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Comprehensive Two-Dimensional HPLC and Informative Data Processing for Pharmaceuticals and Lipids

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Yoshiyuki WATABE, Tetsuo IIDA, Daisuke NAKAYAMA,
Kanya TSUJII, Saki UEDA, Tadayuki YAMAGUCHI,
Junichi MASUDA, Yoshihiro HAYAKAWA
Shimadzu Corporation, Kyoto, Japan

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Introduction

Kakkonto, traditional Chinese drug, is believed to be effective against cold, headache, stiff shoulders, etc. It consists of natural plants and contains many compounds such as ephedrine, glycyrrhizic acid, and cinnamic acid. Nexera-e, comprehensive two-dimensional liquid chromatograph, is useful to separate such a complex matrix. In general, different separation modes are selected for 1st and 2nd dimensions for comprehensive two-dimensional chromatography to obtain high resolution that can not be achieved by each individual separation mode. We tried comprehensive separation using a semi-micro reversed-phase column and neutral pH mobile phase for 1st dimension and an ultra-high speed reversed phase column and acidic pH mobile phase for 2nd dimension. Herbal medicine contains many relatively polar compounds, so pH is an important parameter to modify separation selectivity. We performed differential analysis for commercial Kakkonto products and determined glycyrrhizic acid contained with photodiode array detector (PDA).

In lipidomics, phospholipids are the attractive targets of LC/MS analysis since lipids are important and essential components of biological membranes. However, conventional HPLC system by a single separation mode performs poorly on biological lipid sample, because it contains various kinds of lipids with common moieties that govern their behavior on column. Nexera-e was capable of characterizing phospholipids both quantitatively and qualitatively when coupled with triple quadrupole and iontrap-TOF type of mass spectrometer (MS/MS and IT-TOF) respectively. Reliable identification of lipid species was performed by acquiring *m/z* values of related parent and fragment ions at high accuracy with the IT-TOF and matching the data to commercially available data-base.

Experimental

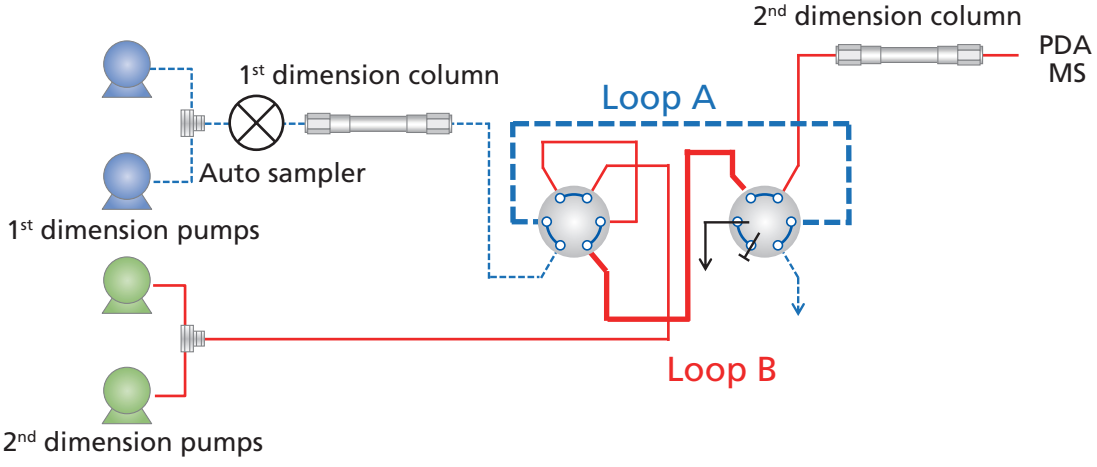

Fig.1 Flow line diagram of "Nexera-e"

