavonoid databases (db) were used; one was created by importing the polyphenols listed in the Phenol-Explorer data base and containing 502 polyphenols [13]; the second was built from 6850 molecules downloaded as 2D MOL files from http://www.metabolome.jp [14]. They were automatically converted to a SQLite database of 3D structures by VEGA ZZ. The 2D to 3D conversion was made by the built-in molecular mechanics engine, performing a mixed approach based on steepest descent and conjugate gradients minimizations, although it was not strictly necessary for the this study, but it could be needed in the next researches in which the 3D properties could be analyzed. During the conversion, some molecular properties (including the monoisotopic mass) were pre-calculated to improve the search performances.

2.4.2. MS/MS fragmentation prediction

Mass Frontier 5.1 (HighChem, Bratislava, Slovakia) software was used to predict MS/MS fragments and fragmentation pathways.

3. Results

LTQ Orbitrap XL is a hybrid mass spectrometer which combines extremely high mass accuracy (<5 ppm with external calibration) and resolution with the capability of multiple levels of fragmentation [15]. All these features are widely used in the proteomic field, and only recently applied for plant constituents identification [16–18].

Fig. 1 outlines the integrated mass spectrometric/informatics approach for the rapid identification of plant phenolics. The LC/UV/ESI-MS performed by using an orbitrap as a MS analyzer represents the first step able to retrieve accurate monoisotopic mass and isotopic mass distribution of the unknown compounds. The monoisotopic mass is then searched in a db of plant phenolics and the output is a list of compounds (list 1), which is then

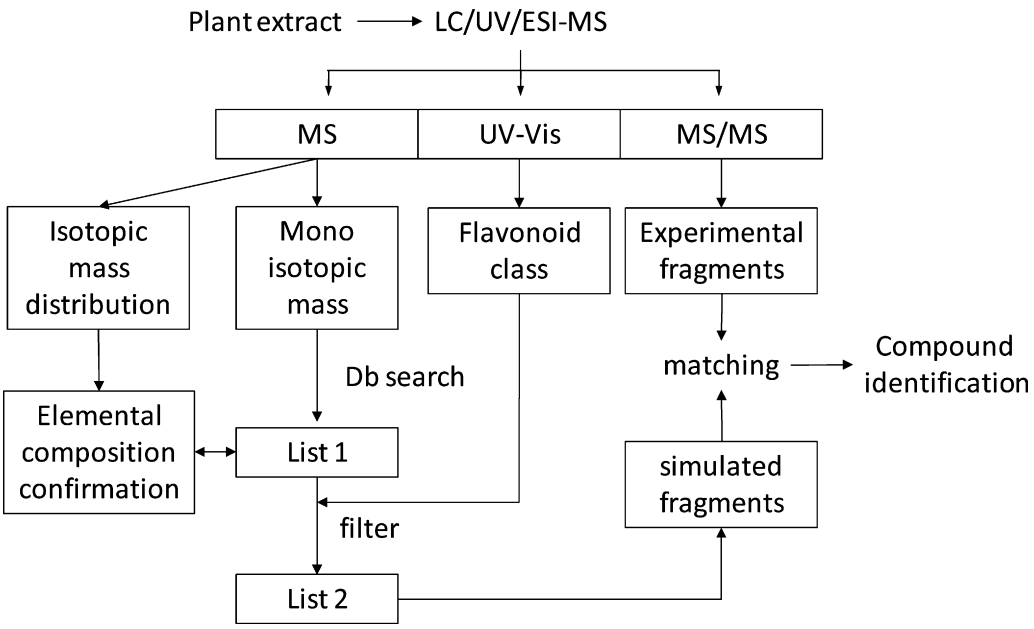

Fig. 1. Schematic representation of the integrated HPLC-UV/ESI-MS and informatics approach for the rapid identification of phenolics in plant extract.

filtered on the basis of the chemical class to which the unknown compound belongs, as determined on the basis of the UV–vis spectrum and on the well established UV absorption bands of phenolics [19]. For each entry belonging to the filtered list (list 2), simulated MS/MS fragments are predicted using the Mass Frontier software and the values are compared with the experimental fragments. The compounds are then ranked on the basis of the matched predicted/experimental fragments and the hit compound at the top of the list identifies the unknown. Final confirmation is then achieved by comparing experimental and simulated isotopic patterns.

