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Differential analysis of natural products using fast polarity switching TOFMS acquisition with high mass accuracy and metabolomics-based approach

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Introduction

The metabolomics technique can rapidly bring information about the similarities and differences within a chromatographic dataset. A metabolomic based approach has been established for metabolite profiling and biomarker discovery, however, it is equally applicable to other research fields including industrial chemical product characterization, food analysis and natural product research.
In the case of natural product research, sample profiling is often a significant challenge due to the intrinsic differences between samples influenced by producing area, cultivation method, extraction method, harvest-time, and local environmental factors in the plant producing area. These natural products show ion signals in both positive and negative ionization. To obtain the complete profile from a sample, it is necessary to run the LC/MS both positive and negative modes.
In this study, an ultra-high-performance liquid chromatography (UHPLC) and a quadrupole ion trap time-of-flight MS was used to generate high accuracy MS^n data on plant extracts taken from different sample classes to determine metabolite profiles and to identify specific endogenous components.

Approach of this study

Strategy of differential analysis using MS-based methods

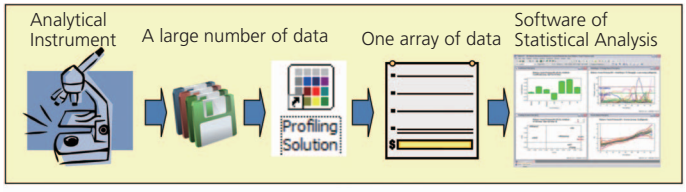


Fig. 1 Procedure of multivariate statistical analysis

Analytical equipment

1) Liquid chromatograph

In natural product research, high throughput and high efficiency analysis is necessary to analyze a complex mixture of samples.
→UHPLC "Nexera" ※1 and "Prominence UFLC "※1

2) Separation column

Column particles which are fine and hard to be clogged up, are necessary to separate a complicated matrix.
→ "Shim-pack XR-ODS" series※1 (1.6 and 2.2 μm particle)

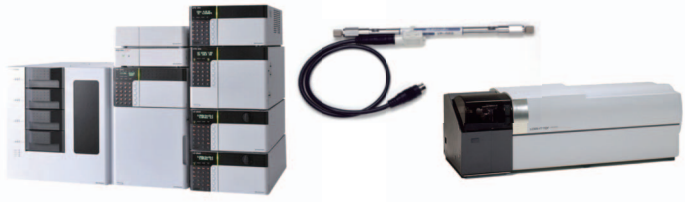
3) Mass spectrometer

Correspondence to UHPLC analysis with fast scanning, fast polarity switching and formula prediction with MS^n data are required.
→ "LCMS-IT-TOF" ※1 (high accuracy MS^n analysis)

4) Software

- "Profiling Solution" ※1 (create an aligned data array)
- SIMCA-P+ ver. 12 ※2 (data mining using multivariate statistical analysis)

※1 Shimadzu, ※2 Umetrics



Experimental

Natural products sample and sample preparation

Extracts of *kakkonto* sample were used as the model samples for complex plant products analysis. *Kakkonto* is an herbal medicine that originated in traditional Chinese medicine (TCM). It is made from a mixture of seven crude drugs (pueraria root , ephedra herb, cinnamon bark, peony root, ginger, jujube and glycyrrhiza). Each crude drug contains a number of recognized active ingredients such as flavonoids, terpenoid, carotenoid, alkaloids and glycosides. Profiling studies for several crude drugs using HPLC and LC/MS were performed for the requirement of quality

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control and index compounds of solvent extracts were determined [1-4].

Four commercially available products of *kakkonto* sample were collected 0.5 g and were suspended in 5 mL of water and were then extracted for 30 min in the ultrasonic bath in order to give the polar component fraction for profiling. After centrifugation the supernatant were filtered using 0.45 um membrane, and was diluted to 20 times with water. Quality control (QC) samples were prepared by pooling aliquots of each sample.

Profiling of metabolites by UHPLC-ESI-QIT/TOFMS

Known compounds of *kakkonto* inside confirmed at retention time, and confirmed mass spectrum by using puerarin, coumarin, paeoniflorin, albiflorin, [6]-gingerol, [6]-shogaol and glycyrrhizin made of a Wako Pure Chemical Industries (osaka, Japan) respectively. Each water extracts were analyzed (n=4), in a randomized order, by LC-MS using a quadrupole ion trap time-of-flight mass spectrometer in electrospray ionization (ESI).

