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Identification of flavonoids using liquid chromatography with electrospray ionization and ion trap tandem mass spectrometry with an MS/MS library

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Searchable MS/MS spectra libraries, constructed using the results of liquid chromatography coupled with electrospray ionization (ESI) tandem mass spectrometry (LC/MS/MS) with data-dependent acquisition on an ion trap mass spectrometer, are presented with regard to the identification and confirmation of a variety of closely related flavonoids in a set of biological samples. Flavonoids were found to exhibit a maximum amount of structurally specific MS/MS spectra at 45% of normalized collision energy on the instrument used, without wideband activation. These MS/MS spectra were then searched automatically against a 297-substance MS/MS library that contains many previously acquired spectra of standard flavonoids. The possible applications of this powerful technique to biological samples are also discussed. Daidzein and genistein were identified through the MS/MS spectra library while searching through LC/MS/MS data for plant and microbial extracts. Moreover, these compounds proved completely distinguishable from other flavonoids of closely related structures in the MS/MS spectra library, using the NIST MS search program. The applicability of the library-searchable spectra at low concentrations was demonstrated by successful identification of daidzein and genistein at 0.05 and 0.5 μg/mL, respectively. Copyright © 2005 John Wiley & Sons, Ltd.

The search for novel pharmacologically active agents via the screening of natural sources, most notably plant extracts, has resulted in the discovery of a host of clinically useful drugs, which now are beginning to play major roles in the treatment of a variety of human diseases. Previous studies have demonstrated that many flavonoid compounds exert protective effects against the development of certain cancers,[1] cardiovascular risk,[2] inflammatory diseases,[3] coronary heart disease, and strokes.[4] Other pharmacological activities have also been attributed to flavonoids, including antimicrobial,[5] antioxidant,[6,7] free radical scavenging properties,[8] and radioprotective effects.[9]

The flavonoids comprise a large class of natural polyphenolic compounds (Table 1), which are known to occur frequently in fruits and vegetables that are regularly consumed by humans. The structural differences among different flavonoid classes are primarily related to the chemistry of the C ring, variations in the number and distribution of phenolic hydroxyl groups across the molecules, and their substitutions.[10] According to the chemical classification system employed, there are between 11 and 26 classes of flavonoids, and easily over 4000 individual compounds. Six flavonoid classes and 20–30 individual compounds are known to occur especially frequently in foods.[11] Although flavonoids exhibit a variety of biological activities, their structures tend to be extremely similar with regard to their backbones, with the main differences arising from hydroxyl group substitutions (Table 2). They are, in fact, so structurally similar that it has proven difficult to differentiate among them with classical analytical methods. Therefore, a better method for the identification and confirmation of individual flavonoids is clearly needed, considering the ever-increasing rise in interest in these compounds.

Nuclear magnetic resonance (NMR) spectroscopy has proven particularly useful in the characterization of the structures of unknown compounds, and has previously been successfully applied to the analysis of metabolites in biological samples.[12] However, under certain circumstances, the ^{1}H NMR spectrum is insufficient on its own for the complete characterization of a given compound. This limitation is obviously the case where analytes contain functional groups that are deficient in protons, or where the protons can readily chemically exchange with the solvent (under such

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Table 1. Subclasses of flavonoids

Class	Flavonoids
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin
Flavones	Luteolin, apigenin
Flavanones	Hesperetin, naringenin, eriodictyol, pentahydroxylflavanone
Flavans	Catechin, gallocatechin, epicatechin, epigallocatechin, dihydrokaempferol, dihydroquercetin, dihydromyricetin
Isoflavones	Daidzein, genistein, glycitein
Anthocyanidins	Cyanidin, delphinidin, malvidin, pelargonidin
Chalcones	Chalcone, tetrahydroxychalcone

circumstances the signals can be broadened beyond the limit of detection). Alternatively, other nuclei can be used in techniques such as ^{13}C NMR.[12]

In contrast, liquid chromatography (LC) is rather well suited for the analysis of complex mixtures, without the necessity for sample derivatization. LC methods with UV detection have questionable specificity for distinguishing compounds of very similar structures as they often exhibit fairly similar UV absorption characteristics. Therefore, with the advent of atmospheric pressure ionization (API) techniques, liquid chromatography/mass spectrometry (LC/MS) has now become a method of choice for such analyses. Structurally informative spectra may be obtained via either collision-induced dissociation (CID) in the source region, or by tandem mass spectrometry (MS/MS). Moreover, mass spectral reproducibility can be obtained using the relatively low-priced quadrupole ion trap instruments with wideband excitation and normalized collision energy, together with the ability of the ion trap to provide fragment ion spectra from mass-selected precursor ions. The universal applicability of mass spectral libraries constructed using LC/MS and LC/MS/MS is a matter under discussion by many of the users and manufacturers of these instruments. Recently, several LC/MS/in-source CID libraries[13] and LC/MS/MS libraries have been constructed for a host of drugs and their metabolites, as well as for pesticides and other natural products.[14,15] However, the scope of these library databases is currently limited, and therefore it will be clearly important in the future to accumulate an inter-laboratory MS/MS spectra library.

In this study we have constructed a flavonoid MS/MS library and, with its help, have developed a rapid method for the screening of various flavonoids from biological samples. A further objective of this research was to generate a novel application for LC/MS/MS technology, specifically in terms of metabolite profiling.

EXPERIMENTAL

Materials

The flavonoid standards used in this study (Table 2) were purchased from IDRTech, Inc. (Daejeon, Korea). The formic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). High-purity grade acetonitrile (Burdick and Jackson, Muskegon, MI, USA) and Milli-Q grade organic-free water were used as solvents.

Preparation of the extracts from plant and microbial sources

Plant extracts from *Puerariae radix* were obtained from the Plant Extract Bank at KRIBB, Daejeon, Korea.[16] The extracts were serially diluted using 10% methanol, in order to achieve concentrations of 1 mg/mL. The microbial culture broth (AH030068 of *Streptomyces armeniacus*) was purchased from the Microbial Resources Bank[17] at KRIBB (Daejeon, Korea). Microbial culture broth extracts were filtered through 0.45 μm mesh after being mixed with equal volumes of acetone. Each of the extracts was analyzed via LC/MS/MS in order to determine its constituents.

