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Use of electrospray ionization ion-trap tandem mass spectrometry and principal component analysis to directly distinguish monosaccharides

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RATIONALE: Carbohydrates are good source of drugs and play important roles in metabolism processes and cellular interactions in organisms. Distinguishing monosaccharide isomers in saccharide derivates is an important and elementary work in investigating saccharides. It is important to develop a fast, simple and direct method for this purpose, which is described in this study.
METHODS: Stock solutions of monosaccharide with a concentration of 400 μM and sodium chloride at a concentration of 10 μM were made in water/methanol (50:50, v/v). The samples were subjected to electrospray ionization ion-trap tandem mass spectrometry (ESI-MS) and the detected $[2M + Na - H_2O]^+$ ions were further investigated by tandem mass spectrometry (MS/MS), followed by applying principal component analysis (PCA) on the obtained MS/MS data sets.
RESULTS: The MS/MS spectra of the $[2M + Na - H_2O]^+$ ions at *m/z* 365 for hexoses and *m/z* 305 for pentoses yielded unambiguous fragment patterns, while rhamnose can be directly identified by its ESI-MS $[M + Na]^+$ ion at *m/z* 187. PCA showed clustering of MS/MS data of identical monosaccharide samples obtained from different experiments. By using this method, the monosaccharide in daucosterol hydrolysate was successfully identified.
CONCLUSIONS: A new strategy was developed for differentiation of the monosaccharides using ESI-MS/MS and PCA. In MS/MS spectra, the $[2M + Na - H_2O]^+$ ions yielded unambiguous distinction. PCA of the archived MS/MS data sets was applied to demonstrate the spatial resolution of the studied samples. This method presented a simple and reliable way for distinguishing monosaccharides by ESI-MS/MS. Copyright © 2012 John Wiley & Sons, Ltd.

Carbohydrates are important compounds and play important roles in a wide variety of metabolism progresses and cellular interactions in organisms.[1,2] They are also an important source of drugs.[3–5] In natural products, saccharides and their derivatives including glycosides and glycolipids account for a large percentage of identified second metabolites. In recent years, research has been ongoing to determine the molecular structures of carbohydrates and saccharide moieties in natural products such as monosaccharide composition, anomeric configuration, their sequence, and linkages.[6] Among them, distinguishing the monosaccharide isomers in carbohydrates and natural products is the most elementary and important task.

Many analytical methods have been introduced for the determination of monosaccharides. As the key tool in molecular structure identification, the nuclear magnetic resonance (NMR) method encountered certain limitations in identifying monosaccharides due to overlap of proton signals and absence of measurable coupling constants, as well as the substantial quantity of sample required. Chromatographic methods are also widely used to separate and identify monosaccharides[7–9] but they are time-consuming and need pre- or post-column derivatization due to the low sensitivity of detecting underivatized saccharide samples. On the other hand, electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS/MS) offer precise mass spectra for chemicals and non-covalent complexes with high sensitivity. They have been adopted in qualitative distinction and quantitation (to some degree) of monosaccharide stereoisomers.[10–13] Although these methods showed good results in the characteristic differentiation of diastereomeric monosaccharides, they usually associated with specific reagents.

In this study, we report a simple, direct and additional assistant reagent-free ESI-MS/MS method for the differentiation of monosaccharides for the first time. Eight very common D-sugar isomers commonly present in natural products including four hexose isomers [glucose (Glc), galactose (Gal), mannose (Man), and fructose (Fru)], three pentose [ribose (Rib), arabinose (Ara) and xylose (Xyl)] and one desose [rhamnose (Rha)] (see Supplementary Scheme 1, Supporting Information) have been subjected to ESI-MS. An interesting product ion of $[2M + Na - H_2O]^+$ was detected and further investigated by MS/MS. Principal component

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analysis was applied and clearly demonstrated the spatial resolution of these studied monosaccharides (hexoses and pentoses). The application of this method in distinguishing monosaccharide in a natural glycoside is also described.

EXPERIMENTAL

Chemicals

All monosaccharide standards (Glc, Gal, Man, Fru, Rib, Ara, Xyl and Rha) used in this study were purchased from Kelong Chemical Reagent (Chengdu, China) with purities over 98%. Daucosterol was separated and identified by our group. Stock solutions of monosaccharide with a concentration of 400 μM and sodium chloride at a concentration of 10 μM were made in water/methanol (50:50, v/v).