The integrated approach was firstly validated by analyzing a mixture of 6 phenols belonging to different classes, namely: apigenin (flavones), daidzein (isoflavones), quercetin (flavonols), naringenin (flavanones), catechin (flavanols), and chlorogenic acid (phenolic acids).

Since in a plant extract the phenolics are contained in a wide concentration range, we firstly investigated the effect of concentration on mass accuracy, thus to establish the tolerance value to be set in db searching parameters. The standards mixture was prepared at four different concentrations ranging from 0.1 to 100 μM and analyzed by LC/UV/ESI-MS, after optimization of the mobile phase composition and separation conditions, thus to provide the best results in terms of peaks shape and ionization efficiency of the analytes.

The results reported in Fig. 2 well indicate that the accuracy at all the concentration levels was much lower than 1 ppm and ranged from 0.2 ppm to −0.7 ppm. Also at the lowest concentration tested, closed to the LOD (1/3 noise/signal ratio) for most of the compounds, the accuracy was significantly lower than 1 ppm. Hence a reliable accurate mass measurement was obtained over a wide range of concentrations. Such an accuracy supported by reliable measure isotopic abundances enables a confident assignment of elemental composition to individual peaks. On the basis of these results, 1 ppm was selected as a default tolerance value in db searching.

The identification of the standard phenols was firstly carried out by using a db created by importing the plant phenols listed in the Phenol-Explorer data base. Phenol-Explorer is the first comprehensive database on phenol content in foods. It includes weighed mean content values for 502 polyphenols in 452 foods and beverages, produced from more than 37,000 original composition data [13].

The first eluting peak (**S1**) displayed in the LC–ESI-MS/UV chromatogram and characterized by a retention time (RT) of 18.8 min, showed a monoisotopic mass at 290.07903 Da. The db search indentified only catechin/epicatechin. The assignment was then confirmed by the UV spectrum (λ_{max} = 280 nm), by the full match among simulated and experimental MS/MS fragments as well as by the isotopic mass distribution (Fig. 3), confirming the elemental composition $C_{15}H_{14}O_6$ (Table 1).

The second peak (**S2**) eluted at a RT of 18.91 min and showed a monoisotopic mass value of 354.09508 Da. Also for this compound, the db searching identified only one entry setting 1 ppm as mass tolerance, which is chlorogenic acid (Table 1). Compound identity was confirmed by the UV–Vis spectrum (290 sh, 326 nm) and by MS/MS spectrum showing the diagnostic product ion at *m*/*z* 163, which was predicted by fragments simulation. Final confirmation was achieved by the perfect match between simulated and experimental isotopic mass distribution confirming the molecular formula $C_{16}H_{18}O_9$.

The **S3** compound (RT 27.64 min) showed a molecular mass of 254.05791 Da and the db searching identified three compounds, two flavones (chrysin and 7,4′-dihydroxyflavone) and one isoflavone (daidzein). Daidzein was easily identified on the basis of UV spectrum and by MS/MS product ions prediction which sorted daidzein at the top of the list (6 fragments predicted), followed by 7,4′-dihydroxyflavone (four product ions) and chrysin (three product ions).

The next peak at a RT of 28.70 min (**S4**) showed a monoisotopic mass at 302.04265 Da. Three compounds were retrieved by db searching, and in particular two flavonols (quercetin and morin) and one flavone (hydroxyluteolin). The UV–vis spectrum excludes hydroxyluteolin and confirms quercetin or morin. MS/MS data provided the same information since only two experimental product ions among seven were predicted for hydroxyluteolin, while 6 peaks matched for both the flavonols. Hence, the similarity of UV and MS data did not allow to distinguish between the two isomers. Quercetin was finally assigned by a manual examination of the MS/MS spectrum, revealing a diagnostic product ion at *m*/*z*