To identify biologically significant components, high mass accuracy MS and MS^n fragment ion information was used to identify the most likely candidate formula.

"Profiling Solution" software was used to create a data array of both positive and negative ion MS data for all sample data; this tool was used to highlight specific components that were statistically different and to export data to "SIMCA-P+ " which data were analyzed by principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA).

Table 1 Analytical conditions of LC-MS

Column	: Shim-pack XR-ODS II (100 mm L. x 2.0 mmI.D., 2.2 um)	Ionization mode	: ESI positive and negative
Flow rate	: 0.45 mL/min	Probe voltage	: +4.5kV/-3.5kV
Column temp.	: 45℃	CDL temperature	: 250℃
Mobile phase	: A) Water containing 0.1% formic acid : B) Acetonitrile containing 0.1% formic acid	BH temperature	: 200℃
		Nebulizing gas flow	: 1.5 L/min
Time prog.	: 2%B (0-0.5min)→40%B (8 min)→ 100%B(15-20 min)→2%B (20.01 min)→ STOP 25 min	Drying gas flow	: 0.1 MPa
		CDL,Q-array voltage	: Default value
		Scan range	: *m/z* 120-1500
injection vol.	: 3 µL		
Mixer vol.	: 50 uL		

Results

Identification of the isolated compounds

From the water extracts of *kakkonto*, about 150 peaks were detected by peak integration function in positive ion TICC and about 140 peaks were detected in negative ion TICC within a 15 min UHPLC separation (Fig. 2). Data will be presented on the MS analysis of known compounds such as puerarin, paeoniflorin, glycyrrhizin resulting in mass accuracy measurements less than 4ppm (using external calibration) acquired with fast polarity switching (switching time of 100msec). These detected peaks were verified using formula prediction software (Shimadzu) that takes into account MS^n information, mass accuracy and isotope modeling. Furthermore, daidzein, formononetin, ephedrine, pseudoephedrine, benzylpaeoniflorin, liquiritin, ononin were tentatively assigned by reference to published literature, by several external databases using high accuracy MS^n data.

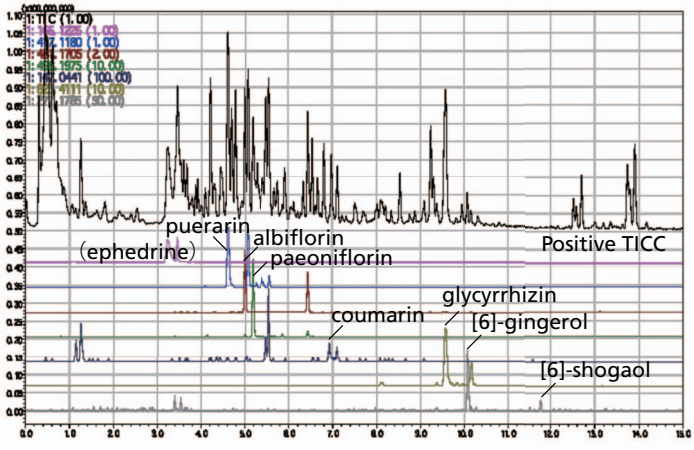
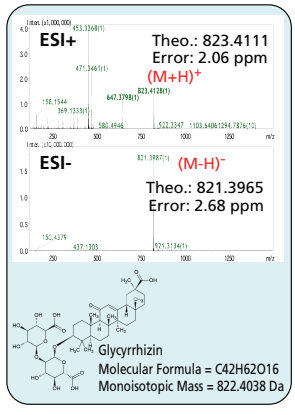


Fig. 2 UHPLC-MS chromatogram of water extracts from *Kakkonto* sample A
The major known compounds were identified using authentic standards.



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Principal Component Analysis

Pooled QC sample analysis was used to assess the performance of the system by repeatedly injecting the QC sample. PCA of the data set resulting from the profiling of the *kakkonto* samples showed the QC samples to be tightly clustered together for both positive and negative ESI data (Fig. 3). Profiling solution software produced a data array of retention time and *m/z* pairs. About ten thousand ions (6400 ions detected in positive ion, 4500 ions detected in negative ion) were detected.