Glycoside hydrolysis

Daucosterol (0.5 mg) dispersed in MeOH (5 mL) was heated with 2 mol/L HCl (6 mL) under reflux for 15 h. The reaction mixture was diluted with water (15 mL) and extracted with $CHCl_3$ (3 × 15 mL) to remove the aglycon. The aqueous layer was directly subjected to ESI-MS analysis.

Mass spectrometry

ESI-MS and ESI-MS/MS analyses were performed on a Finnigan LCQ^{DECA} (San Jose, CA, USA) ion-trap mass spectrometer equipped with an ESI source. The ESI spray voltage was held at 4.5 kV, and the spray was stabilized with nitrogen sheath gas. The heated capillary temperature was set to 200 °C. Helium was introduced into the ion trap as the collision gas for collision-induced dissociation (CID). The maximum ion injection time of 50 ms and three 'microscans' per analytical scan was set. The instrument was operated in the positive ion mode with the experimental parameters as follows: sheath gas flow rate, 35 arbitrary units (arb); capillary voltage, 30 V.

The CID MS/MS experiments were carried out by increasing the normalized collision energy from 10% to 40% with a step size of 5%, and the mass range was set to *m/z* 100–500. Samples were continuously introduced into the ESI source chamber for analysis with a syringe pump at a flow rate of 5 μL/min. To deposit insoluble substances, all samples were centrifuged before analysis at a rotate rate of 5000 rpm for 10 min. All experiments were repeated at least three times on different days. Each spectrum used in analysis was the result of at least 30 scan accumulations.

High-resolution ESI-MS/MS (HR-ESI-MS/MS) was performed on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, USA) to verify and help interpret the results obtained from the low-resolution ESI ion-trap mass spectrometer. The mass analyzer was calibrated before measurements according to the manufacturer's instructions to ensure operation within the <3 ppm instrument specifications. The instrument was operated in the positive ion mode with the experimental parameters as follows: sheath gas flow rate, 30 arb; auxiliary gas flow rate, 8 arb; capillary voltage, 30 V; capillary temperature, 200 °C; spray voltage, 4.5 kV. A normalized collision energy of 30% was adopted to perform CID MS/MS experiments and the mass range was set to *m/z* 100–500 with an isolation width of 2.0 *m/z* units. A stable sample flow of 5 μL/min was introduced into the mass spectrometer by syringe pump.

RESULTS AND DISCUSSION

ESI-MS and ESI-MS/MS of monosaccharides

In our work, ESI-MS spectra of monosaccharides in water/methanol (50:50, v/v) were obtained. Typical full ESI-MS spectra of hexose and pentose can be seen in Fig. 1. A remarkable phenomenon was observed in our study that sodiated monosaccharide dimer ($[2M + Na]^+$, *m/z* 383) and dimer dehydrate ($[2M + Na - H_2O]^+$, *m/z* 365) ions were formed in the ESI process; this phenomenon was also repeatable on the LTQ Orbitrap LX instrument and these ions were confirmed to be sodiated monosaccharide dimers and dimer dehydrates according to the HR-MS data.

A source CID test was performed to inspect the origin of the $[2M + Na - H_2O]^+$ ions by raising the cone voltage from 20 to 60 V in step sizes of 10 V. The signal intensity of $[2M + Na - H_2O]^+$ ions increased and that of $[2M + Na]^+$ ions decreased with increasing the cone voltage. CID MS/MS experiments were also employed for the $[2M + Na]^+$ ions and peaks with a mass loss of 18 Da were observed. The results confirm that $[2M + Na - H_2O]^+$ was formed from the dehydration process of $[2M + Na]^+$, and might be formed via