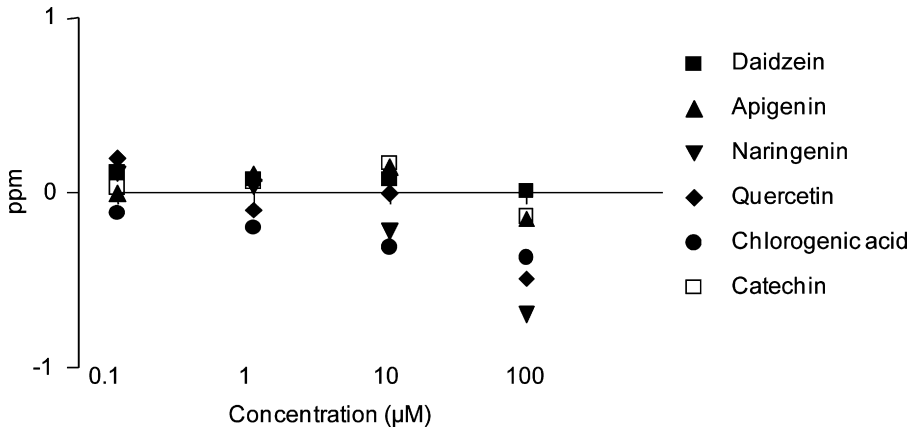

Fig. 2. Mass accuracy of standard plant phenols at four different concentrations (0.1–100 μM).

219 $[M+H-2C_2H_2O]^+$ which was reported to be characteristic for quercetin only [20].

The db searching of the compound **S5** (RT 30.8 min; monoisotopic mass at 272.06847 Da) identified two candidates characterized by the molecular formula $C_{15}H_{12}O_5$, namely butein and naringenin. Butein (chalcones) was easily excluded on the basis of the UV spectrum, while naringenin was correctly identified on the basis of the UV spectrum and MS/MS fragmentation study, showing that all the eight experimental product ions were predicted.

The last peak (**S6**; RT of 31.2 min) showed a monoisotopic mass at 270.05282 Da and the db search identified 5 hits bearing the same molecular formula ($C_{15}H_{10}O_5$), which was then confirmed by isotopic mass distribution. The UV spectrum of the unknown compound, being characterized by two intense peaks at 267 and 336 nm, indicated a flavone derivative. List 1 was then filtered on the basis of UV–vis data and galangin (flavonol) and genistein (isoflavone) were excluded. As shown in Table 1, the filtered list 2 contains three entries, apigenin, baicalein and 7,3′,4′-trihydroxyflavone and the MS/MS product ions for each of these compounds were then predicted. The similarity between predicted and experimental product ions sorted apigenin at the top of the list (three product ions matched, while for both baicalein and 7,3′,4′-trihydroxyflavone only one). On the basis of the data, the peak at 31.2 min was correctly identified as apigenin.

The method was then applied for the identification of phenolics contained in an EtOH extract of *A. keiskei*. We recently reported the identification of *A. keiskei* phenol compounds by using a conventional LC/UV/ESI-MS approach and using an ion-trap as MS analyzer [10]. Phenolics identification was achieved on the basis of the following data: nominal mass, UV and MS/MS data. Structure assignment was time consuming since it required a deep skill in MS/MS fragmentation rules and in general a strong background