From the PCA, LC/MS data of these extracts clearly enabled differentiation of *kakkonto* samples, four experimental groups were classified into three major clusters in negative mode (Fig. 3b). This results suggest that LC/MS approach could be applied to understand the characteristics of sample, for example the producing area of herbal plants.

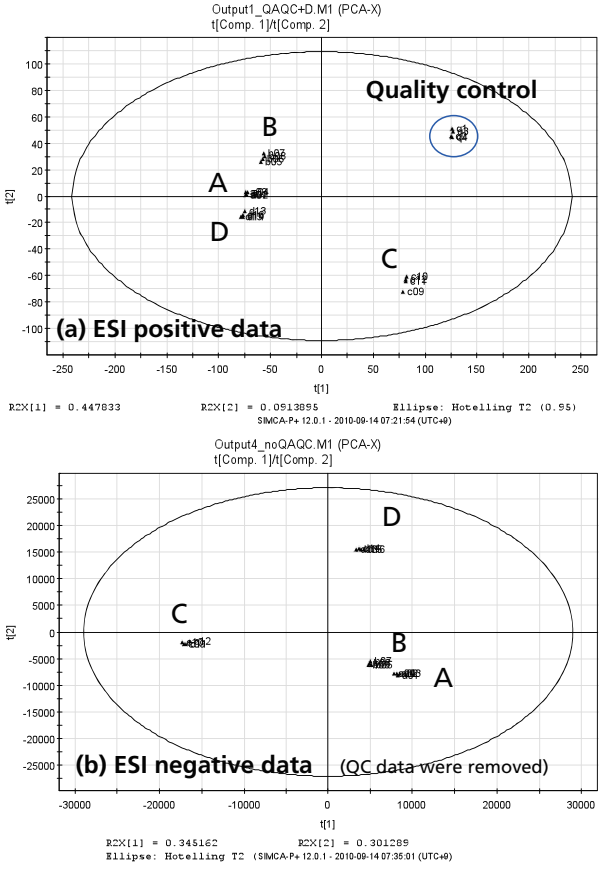

Fig. 3 Multivariate statistics were performed on the aligned data set of positive and negative ions using Umetrics SIMCA-P+ sofware.

Differential analysis using degradation model sample

In addition, this system was applied to the confirmation of quality deterioration of natural products sample. The water extracts were heated at sixty degrees centigrade for one week, and analyzed in the same system. Multivariate statistical analysis were applied to compare the LC/MS data set of samples before and after heating. Fig. 4 shows the OPLS-DA score plot and S-plot of the extracted material about three samples. Several constituents were found as the most differentiating components between heating and non-heating (Fig. 5). This results suggest that LC/MS approach could be applied to evaluate the content of degradation of industrial products.

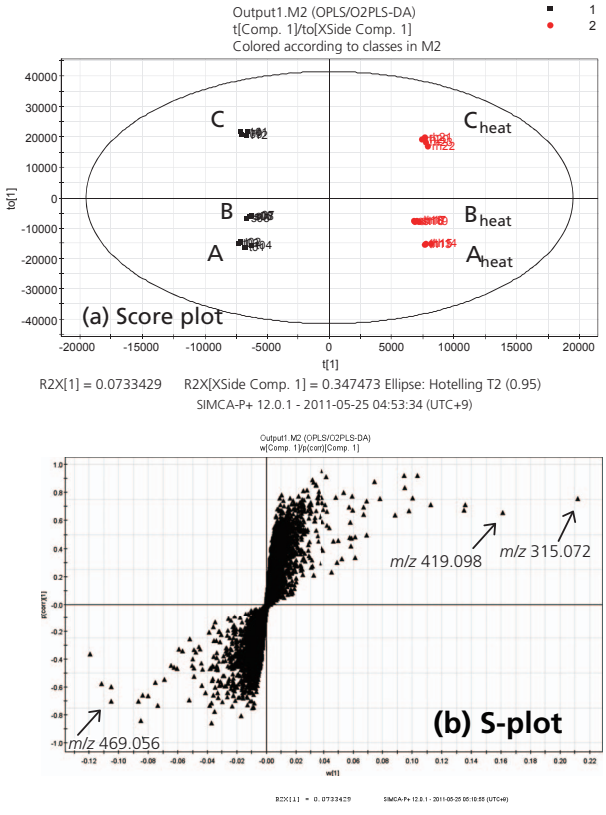

Fig. 4 OPLS-DA score plot and S-plot obtained from degradation model data on negative ion mode