Analytical system

Mass spectra were obtained via either direct infusion or liquid chromatographic introduction into a Finnigan LCQ Advantage MAX ion trap mass spectrometer, equipped with a Finnigan Surveyor Modular HPLC system (Thermo Electron Co., USA). The chromatographic separation of the compounds was achieved using a YMC-Hydrosphere C18 column (2.0 × 50 mm, 5 μm) at a flow rate of 0.2 mL/min. Mobile phases A and B were water and acetonitrile, respectively, both containing 0.1% formic acid. Gradient elution was conducted as follows: 0–30 min for 5–60% B with a linear gradient, followed by 30–45 min of 100% B. The MS/MS system was operated in electrospray ionization (ESI) mode. The typical operating parameters were as follows: spray needle voltage, 5 kV; ion transfer capillary temperature, 200°C; nitrogen sheath gas, 60; and auxiliary gas, 5 (arbitrary units). The ion trap contained helium damping gas which was introduced in accordance with the manufacturer's recommendations. Mass spectra were acquired in a *m/z* range of 50–1000, with 3 microscans and a maximum ion injection time of 200 ms. It was possible to acquire data in positive and negative modes in a single LC run, using the continuous polarity switching ability of the mass spectrometer. In order to calibrate the instruments for quantitative measurement of daidzein ($[M+H]^+$ *m/z* 255) and genistein ($[M-H]^-$ *m/z* 269), the instruments were operated in the selected ion monitoring (SIM) mode. Data-dependent experiments were controlled by the menu-driven software provided with the system. All of the experiments were conducted under automatic gain control conditions.

Optimization of MS/MS fragment spectrum

For the optimization of the MS/MS fragmentation, the analytes were directly infused into the source at 5 μL/min by means of an integrated syringe pump; the MS/MS spectra were acquired in a data-dependent scan mode that used criteria from the previous MS^1 scan to select the target precursor peak. The SIM analysis was a narrow scan event that monitored the *m/z* value of the selected ion, in a range of 1.5 Th centred on the peak for the molecular ion; this function was used both in the quantitative analyses and as the means to isolate molecular ions of the flavonoids for MS/MS in positive and negative ESI modes. The MS/MS fragment spectra were produced using normalized collision energies with an increment of 5% from 30% to 60%, and also with wideband activation 'ON' and 'OFF'. The laboratory-frame collision energy was optimized for each of the precursor ions, on the

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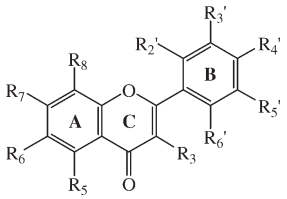
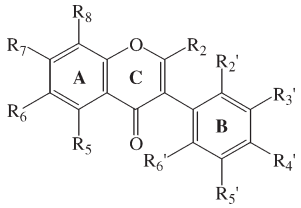
Table 2. Chemical structures and acquisition parameters for closely related flavonoids used in this paper



Compound No.	R_3	R_5	R_6	R_7	R_8	R_2'	R_3'	R_4'	R_5'	R_6'	MW	Polarity*	Retention time (min)
1				OH					OH		254	+/−	16.03
2			OH					OH			254	+/−	16.03
3			OH			OH					254	+/−	18.08
4		OH				OH					254	+/−	25.10
5	OH					OH					254	+/−	19.86
6			OH	OH							254	+/−	17.65
7		OH						OH			254	−	23.32
8	OH		OH								254	−	22.54
9				OH				OH			254	+/−	15.20
10		OH		OH							254	+/−	23.54
11		OH		OH		OH					270	+/−	20.26
12				OH	OH			OH			270	+/−	12.76
13				OH			OH	OH			270	+/−	13.34
14				OH	OH	OH					270	+/−	13.60
15	OH		OH					OH			270	+/−	16.90
16		OH		OH				OH			270	+/−	18.76
17	OH	OH						OH			270	+/−	16.75
18		OH	OH	OH				OH			286	+/−	15.07
19	OH		OH			OH		OH			286	+/−	13.75
20	OH		OH				OH	OH			286	+/−	14.23
21	OH			OH			OH	OH			286	+/−	14.03
22				OH	OH		OH	OH			286	+/−	11.32
23	OH	OH		OH				OH			286	−	19.21
24		OH		OH			OH	OH			286	+/−	16.69
25	OH			OH			OH	OH	OH		302	+/−	11.96
26		OH		OH			OH	OH	OH		302	+/−	14.42
27	OH	OH		OH		OH		OH			302	−	15.31
28	OH	OH	OH	OH			OH	OH			318	−	13.29
29	OH	OH		OH	OH		OH	OH			318	+/−	13.80
30	OH	OH		OH			OH	OH	OH		318	−	14.14



Compound No.	R_3	R_5	R_6	R_7	R_8	R_2'	R_3'	R_4'	R_5'	R_6'	MW	Polarity*	Retention time (min)
31				OH				OH			254	+/−	15.23
32				OH			OH	OH			270	+/−	13.23
33		OH		OH				OH			270	−	18.43
34				OH				OH	OH		270	+/−	13.22
35		OH		OH				OH	OH		286	+/−	15.78

*+/−, positive and negative ion MS/MS spectra included in database; +, only positive MS/MS spectrum included; −, only negative MS/MS spectrum included.

basis of overall spectral intensity and on production of useful fragment ions.

Spectral library building and searching

All library building and searching operations were conducted using the Xcalibur data system (version 1.4 SR1, Thermo Electron Co., USA) and the NIST mass spectral search program (version 2.0, FairCom Co., USA), running on a Dell OptiPlex GX270 system (Dell Inc.). Mass spectral searching utilized the algorithms established by the National Institute of Standards and Technology, as implemented in the Xcalibur data system.

Method validation

Calibration curves of the standard compounds (daidzein and genistein) were prepared using standard solutions in a

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concentration range between 0.5–10 000 ng/mL. The linearity of the calibration curves was validated using measurements in triplicate. The limit of detection (LOD) was determined as the concentration giving a signal-to-noise (S/N) ratio of 3:1. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the standard curve at which the percentage coefficient of variation (%CV) was below 15%. Specificity was established by the lack of interference peaks at the retention times for the standards. Linearity was evaluated at ten concentration levels, encompassing a range between 1 ng/mL and 10 μg/mL. The linear regression equations and correlation coefficients were calculated via the least-squares method.