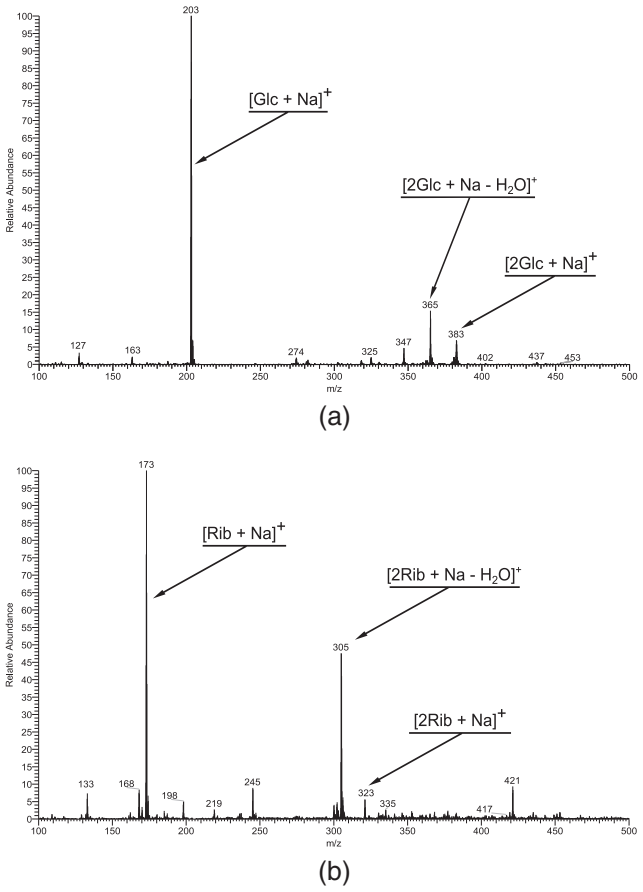

Figure 1. ESI-MS spectra of (a) glucose and (b) ribose in a methanol/water solution of NaCl.

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a similar way to the formation of protonated disaccharides from monosaccharide proton-bound dimer dehydration mentioned by Zapfe and Mu.[14] Although $[2M + Na - H_2O]^+$ ions can be easily observed during MS/MS of $[2M + Na]^+$, the signal intensity was low and the CID procedure mainly produces sodiated molecules, as was reported by March and Stadey.[15] In addition, MS/MS spectra of $[M + Na]^+$ ions showed a slight difference between Gal, Glc and Man as was reported by Zhu and Sato.[12] Thus $[2M + Na]^+$ and $[M + Na]^+$ ions were not chosen to distinguish monosaccharide isomers.

Formation of non-covalent dimers during ESI of saccharides has already been observed and was found to be very often a concentration-related phenomenon.[16,17] To find the proper concentration of monosaccharide samples, LOD (limit of detection) tests for the $[2M + Na - H_2O]^+$ ions were performed for all monosaccharide samples by observing the absence of $[2M + Na - H_2O]^+$ ions with a sample concentration increase in steps of 50 μM starting from 50 μM; the LOD tests showed that samples with concentration of 350 μM were capable of forming $[2M + Na - H_2O]^+$ ions for all samples on the Finnigan LCQ^{DECA} instrument. To obtain better $[2M + Na - H_2O]^+$ ion signal intensity, a sample concentration of 400 μM was finally set in our experiments.

The mass spectra of hexoses and pentoses are shown in Fig. 1; in the case of hexoses, the ions $[M + Na]^+$ (*m/z* 203), $[2M + Na]^+$ (*m/z* 383) and $[2M + Na - H_2O]^+$ (*m/z* 365) were observed, while pentoses showed ions at *m/z* 173 ($[M + Na]^+$), *m/z* 323 ($[2M + Na]^+$) and *m/z* 305 ($[2M + Na - H_2O]^+$), and for desose they were *m/z* 187 ($[M + Na]^+$), *m/z* 351 ($[2M + Na]^+$) and *m/z* 333 ($[2M + Na - H_2O]^+$). As the only desose, Rha can be identified directly by its quasi-molecular ion from the MS mode. To distinguish different hexoses and pentoses, MS/MS was adopted to investigate the dimer dehydrate ions. Breakdown curves of the MS/MS ions were generated for the dimer dehydrate ions of all the monosaccharides (see Supplementary Fig. 1, Supporting Information). With a collision energy lower than 25%, the relative abundances of the product ions of these dimer dehydrate ions were low compare to that of their precursor ions (base peaks), while the breakdown curves for all but the precursor ions became flat when the precursor ions were fragmented with a collision energy higher than 30%. This means that a better MS/MS spectra reproducibility could be obtained when the experiments were carried out with a collision energy higher than 30%, and this is why the product ions of $[2M + Na - H_2O]^+$ and 30% normalized collision energy were chosen for differentiation of monosaccharides.