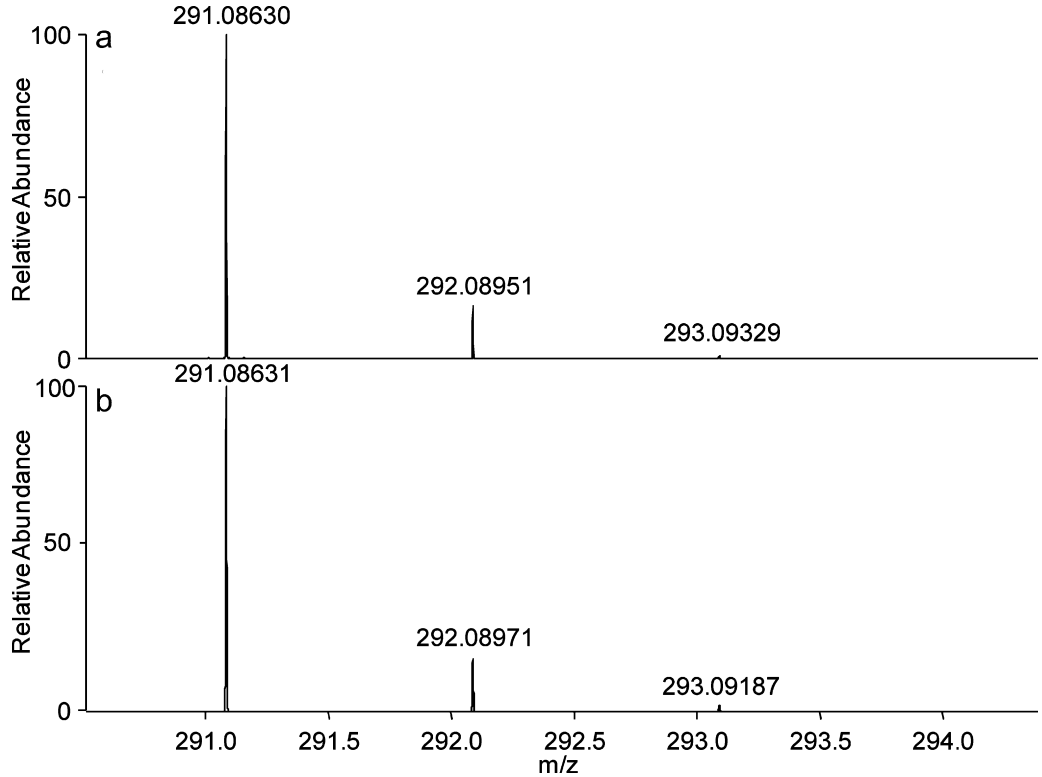

Fig. 3. Experimental (a) and simulated (b) isotopic mass distribution of catechin. The simulated spectrum (60,000 resolving power) was calculated by considering the formula $[C_{15}H_{14}O_6+H]^+$. The simulated spectrum was obtained by the isotope simulation feature of Qual Browser v. 2.0.7.

Table 1
Db searching, UV–vis data and experimental and predicted MS/MS data for flavonoids and phenolic acid standards.

Peak	Mono-isotopic mass value (Da)	List 1	UV–vis (nm)	List 2	Matched experimental/simulated MS/MS fragments	Hit compound
S1	290.07903	• (+)-Catechin	234, 279	• (+)-Catechin	• 119, 123, 139, 151, 165, 249, 273	(+)-Catechin
		• (−)-Epicatechin		• (−)-Epicatechin	• 119, 123, 139, 151, 165, 249, 273	(−)-Epicatechin
S2	354.09508	• Chlorogenic acid	240, 298 sh, 326	• Chlorogenic acid	• 163	Chlorogenic acid
S3	254.05791	• 7,4′-Dihydroxyflavone	261(sh), 301	• Daidzein	• 119, 121, 137, 227, 237, 199	Daidzein
		• Chrysin				
		• Daidzein				
S4	302.04265	• 6-Hydroxyluteolin	256,266(sh)370	• Morin	• 109, 137, 153, 165, 247, 275, 285	Quercetin
		• Morin		• Quercetin	• 109, 137, 153, 165, 247, 275, 285	
		• Quercetin				
S5	272.06847	• Butein	288, 331(sh)	• Naringenin	• 119, 147,153, 163, 179, 189, 231, 255	Naringenin
		• Naringenin				
S6	270.05282	• Galangin	267, 336	• 7,3′,4′-Trihydroxyflavone	• 243	Apigenin
		• Genistein		• Apigenin	• 119, 153, 243	
		• 7,3′,4′-Trihydroxyflavone		• Baicalein	• 243	
		• Apigenin				
		• Baicalein				

in plant phenols chemistry. In the present study, the EtOH extract of *A. keiskei* was re-analyzed by using an orbitrap as MS analyzer and phenolics identification was carried out by the novel approach here proposed. LC–ESI-MS and UV chromatograms identified 8 compounds (two more peaks in respect to the previous analysis, namely **a7** and **a8**). Db searching results and hit identification on the basis of MS/MS studies are reported in Table 2. The peak **a1** eluting at 21.9 min was easily identified as chlorogenic acid as above reported. The peak **a2** eluting with a RT of 24.9 min showed an isotopic mass at 594.15825 Da. Db searching identified 5 flavonoids, two flavones and three flavonols. As reported in Table 2, the UV–vis spectrum excluded the two flavonols thus reducing the list 2 to three compounds, namely apigenin 6,8-di-C-glucoside, chrysoeriol 7-O-apiosyl-glucoside and luteolin 7-O-rutinoside. For each of these compounds the MS/MS product ions were predicted and compared with the experimental MS/MS spectrum characterized by two main product ions at *m*/*z* 287 and 449. Luteolin 7-O-rutinoside was the only compound for which both the two experimental product ions were predicted, while for the other two compounds only the product ion at *m*/*z* 449 matched the simulated values. By using this approach, quercetin 3-O-glycoside/quercetin 3-O-galactoside (**a3**) and luteolin C-glycoside (**a4**) were easily identified. By contrast, for compounds **a5**–**a8** eluting at RT 43.1. 43.2, 43.6 and 44.7 min and characterized by monoistopic masses of 338.15189, 392.19895, 408.19361 and 406.21451 Da respectively, no entries were retrieved by Db searching. We then searched the monoisotopic masses by using a larger db containing 6850 entries [14]. In such a case the db searching identified several entries for each compound as listed in Table 2. The list 1 was then filtered on the basis of the UV–vis spectra. All the compounds showed a UV spectrum, characteristic of a chalcone moiety (λmax = 239, 366 nm), and on the basis of these information, list 1 was filtered to generate list 2 containing much less entries. Then, for each entry of list 2 the predicted MS/MS data were generated and matched with the experimental values for unknowns identification. The db searching for compound **a5** (monoisotopic mass of 338.15189 Da) generated a list 1 containing twenty entries which were reduced to five entries by considering only those compounds bearing a chalcone moiety. Then, for each compound belonging to list 2, the MS/MS product ions were predicted and compared to the experimental MS/MS spectrum. 4-Hydroxyderricin was easily identified because it was the only compound for which all the four experimental product ions were predicted.