With regard to qualitative identification, search accuracy was expressed as the reverse search matching score (RSI), using the Xcalibur data system with the constructed MS/MS spectra library.

RESULTS AND DISCUSSION

Investigation of MS/MS parameters for matching MS/MS spectra

The applicability of mass spectra is of ever-increasing importance in the field of structural chemistry. Several mathematical approaches have already been developed for the search of an 'unknown' spectrum against a library of previously acquired spectra for GC/EIMS.[18,19] In the case of LC/MS and LC/MS/MS using soft ionization techniques, however, the implementation of library searching has been limited by a lack of fragmentation pattern reproducibility. Recently, the development of wideband excitation and normalized collision energy in ion traps by Lopez *et al*.[20] has resulted in the possibility of obtaining highly reproducible mass spectra which can then be searched using the NIST algorithm; this clearly facilitates inter-instrument comparability.[14,15] In this study we investigated a set of very closely related flavonoids, many of which were stereo or positional isomers of one another, as this would constitute an appropriately challenging test to determine whether the tested spectral reproducibility and library searching algorithms were adequate for the differentiation of extremely similar compounds. In order to determine the practical utility of the libraries, we developed a rapid system for the screening of various flavonoids from biological samples, including plant and microbial extracts.

Although a variety of flavonoid subclasses exists, e.g., flavones, flavonols, and isoflavones (Table 1), we attempted to determine the fragmentation conditions applicable to the identification of each of the flavonoid compounds. Several spectra of compound **12** (MW 270) are shown as examples in Fig. 1. These MS/MS fragmentation spectra were produced under conditions involving a range of normalized collision energies (at 5% increments from 30 to 60%). The dissociation of the molecular ion peak ($[M+H]^+$ or $[M-H]^-$) was not observed at collision energies between 30 and 40%. Also, unexpected adduct ions (*m/z* 288, 300) made a rather abrupt appearance on increasing the collision energy, and overall ion signal intensity was relatively low at collision energies in excess of 50%. We did not investigate the nature and origin of these adduct ions. A normalized collision energy of 45% was chosen in both positive and negative ionization modes to generate informative and reproducible fragment ion spectra.

We also observed that the fragmentation patterns were more informative and more highly reproducible in the wideband activation 'OFF' mode (Fig. 1). In the wideband activation 'ON' mode, the product ion signals were low in both positive and negative ion modes at 45% collision energy (Figs. 1(A) and 1(B)). In cases in which wideband activation was applied, the $[M+H-H_2O]^+$ ion (*m/z* 253) vanished completely, whereas the $[M+H]^+$ ion (*m/z* 271) and the water loss ion (*m/z* 253) were clearly observed at 45% normalized collision energy in the wideband activation 'OFF' mode. In general, the implementation of wideband activation has been found to generate more structurally informative MS/MS spectra, producing more lower *m/z* ions in MS^2 experiments.[20] In contrast, in the present work informative spectral data for the flavonoids were recorded most readily in wideband activation 'OFF' mode. This unusual MS/MS fragmentation behavior has already been reported in studies of fragmentations of steroids.[20]

However, we decided to construct a wideband activation MS/MS library using several standard compounds in addition to the existing narrowband MS/MS library. The biological samples were reanalyzed in triplicate using both wide- and narrowband activation modes. The wideband MS/MS spectra were produced using 50% normalized collision energy, as this condition was previously reported by Baumann *et al*.[14] We investigated the matching factor differences using the daidzein (**31**) and genistein (**33**) data against both the narrow- and wideband library entries. As a result, we reconfirmed that the narrowband mode provided higher matching factor values (Table 3).

We determined that, in wideband excitation 'OFF' mode, our techniques were sufficient for the construction of an MS/MS flavonoid spectral library. These results suggested that an ion trap platform could provide the conditions suitable for the universal mass spectrometry of flavonoids. As a consequence, the acquisition condition for MS/MS spectra for library construction was optimized at 45% normalized collision energy, without wideband activation.

It seemed worthwhile to attempt to predict theoretically the possible product ion fragmentation patterns of our analytes. Figure 2 depicts the proposed fragmentation pattern for compound **12**, obtained using the Mass Frontier (v4.0, HighChem Ltd., Slovak) software. The fragmentation pattern suggested by the software appears to be consistent with the experimental results shown in Fig. 1(A).

Flavonoids exhibit closely similar structures, substituted with hydroxyl groups in varying locations; some such substances have identical molecular weights, i.e., in our case **11**–**17**, **32**, and **33**. In order to distinguish between flavonoids with identical molecular weights, they can be differentiated according to their LC retention times as well as by their MS/MS spectra. Each of these analytes yielded different results, generating information sufficient for the differentiation of individual flavonoids, under optimized instrumental conditions (Fig. 3). For example, compounds **15** and **17** exhibited similar LC retention times (Fig. 3(A)), but their MS/MS spectra were remarkably different, and these flavonoid isomers (Table 2) could thus be readily differentiated