The MS/MS spectra generated from $[2M + Na - H_2O]^+$ ions at *m/z* 365 (hexoses) and 305 (pentoses) presented a series of product ions as shown in Fig. 2(a) (for four hexoses) and Fig. 2(b) (for three pentoses). The hexose isomers and pentose isomers gave similar product ions with different relative abundant ratios, respectively. The product ions were detected at *m/z* 185, 203, 245, 275, 305 and 347 for hexose isomers (except for Fru), and *m/z* 173, 215, 245 and 287 for pentose isomers. It should be noticed that, as a ketose, Fru presented a special fragmentation pattern; the lack of peaks at *m/z* 245 and 305 made it different from the other three hexoses. Although $[2M + Na - H_2O]^+$ ions of hexoses (Glc, Man and Gal) and pentoses (Rib, Ara and Xyl) showed similar fragmentation patterns, the relative abundances of their product ions showed the potential to distinguish the saccharide isomers. Glc could be easily recognized from hexoses (Glc, Man and Gal) according to its relative abundance ratio of ions at *m/z* 347 to 275 (1.41 ± 0.042 for Glc, 0.68 ± 0.023 for Man and 0.59 ± 0.012 for Gal). Although the MS/MS spectra for Man and Gal seemed quite similar to each other at first glance, they maintained different relative abundance ratios of ions at *m/z* 275 to 245 (2.47 ± 0.024 for Man and 3.77 ± 0.016 for Gal) and thus could also be distinguished. As for the three pentoses (Rib, Ara and Xyl), they could be distinguished from each other by comparing the relative abundance ratios of ions at *m/z* 245 to 215 (0.40 ± 1.23 for Ara, 1.09 ± 0.12 for Rib and 6.93 ± 0.034 for Xyl).

Fragmentation mechanism

The $[2M + Na - H_2O]^+$ ions are in essence sodium-ionized disaccharides, so the fragmentation pattern of the MS/MS spectrum of $[2M + Na - H_2O]^+$ ions can be explained as that of the disaccharides. It is clear that the ions at *m/z* 347 and 287 were formed due to loss of water of hexoses and pentoses, respectively, and the other fragment ions may arise from the precursor ion as shown in Supplementary Fig. 2 (see Supporting Information).

Mechanisms of the generation of MS/MS fragment ions from the precursor $[2M + Na - H_2O]^+$ ions of hexoses (except for Fru) and pentoses have been proposed. Glucose and arabinose were chosen as examples to demonstrate the fragmentation pathways for hexoses and pentoses, respectively. Supplementary Scheme 2 (see Supporting Information) shows the fragmentation mechanism of glucose. In the MS/MS spectra, a loss of 60 Da ($C_2O_2H_4$, HOCH = CHOH) was observed from $[2Glc + Na - H_2O]$ at *m/z* 365, which was formed due to the fragmentation from *m/z* 365 → 305. A further fragmentation from *m/z* 305 → 275 and *m/z* 275 → 245 showed the successively losses of 30 Da (HCHO). In addition, the fragmentation mechanism of arabinose can be found in Supplementary Scheme 3 (see Supporting Information). Similar to glucose, a loss of 60 Da ($C_2O_2H_4$, HOCH = CHOH) from the precursor ion $[2Ara + Na - H_2O]^+$ at *m/z* 305 gave the fragment ion at *m/z* 245, which was further fragmented and produced the ion at *m/z* 215 corresponding to a loss of 30 Da (HCHO). All these results have been confirmed by HR-ESI-MS/MS obtained from a LTQ Orbitrap LX mass spectrometer (Supplementary Table 1 (see Supporting Information) and Table 2), and conform to the results reported by Hofmeister *et al.*[18] and Salpin and Tortajada.[17]

Principal component analysis

PCA was employed to analyze the MS/MS data obtained from the $[2M + Na - H_2O]^+$ ions of different monosaccharide isomers (for hexoses they were Glc, Man and Gal, while, for pentose, they were Rib, Ara and Xyl) with the software SIMICA-P + (version 11.0, Umetric, Umea, Sweden). Intensities of the MS/MS ions of $[2M + Na - H_2O]^+$ (*m/z* 185, 245, 275, 305 and 345 for hexose isomers and *m/z* 173, 215, 245 and 287 for pentose isomers) were chosen as components for PCA. Good spatial resolution was obtained in the PCA plot (Fig. 3), which showed clustering of MS/MS data of identical monosaccharide samples obtained from different experiments.