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Kakkonto

1D Column	: Shim-pack XR ODSII (100 mm L. x 1.5 mm I.D., 2.2 µm)
Mobile Phase	: A; 10 mmol/L (sodium) phosphate buffer pH = 6.8 B; acetonitrile
Flow Rate	: 0.05 mL/min
Time Program	: B Conc. 5% (0 min) → 30% (70 min) → 90% (80 min) → 90% (90 min) → 50% (90.1 min) → STOP (110 min)
Column Temp.	: 40 °C
Injection vol.	: 2 µL
Loop vol.	: 50 µL (Modulation time : 60 sec)
2D Column	: Kinetex (50 mm L. x 3 mm I.D., 2.6 µm)
Mobile Phase	: A; 10 mmol/L (sodium) phosphate buffer pH= 2.6 B; acetonitrile
Flow Rate	: 2 mL/min
Time Program	: Without Auto-gradient; B Conc. 5% (0 min) → 60% (0.75 min) → 5%(0.76 min) → STOP (1 min) With Auto-gradient; Initial.B Conc. 5% (0 min) → 40% (0.75 min) → 5%(0.76 min) → STOP (1 min) Final.B Conc. 20% (0 min) → 60% (0.75 min) → 20%(0.76 min) → STOP (1 min) The initial and final B conc. has been changed by a stepwise method
Detector	: SPD-M30A Photo diode array detector (PDA, standard cell 1µL, wavelength = 254 nm)

Glycerophospholipids

1D Colum	: Nucleosil SIL (150 mm L. X 1.0 mm I.D., 3 µm)
Mobile Phase	: A; isooctane/acetone/ethyl acetate/acetic acid = 40/20/20/0.03 (v/v/v/v) B; isooctane/2-propanol/water/acetic acid/28% ammonia aq.sol.= 40/51/9/0.03/0.03 (v/v/v/v/v)
Flow Rate	: 0.02 mL/min
Time Program	: B Conc. 30% (0 min) → 40% (25 min) → 100% (40 min) → 100% (55 min) → 30% (55.1 min) → STOP (70 min)
Column Temp.	: 40 °C
Injection vol.	: 5 µL
Loop vol.	: 20 µL
2D Column	: Phenomenex Kinetex C18 (50 mm L. X 4.6 mm I.D., 2.6 µm)
Mobile Phase	: A; methanol/water/acetic acid/28% ammonia aq.sol. = 90/10/0.05/0.05 (v/v/v/v) B; 2-propanol/acetic acid/28% ammonium hydroxide = 100/0.05/0.05 (V/V/V)
Flow Rate	: 3.5 mL/min (50% split for MS)
Time Program	: B Conc. 10% (0 min) → 50% (0.75 min) → 10%(0.76 min) → STOP (1 min) The initial B conc. has been changed by a stepwise method
Detector	: Shimadzu LCMS-8050 (MS/MS, ESI positive , MRM mode) Shimadzu LCMS-IT-TOF (IT-TOF, ESI positive/negative scan, *m/z* :700-800

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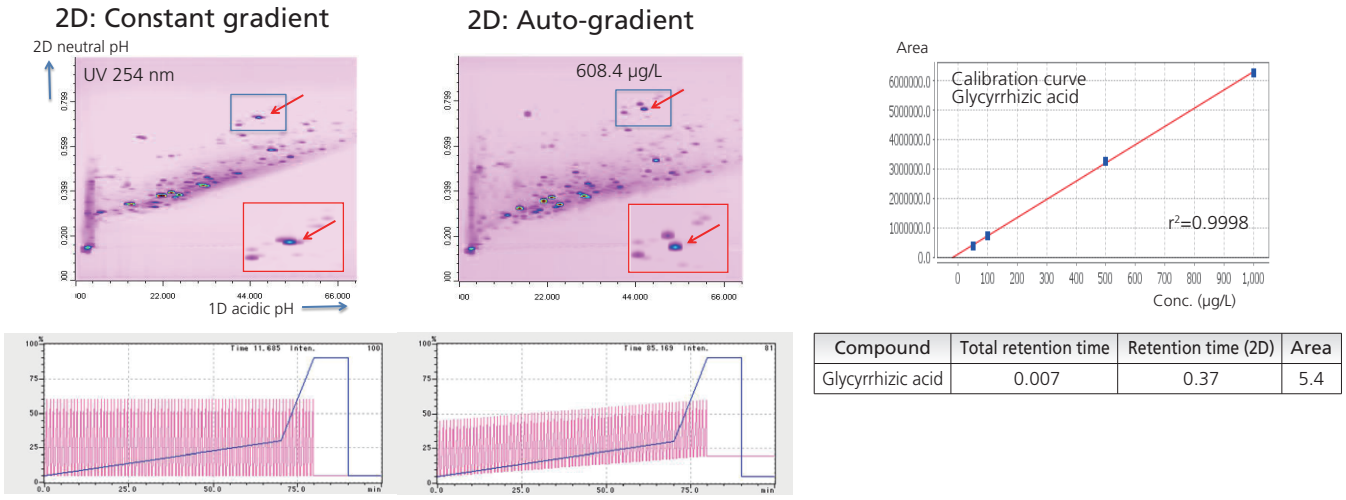
Results