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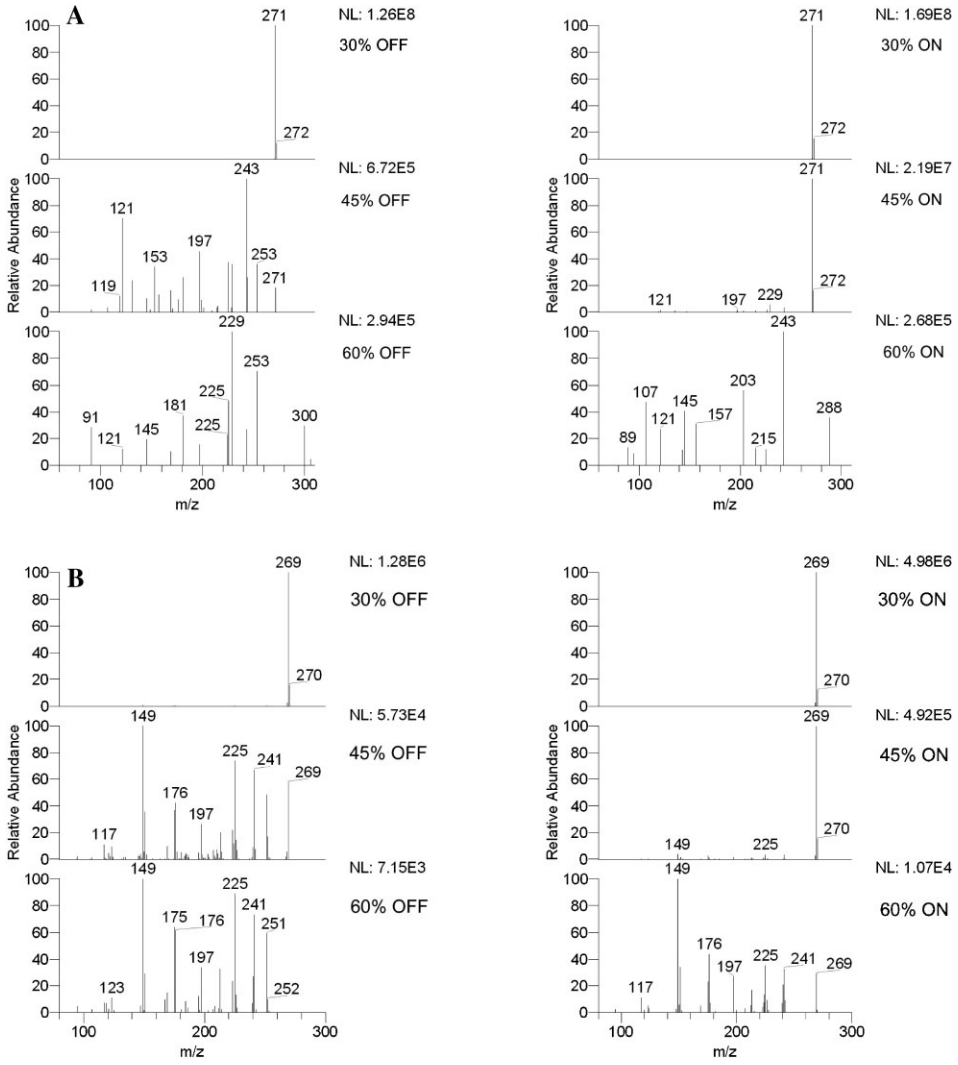

Figure 1. ESI-MS/MS spectra of compound **12** (MW 270), obtained at different normalized collision energies (%) and wideband activation conditions ('ON' vs. 'OFF'). The spectra were generated in (A) positive and (B) negative ionization mode. Left column: wideband activation 'OFF'; right column: wideband activation 'ON'.

(Fig. 3(B)). This is a noteworthy result, considering that many of these compounds are quite closely structurally related, often differing only in the sense that they are stereo or positional isomers of one another. The search algorithm used in this study successfully differentiated among all of the tested flavonoid compounds, allowing us to construct a meaningful MS/MS library by using optimized conditions.

Sensitivity

Each of the standard solutions used in this study was analyzed five times. The detector response (peak area) was linear from 0.001 to 10 μg/mL for both daidzein ($y = 7.68031E7x + 3.81599E6$, $R^2 = 0.9994$) and genistein ($y = 2.61129E7x + 6.29415E5$, $R^2 = 0.9998$). The LODs for daidzein and genistein were both 1.0 ng/mL, and their LLOQs were 1.0 and 2.0 ng/mL, respectively (Table 4). Table 4 also shows the percentage coefficient of variation (%CV) and match accuracy of the two tested flavonoids.

The reverse search matching score (RSI) represents the computed match value obtained by ignoring any peaks in the unknown spectrum that are not in the library spectrum; a perfect match is assigned a value of 1000. As a general guide, a value of 900 or greater is an excellent match, 800–900 is a good match, and 700–800 represents a fair match. On this basis (i.e., an RSI matching factor in excess of 700) we determined that the minimal concentration of daidzein required for a successful library search of daidzein was 0.05 μg/mL, and that for genistein was 0.5 μg/mL. This MS/MS library searching method is approximately ten times as sensitive as the method using capillary electrophoresis reported previously.[21]

Library building and searching

The construction of an MS/MS library is achieved by simply entering the collected spectral data into the NIST MS search program on the Xcalibur data system. Figure 4 shows an example of an entry in the constructed MS/MS library that includes compound structure, molecular formula, CAS registry number, database class, and information regarding the ten most intense peaks (Fig. 4(D)). The database was divided

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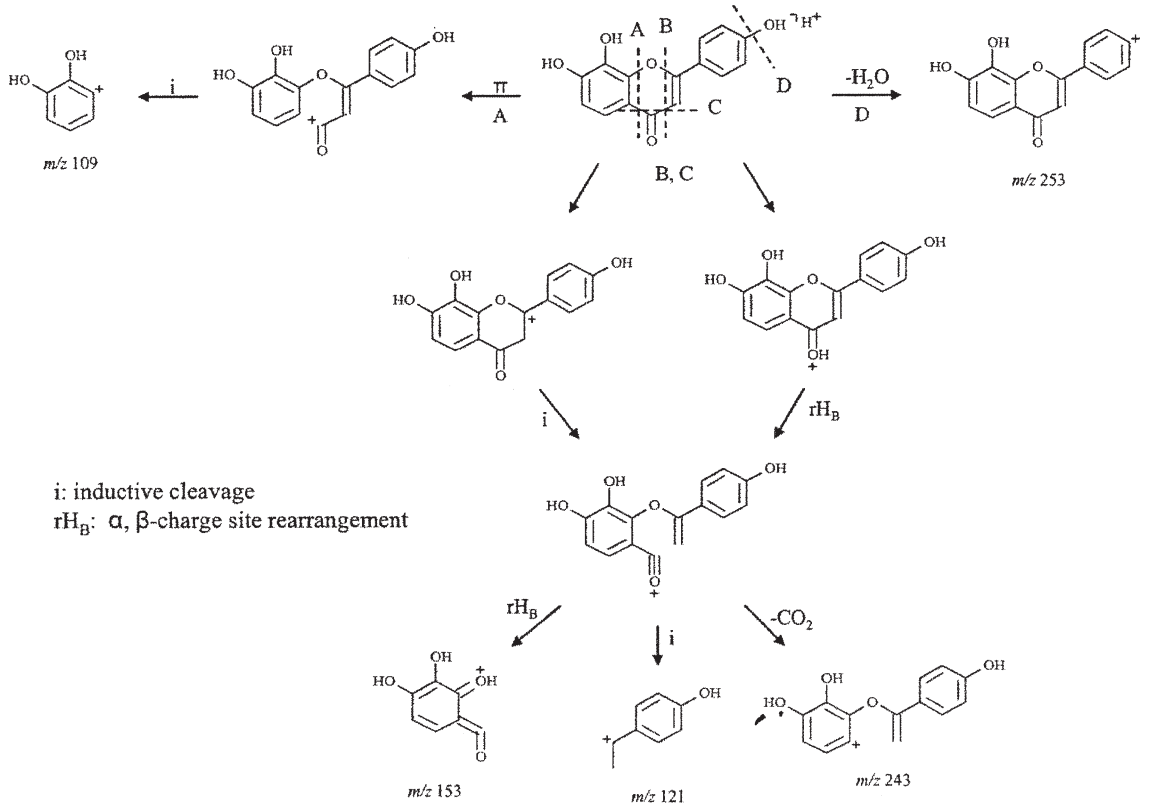