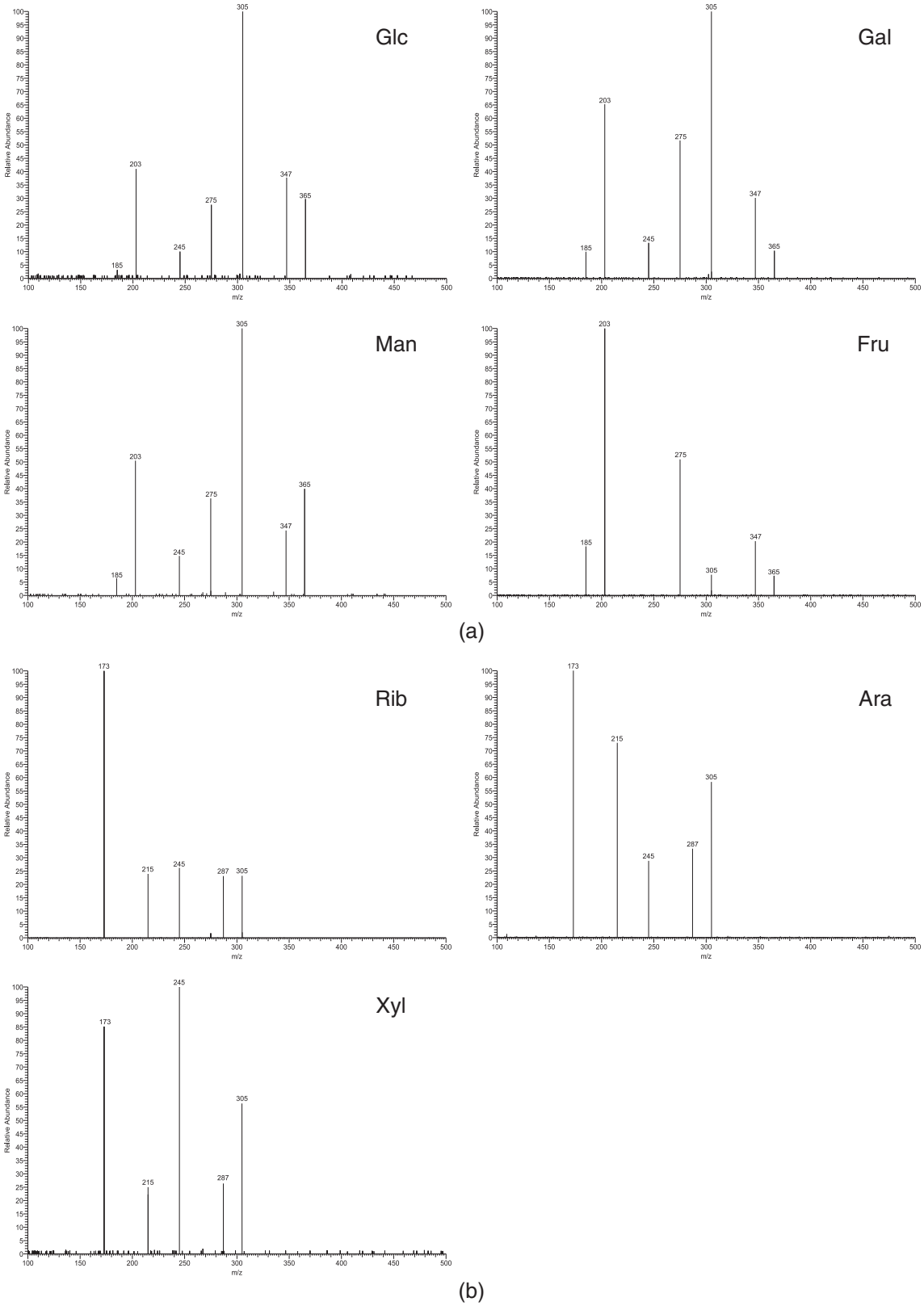

Figure 2. Positive ESI-MS/MS spectra of (a) four hexose isomers (Glc, Gal, Man and Fru) and (b) three pentose isomers (Rib, Ara and Xyl) generated from the precursor ion $[2M + Na - H_2O]^+$ (*m/z* 365). Fragmentation energy was 30%, label threshold was set to 3%.