Db searching of compound **a6** identified 33 entries, all with the same elemental composition ($C_{25}H_{28}O_4$) which were then filtered to 12 by considering only those compounds containing the chalcone moiety. The experimental MS/MS spectrum of **6a** is shown in Fig. 4 and is characterized by four product ions at *m*/*z* 269, 273, 283 and 337. MS/MS prediction identified two structurally similar compounds for which all the four experimental fragments were matched, xanthoangelol and 4′-geranyloxy-4,2′-dihydroxychalcone (Fig. 4). Fragmentation pathway simulation of both the two compounds permitted to chose the product ion at *m*/*z* 283 as target ion for MS^3 in order to distinguish between the two structure related entries. The MS^3 spectrum contained a diagnostic product ion at *m*/*z* 255 which was predicted only for xanthoangelol. The simulated MS/MS spectrum of the ion at *m*/*z* 283 arising from 4′-geranyloxy-4,2′-dihydroxychalcone generated in fact a daughter ion at *m*/*z* 239. Hence compound **6a** was identified as xanthoangelol.

Compounds **a7** and **a8** which were not previously found by using a conventional LC/UV/ESI-MS approach and an ion-trap as MS analyzer [10], were easily identified as xanthoangelol b and f as shown in Table 2.

It should be noted that the results achieved by the approach herein described can be reached only by an high resolution MS analyzer. In fact, by setting the mass tolerance to 250 ppm (a mass accuracy reported for ion trap) a large list of compounds characterized by different elemental compositions was obtained. For instance, the db search setting the monoisotopic mass value to 338.15189 Da and 1 ppm as tolerance (4-hydroxyderricin) retrieved 21 entries characterized by the same elemental composition. By contrast, when 250 ppm was set as mass tolerance, the number of compounds retrieved increased to 53 and were characterized by different elemental compositions. It is clear that in such conditions, the proposed approach cannot be used due to the large number of entries or it is greatly limited since it requires a time-consuming work to predict the MS/MS spectra of the compounds in list 2.

MS/MS product ions prediction obtained by commercially available software was found to be a potent diagnostic tool for compounds identification. For all the standard compounds tested

Table 2
Db searching, UV–vis data and experimental and predicted MS/MS data for flavonoids identified in *Angelica keiskei* EtOH extract. Compounds **a1**, **a2**, **a3**, **a4** were identified by using the Phenol-Explorer data base and then confirmed by Metabolomics.jp. Compounds **a5**, **a6**, **a7**, **a8** were identified only using the Metabolomics.jp db.