Figure 2. Proposed fragmentation pathways of compound **12** ($[M+H]^+$, *m/z* 271) generated at 45% normalized collision energy and with wideband activation 'OFF'. The fragments were predicted using Mass Frontier software.

into two categories, the 'IDRPO (positive ion spectra library)' and the 'IDRNE (negative ion spectra library)', for convenience (Fig. 4(B)). Figure 5 shows the search results using the constructed flavonoid MS/MS library, and shows that the highest probability of identification of this constituent, detected in an extract of *Puerariae radix*, was that for daidzein.

The library database can be readily updated, and new spectra can easily be added directly to the NIST MS search program, i.e., databases on online systems may be continuously maintained, updated, and expanded. The PC database is thus linked to sophisticated software that takes only seconds to produce the search results. The NIST search program is capable of recommending a candidate with a similar MS/MS spectrum, even though the unknown sample has not been registered in the database. Such recommendations allowed us to draw structural deductions with only partial information regarding unknown structures. Our MS/MS spectral library database now contains the spectra of more than 500 flavonoids, and more than 430 microbial metabolites.

Analyses of plant extracts and microbial metabolites

In this study, we constructed an MS/MS library which now contains the MS/MS spectra of more than 930 identified compounds. In order to test our procedures, we further attempted

Table 3. The reverse search scores (RSI) from cross searches using narrow band and wide band spectra vs. narrow band and wide band library entries

		MS/MS library RSI[a] (±SD[b])	
Compound	Activation mode	Narrowband	Wideband
Daidzein of *Puerariae radix* extract	Narrowband	**854** (±16)	287 (±26)
	Wideband	630 (±67)	**654** (±59)
Genistein of *Streptomyces armeniacus* extract	Narrowband	**868** (±42)	468 (±86)
	Wideband	651 (±37)	**795** (±57)

[a] Reverse search matching factor.
[b] SD, standard deviation (n = 3).
The normalized collision energies were 45% and 50%, for narrow band and wide band activation, respectively. The matching factor was calculated using the Xcalibur data system (v1.4 SR1). Entries in bold font represent scores obtained when an experimental spectrum was searched against the library obtained using the same activation condition (narrow- vs. wideband).

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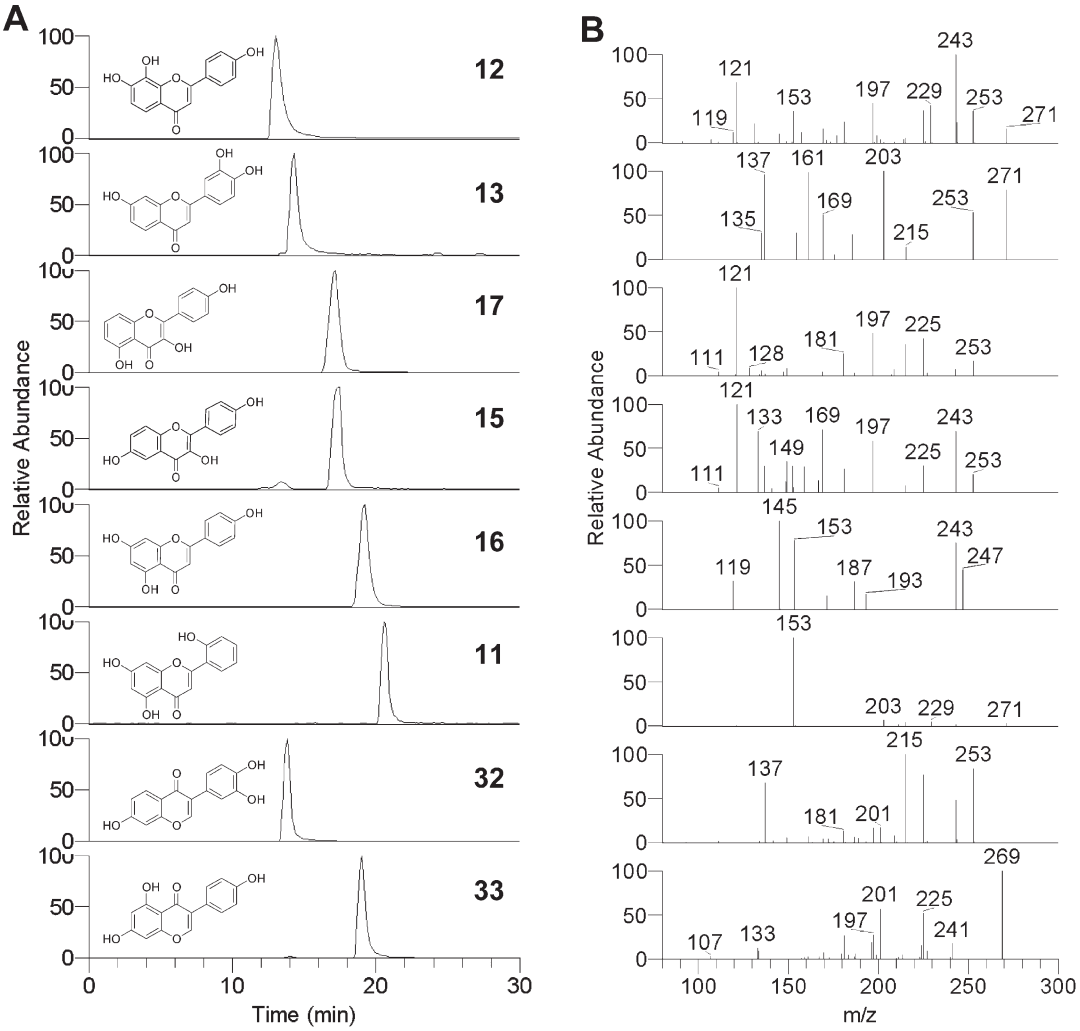