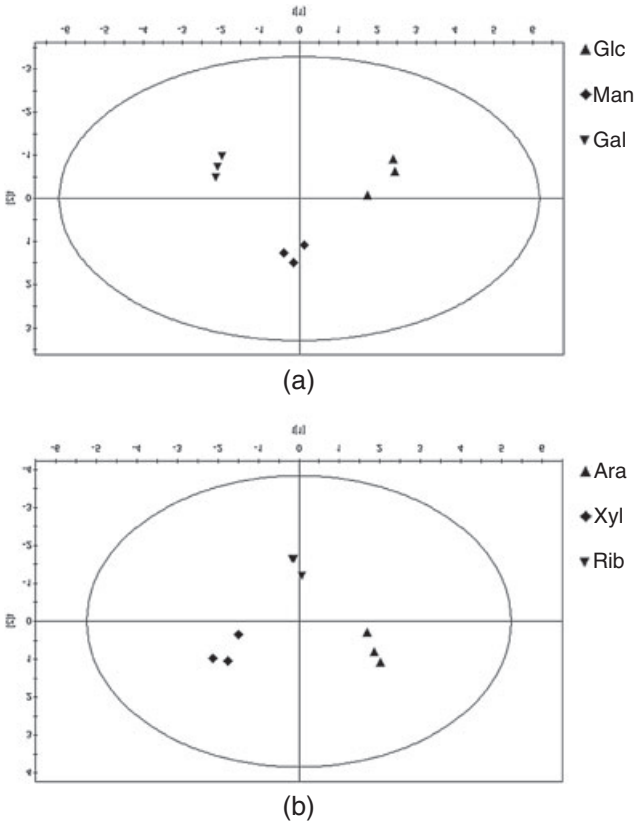

Figure 3. PCA plot of (a) three hexose isomers (Glc, Gal and Man) and (b) three pentose isomers (Rib, Ara and Xyl).

Analysis of the hydrolysate of daucosterol

The presence of ions at *m/z* 365 and 383 in ESI-MS of the hydrolysate of daucosterol indicated the existence of a hexose moiety in the sample. MS/MS data obtained from the precursor ion at *m/z* 365 were thus analyzed. The relative abundance ratio of ions at *m/z* 347 to 275 was calculated to 1.43, which indicated the presence of glucose. The grouping of the MS/MS data with that of glucose in PCA also supported the conclusion. By comparing its NMR data, daucosterol is confirmed to contain a glucose.

CONCLUSIONS

This study showed that monosaccharides of four commonly found hexoses (Glc, Gal, Man, and Fru) and three pentoses (Rib, Ara and Xyl) can be distinguished using ESI-MS/MS on an ion-trap instrument. The ions of $[2M + Na - H_2O]^+$ were detected for all monosaccharides and further investigated by MS/MS. Rha was directly identified from ESI-MS based on its quasi-molecular ion. Absence of peaks at *m/z* 245 and 305 in the MS/MS spectra of the precursor at *m/z* 365 is a characteristic of Fru. In MS/MS spectra, the $[2M + Na - H_2O]^+$ ion at *m/z* 365 (for Glc, Gal and Man) and *m/z* 305 (for Rib, Ara and Xyl) yielded unambiguous distinction. PCA methods were further applied to analyze the archived MS/MS data of the monosaccharide standards and monosaccharides in the hydrolyzed sample of daucosterol; the related PCA plots demonstrated the spatial resolution of the MS/MS spectra of these studied monosaccharides and the clustering with that of the glycoside sample. This method presented a simple and reliable way for distinguishing monosaccharides without derivatization by direct injection to ESI-MS/MS analysis.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Acknowledgements

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