Peak	Mono-isotopic mass value (Da)	List 1	UV–vis (nm)	List 2	Matched experimental/simulated MS/MS fragments	Hit compound
a1	354.09510	• Chlorogenic acid	240, 298 sh, 326	• Chlorogenic acid	• 163	Chlorogenic acid
a2	594.15825	• Chrysoeriol 7-O-apiosyl-glucoside	251, 268, 346	• Chrysoeriol 7-O-apiosyl-glucoside	• 449	
		• Luteolin 7-O-rutinoside		• Luteolin 7-O-rutinoside		
		Apigenin 6,8-di-C-glucoside		• Apigenin 6,8-di-C-glucoside	• 287, 449	Luteolin 7-O-rutinoside
		• Kaempferol 3-O-galactoside 7-O-rhamnoside			• –	
		• Kaempferol 3-O-rutinoside				
a3	464.09565	• Ellagic acid glucoside	255, 266 sh, 352	• Myricetin 3-O-rhamnoside	• –	
		• Myricetin 3-O-rhamnoside		• Quercetin 3-O-galactoside	• 303	Quercetin 3-O-galactoside
		• Quercetin 3-O-galactoside		• Quercetin 3-O-glucoside	• 303	Quercetin 3-O-glucoside
		• Quercetin 3-O-glucoside				
a4	448.10084	• 6-Hydroxyluteolin 7-O-rhamnoside	255, 268 sh, 346	• 6-Hydroxyluteolin 7-O-rhamnoside	• –	
		• Luteolin-C-glucoside		• Luteolin-C-glucoside		
		• Kaempferol-O-galactoside			• 287	Luteolin-C-glucoside
		• Kaempferol-O-glucoside				
		• Quercetin 3-O-rhamnoside				
a5	338.15189	• 4-Hydroxyderricin	239, 366	• 4-Hydroxyderricin	• 119, 147, 219, 283	4-Hydroxyderricin
		• 2′,4′-Dihydroxy-6′-methoxy-3′-prenylchalcone		• 2′,4′-Dihydroxy-6′-methoxy-3′-prenylchalcone	• 283	
		• 2′-Hydroxy-6′-methoxy-4′-prenyloxychalcone		• 2′-Hydroxy-6′-methoxy-4′-prenyloxychalcone		
		• Licochalcone C		• Licochalcone C	• 283	
		• Licochalcone A		• Licochalcone A		
		• Crotaramin			• 147, 283	
		• Bavachinin			• 147	
		• 4′-Hydroxyisoderricin				
		• Falciformin				
		• 5-Hydroxy-7-methoxy-6-C-prenylflavanone				
		• Tephrinone				
		• 5-Methoxy-7-prenyloxyflavanone				
		• 6-C-Prenyl-8-C-methylpinocembrin				
		• 5,7-Dihydroxy-6-methyl-8-prenylflavanone				
		• 7-O-Prenylcryptostrobin				
		• Hildgardtol A				
		• Hildgardtol B				
		• 2′-O-Methylphaseollinisoflavan				
		• 4′-O-Methylglabridin				
		• Sandwicensin				
a6	392.19895	• Abyssinone VI	239, 366	• Abyssinone VI	• 283, 337	
		• Kanzonol C		• Kanzonol C	• 337	
		• 4,2′,4′-Trihydroxy-3′,5′-diprenylchalcone		• 4,2′,4′-Trihydroxy-3′,5′-diprenylchalcone	• 273, 337	
		• 4′-Geranyloxy-4,2′-dihydroxychalcone		• 4′-Geranyloxy-4,2′-dihydroxychalcone		
		• Xanthoangelol		• Xanthoangelol	• 269, 273, 283, 337	
		• Stipulin		• Stipulin		
		• Artoindonesianin J		• Artoindonesianin J	• 269, 273, 283, 337	Xanthoangelol
		• Bis(6″,6″-dimethyl-4″,5″-dihydropyrano)		• Bis(6″,6″-dimethyl-4″,5″-dihydropyrano)[2″,3″:4′,5′][2″,3″:4,3]-2′-hydroxychalcone	• 337	
		• [2″,3″:4′,5′][2″,3″:4,3]-2′-hydroxychalcone		• Flemiwallichin E	• 269.337	
		• Flemiwallichin E		• 3′-Geranyl-2′,4′,6′-trihydroxychalcone	• 269, 283, 337	
		• 3′-Geranyl-2′,4′,6′-trihydroxychalcone		• 3′-Neryl-2′,4′,6′-trihydroxychalcone		
		• 3′-Neryl-2′,4′,6′-trihydroxychalcone		• Linderachalcone		
		• Linderachalcone				
		• 7-Prenyloxy-8-C-(3-hydroxy-3-methyl-trans-buten-1-yl)flavanone			• 269	
		• 7,4′-Dihydroxy-6,8-di-C-prenylflavanone			• 269, 283. 337	
		• Abyssinone IV				
		• Glabrol			• 269, 283. 337	

Table 2 (*Continued*)