Figure 3. LC/ESI-MS chromatograms (A) and MS/MS spectra (B) of eight flavonoids, obtained under optimized instrumental conditions.

to determine whether the library was applicable to the identification of unknown compounds in both plant and microbial sources.

Puerariae radix is known to exert both sedative and antipyretic effects, and has often been used in the treatment of influenza, wrist stiffness, and headaches. The determination of flavonoids within *P. radix* turned out to be rather difficult, owing primarily to the complexity of the components in the derived extract. In order to identify structurally similar flavonoids from the *P. radix* extracts, we analyzed this commonly used Korean herb using data-dependent LC/MS/MS in both positive and negative ion modes, under our

Table 4. Summary of results obtained for standard samples used for quantitative calibration and library search

	Daidzein			Genistein		
Concentration (μg/mL)	Conc. found (μg/mL)	CV (%)	Match accuracy (RSI[a]±SD)	Conc. found (μg/mL)	CV (%)	Match accuracy (RSI±SD)
0.001	0.00053 ± 0.00014	7.03	n.s[b]	0.00094 ± 0.00029	23.96	n.s
0.002	0.00135 ± 0.00029	10.22	n.s	0.00212 ± 0.00030	14.01	n.s
0.005	0.00550 ± 0.00022	3.21	433.5 ± 242.5	0.00496 ± 0.00076	14.58	n.s
0.01	0.11144 ± 0.00108	8.40	603.3 ± 48.4	0.01057 ± 0.00087	7.98	n.s
0.05	0.05093 ± 0.00162	3.09	733.5 ± 14.8	0.04992 ± 0.00167	3.32	575.0 ± 71.0
0.1	0.09939 ± 0.00473	4.69	786.3 ± 29.4	0.09999 ± 0.00397	3.96	640.3 ± 22.6
0.5	0.53182 ± 0.01170	2.01	860.0 ± 49.5	0.49492 ± 0.03703	7.18	755.0 ± 49.6
1	1.13261 ± 0.04778	4.04	833.7 ± 40.6	1.17136 ± 0.01655	1.39	775.0 ± 13.2
5	5.27227 ± 0.07406	1.39	858.7 ± 43.0	4.88082 ± 0.04486	0.91	764.0 ± 22.1
10	9.84945 ± 0.20188	2.04	852.3 ± 22.1	10.04265 ± 0.06544	0.65	788.3 ± 15.1

[a] Reverse search matching factor.
[b] Not searched.

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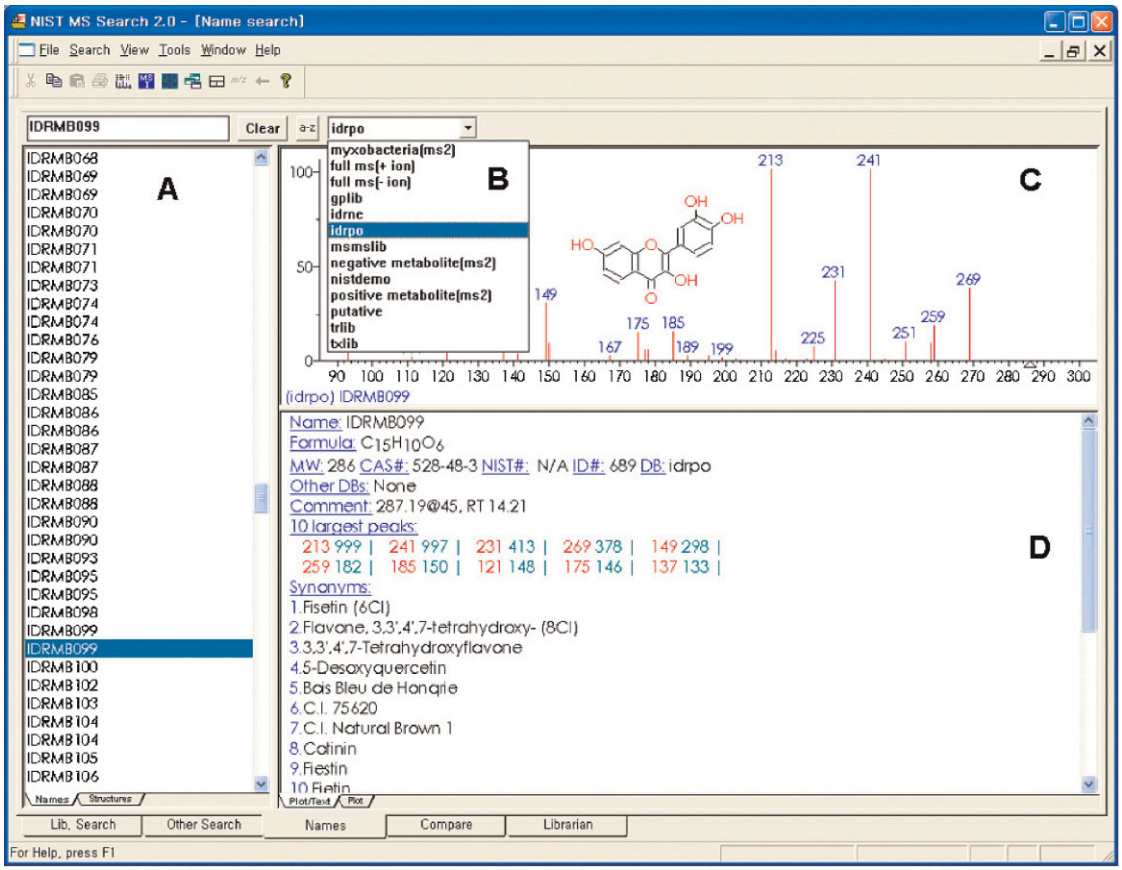