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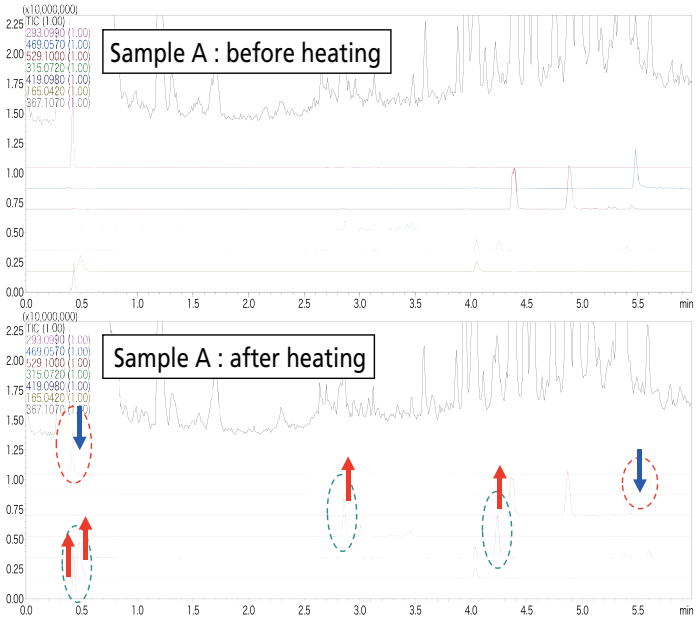

Fig. 5 MS chromatograms of samples before and after heating on negative ion mode. Several constituents were found that have relative changes in ion signal intensity.

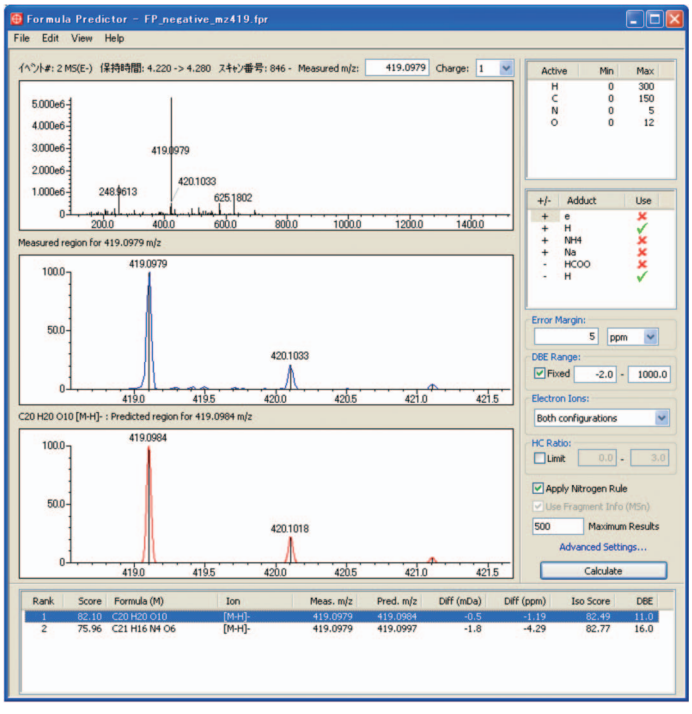

Fig. 6 The formula prediction software results on the *m/z* = 419.0979 ion are displayed. The highest score calculated corresponds to the molecular formula $C_{20}H_{20}O_{10}$.

Conclusions

- ✓ Mass spectrometry-based metabolite profiling was used to identify changes in chemical component levels in natural product model using fast polarity switching TOFMS analysis.
- ✓ Bioactive marker compounds were measured and identified using a combination of high accuracy MS^n data and verified by reference to authentic standards and to internal and external databases.
- ✓ Established approach is useful for the rapid determination of subtle differences and exploring the potential markers for quality control within the natural products.

References

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