Peak	Mono-isotopic mass value (Da)	List 1	UV–vis (nm)	List 2	Matched experimental/simulated MS/MS fragments	Hit compound
		• Prostratol F • Euchrenone a17 • Dorsmanin B • 5,7-Dihydroxy-8-C-geranylflavanone • 5-Hydroxy-7-prenyloxy-8-C-prenylflavanone • 5,7-Dihydroxy-6,8-di-C-prenylflavanone • 5-Hydroxy-7-O-nerylflavanone • Linderatone • Kazinol B • Hispaglabridin A • Lespein • Lespedezin • Ficifolinol • Erythrabyssin II • Erybraedin C • Erybraedin A			• – • – • – • – • – • – • – • – • – • – • – • – • –	
a7	408.19361	• Bartericin C • Bartericin B • Xanthoangelol B • Bartericin D • Bartericin A • Paratocarpin D • Paratocarpin E • Anthyllin • 3′-Geranyl-3,4,2′,4′-tetrahydroxychalcone • Kuwanol D • Ammothamnidin • Cedrediprenone • 3′-Geranylchalconaringenin • Glyinflanin A • 4,2′,4′-Trihydroxy-6″-methyl-6″-(4-methyl-3-pentenyl)pyrano[2″,3″:3,2]dihydrochalcone • Lehmannin • Kushenol A • Abyssinone V • 6,8-Diprenylnaringenin • Euchrestaflavanone A • Bonannione A • Sophoraflavanone A • 5,7-Dihydroxy-4′-geranyloxyflavanone • Leachianone E • Macarangaflavanone B • Macarangaflavanone A • Remangiflavanone A • Paratocarpin J • Euchrenone a16 • 3-Hydroxyglabrol • (2R,3S)-3,5,7-Trihydroxy-6-geranylflavanone • 2-Prenyl-6a-hydroxyphaseollidin	239, 366	• Bartericin C • Bartericin B • Xanthoangelol B • Bartericin D • Bartericin A • Paratocarpin D • Paratocarpin E • Anthyllin • 3′-Geranyl-3,4,2′,4′-tetrahydroxychalcone • Kuwanol D • Ammothamnidin • Cedrediprenone • 3′-Geranylchalconaringenin • Glyinflanin A	• 391 • 391 • 269, 271, 289, 391 • 391 • 391 • 391 • 391 • 271 • 271, 391 • 271, 391 • 269, 391 • 391 • 391 • 271, 289, 391 • –	Xanthoangelol B
a8	406.21451	• Xanthoangelol F • 2′,6′-Dihydroxy-3′-(1-p-menthen-3-yl)-4′-methoxychalcone • Tunicatachalcone • Adunctin B • Adunctin C • Adunctin D • Grenoblone • 5-Methoxy-7-prenyloxy-8-C-prenylflavanone • Heminitidulan • Erycristin	239, 366	• Xanthoangelol F • 2′,6′-Dihydroxy-3′-(1-p-menthen-3-yl)-4′-methoxychalcone	• 287, 313, 291 • 391	Xanthoangelol F