Figure 4. Example of a page of the MS/MS spectral library: (A) compound list, (B) separated databases (IDRPO, IDRNE) and other databases, (C) spectrum and compound structure, and (D) compound information.

optimized instrumental conditions (Fig. 6(A)). The *m/z* values of the molecular ions and MS/MS spectra of each of them were then searched, using the MS/MS spectra library. As a result we identified daidzein (**31**) as the 15.43 min peak (Fig. 5); this result is consistent with the report of He *et al.*[22] The MS/MS spectrum of the *m/z* 255 precursor was dominated by *m/z* 227, 199, and 137, with comparable relative intensities. In addition, the daidzein isomers (compounds **1**, **2**, and **3**) were distinguished independently via the MS/MS library.

Previously, Wang *et al.*[21] reported detection of puerarin, daidzin, biochanin A, and genistein in the *P. radix* extracts. We found several peaks, presumably representing flavonoids, with molecular ions at *m/z* 416, 284, and 270, in the *P. radix* extracts (data not shown).

In order to analyze the flavonoids in *P. radix*, extraction procedures with organic solvents have been conducted by several researchers, with subsequent analysis by high-performance liquid chromatography (HPLC) coupled with mass spectrometry,[23] and also by capillary electrophoresis;[21,24] the detection limits of these methods were reported to be 1.77 mg/mL for CE with UV detection,[21] and 0.28 mg/mL with electrochemical detection.[25] However, the conventional extraction methods were also associated with some decided disadvantages, including the requirement of large amounts of toxic and flammable organic solvents, and the time-intensive pre-concentration procedure. However, LC/MS/MS combined with a library search was associated with a remarkable set of advantages, including rapid identification, high sensitivity, and the requirement of only a small quantity of organic solvents.

We also conducted an analysis of microbial metabolites using the established library. *Streptomyces armeniacus* AH030068 extract was analyzed via data-dependent LC/MS/MS at 45% collision energy, without wideband activation. We observed three LC peaks in the raw data (not shown). Figure 6(B) shows the LC chromatogram, and mass and MS/MS spectra of the selected precursor (*m/z* 271, $[M+H]^+$). This unknown constituent was identified clearly as genistein (**33**) by a library search (Fig. 7). This result was based on high match factors, which have been discussed in detail by other workers,[14] and high probability values (in excess of 90%).

CONCLUSIONS

In our analysis of the tested flavonoids, we achieved high MS/MS spectral reproducibility using the ion trap instrument with wideband activation 'OFF' and 45% normalized collision energy. The quality and reproducibility of the product ion spectra produced in this way was sufficient to reliably differentiate between closely related structures. As expected, the tightest library fits can be achieved in cases in which a spectrum is acquired under conditions identical to

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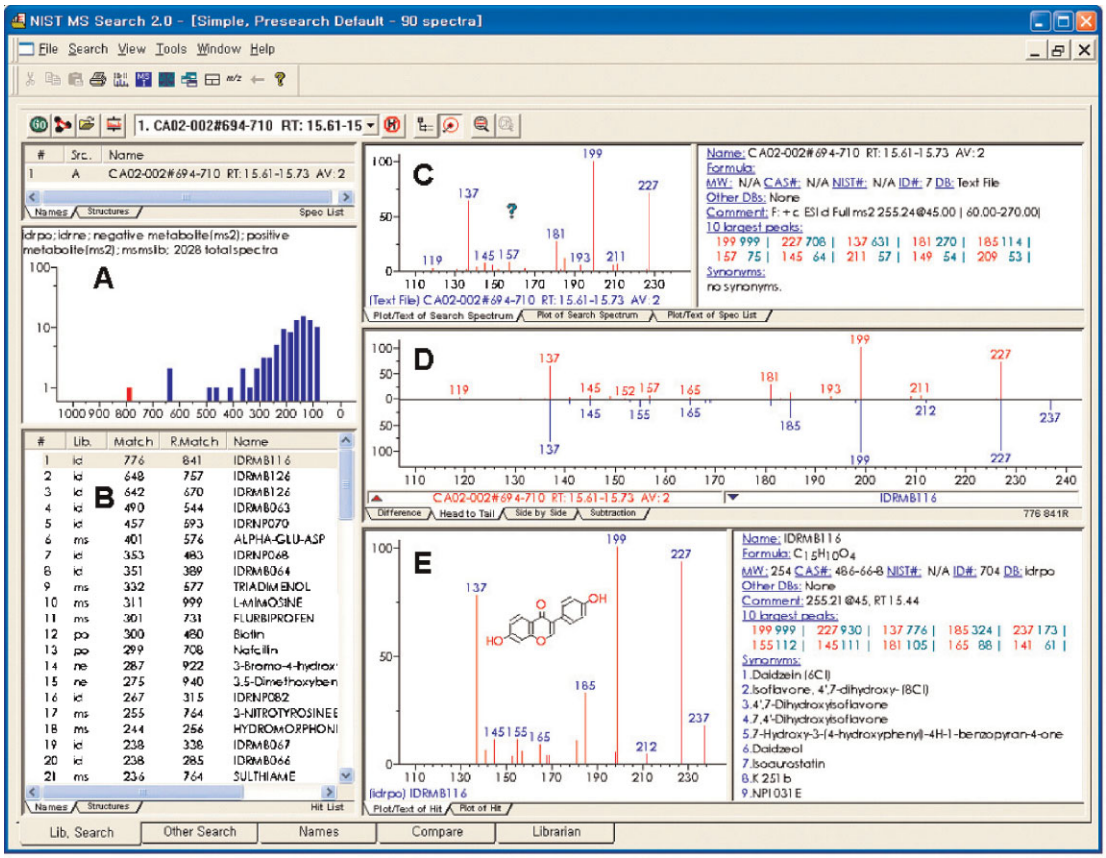

Figure 5. An example of library search results showing the greatest probability of the MS^2 spectrum obtained after the LC/MS/MS analysis of the *P. radix* extract: (A) match-based score plot, (B) partial report obtained after matching this spectrum against a user library, (C) acquired sample spectrum, (D) difference spectrum, and (E) structure and information for the identified molecule.

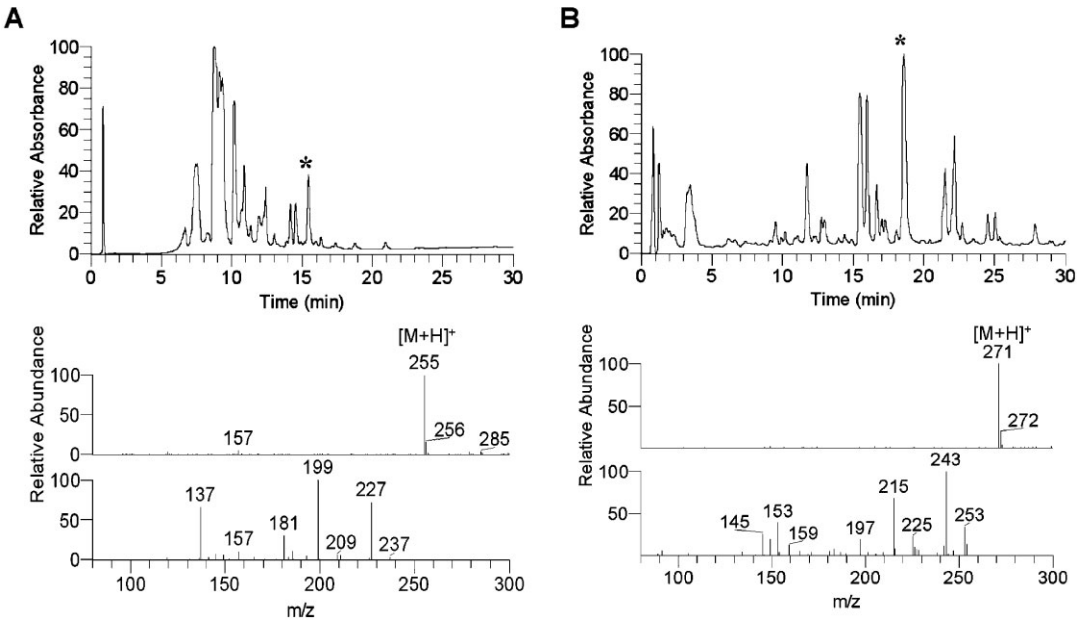

Figure 6. LC chromatograms, full-scan mass spectra, and data-dependent product ion scan mass spectra of the $[M+H]^+$ ions obtained during the LC/MS/MS analysis of (A) *Puerariae radix* and (B) *Streptomyces armeniacus* extracts. * LC peak selected for the MS^1 and MS/MS analyses.

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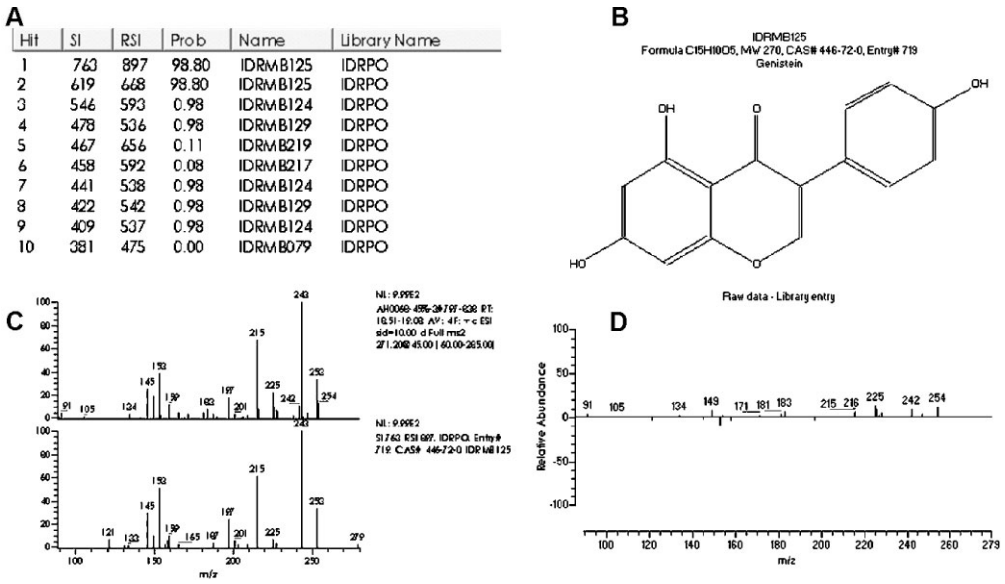

Figure 7. Library search results for the MS/MS spectrum (*m/z* 271→) obtained using data-dependent acquisition in the direct LC/MS/MS analysis of *Streptomyces armeniacus* AH030068 extracts: (a) partial report obtained after the matching of this spectrum against a user library, (b) the structure of the identified molecule, (c) the analyte and library spectra, and (d) the difference spectrum.

those employed to create a reference spectrum within a library. This developed method was then successfully applied to the identification of flavonoids in complex biological samples, using small sample amounts (1 mg/mL, 10 μL) and low concentrations (e.g., 0.05 μg/mL for daidzein).

The objective of this study was to develop a rapid and retention time-independent technique for the identification of constituents within biological samples. A fast and sensitive method for the differentiation of flavonoid homologues using the MS/MS spectra library has been developed, and its applicability has been demonstrated. We anticipate that this system might facilitate metabolomics, as well as the study of chemical screening, in a variety of ways. The lead targets could be evaluated rapidly, without incurring the loss of a large quantity of biological metabolites.

We hope to continue the construction of a large database in the near future, built in part by sharing and combination of inter-laboratory libraries. With such a large library, a variety of metabolites could be routinely identified in a single LC run.

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