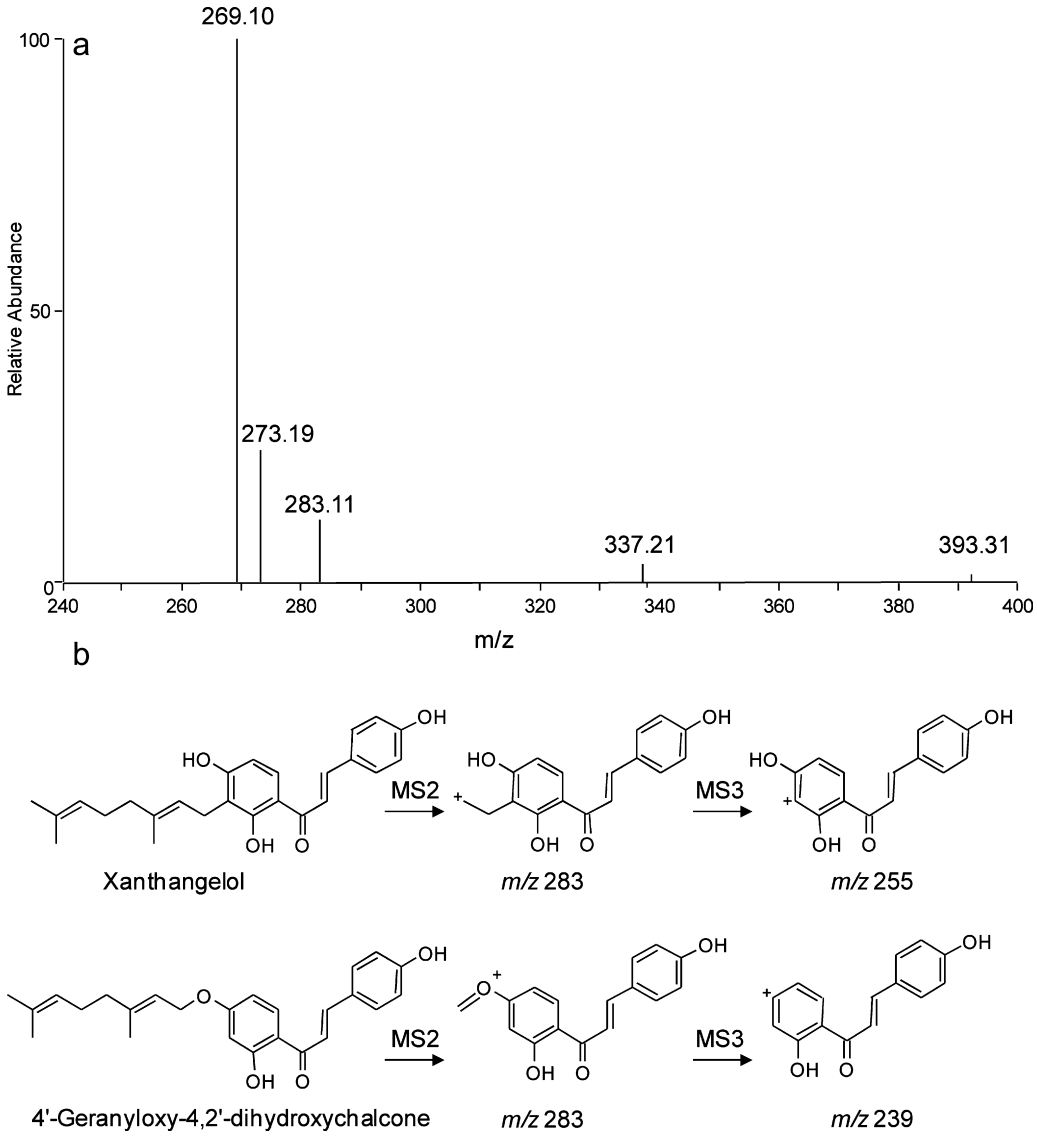

Fig. 4. (Panel A) Experimental MS2 spectrum of the ion at *m/z* 393.21. (Panel B) The predicted structures of the ion at *m/z* 283 arising from xanthoangelol and 4′-geranyloxy-4,2′-dihydroxychalcone, and the corresponding daughter ions (MS3).

we observed that the prediction was highly reliable and permitted an uncertain structure assignment. The software, beside to generate predicted software product ions on the basis of well known fragmentation rules, also furnish fragments structures and fragmentation mechanisms. Such additional information was found of great value since they permitted to unequivocally select product ions to be further collisionally dissociated (MS^3), in order to distinguish among isomers or structurally related compounds.

Beside MS data, also the UV–vis spectra were of great diagnostic value since permitted to identify the phenolics class to which the unknown belongs. Such a piece of information permits to filter the list of compounds identified on the basis of the molecular mass and to significantly reduce the number of compounds for which the simulated MS/MS spectra are generated. It should be considered that the approach here proposed requires an efficient chromatographic separation of the plant phenolic constituents in order to acquire reliable UV spectra of the unknown compounds. The efficient separation of the selected standards as well as of the phenols from *A. keiskei* was suitably achieved by conventional reversed phase chromatography. However, in case where the method is applied to more complex mixtures or in case of co-eluting peaks, more innovative chromatographic separations are needed.

Finally it should be noted that the proposed approach is limited for the identification of optical isomers since they are characterized by the same MS/MS fragmentation pathway (as in the case of catechin/epicatechin) as well as for unknown compounds not reported in the db.

4. Conclusion

The proposed approach based on high resolution MS analyzer together with UV–vis spectra and MS/MS fragmentation prediction was found to be a new, reliable and potent tool for a easy and rapid identification of plant phenols. Moreover, such an approach can be successfully used without a strong background in plant phenols chemistry and MS/MS fragmentation knowledge, thus to greatly facilitate the phytochemical characterization of plant extracts.

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