Kakkonto (Chinese crude drug): RP×RP-PDA

Effect of auto gradient function and determination of Glycyrrhzic acid



Compound	Total retention time	Retention time (2D)	Area
Glycyrrhizic acid	0.007	0.37	5.4

Fig.2 Comprehensive-2D separation of commercial Kakkonto product with/without "Auto gradient program" function, linearity of 50-1000 mg/L and repeatability of 5 replicated analyses of real sample

Differential analysis between two kakkonto products

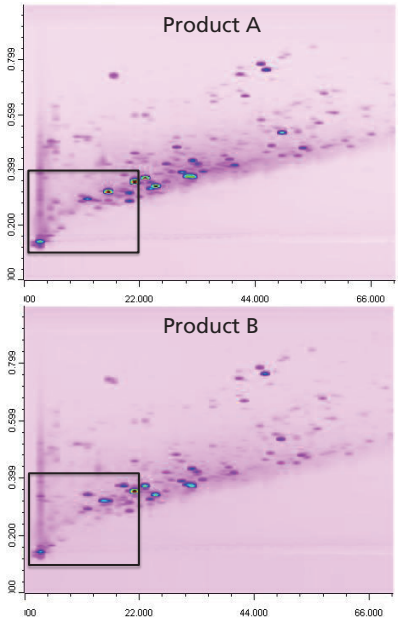


Fig.3 Comparison of comprehensive contour plots of two Kakkonto products

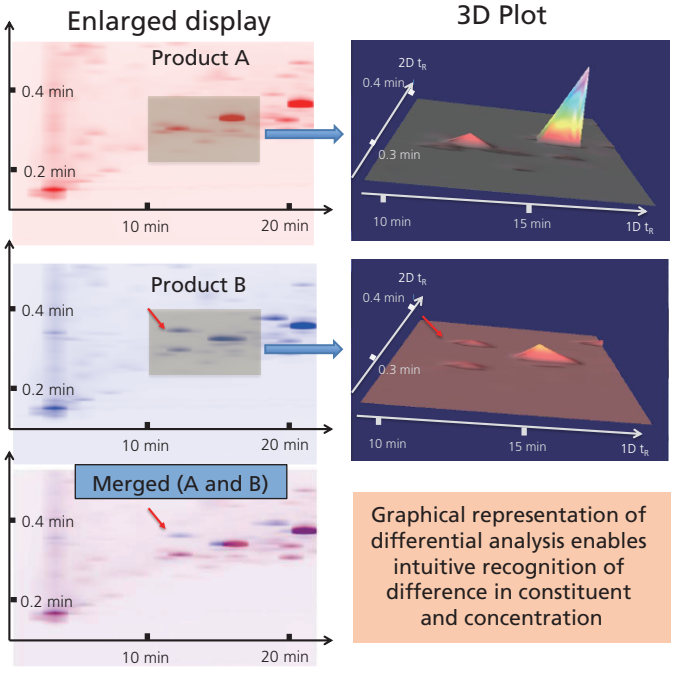


Fig.4 Differential analysis between A and B products

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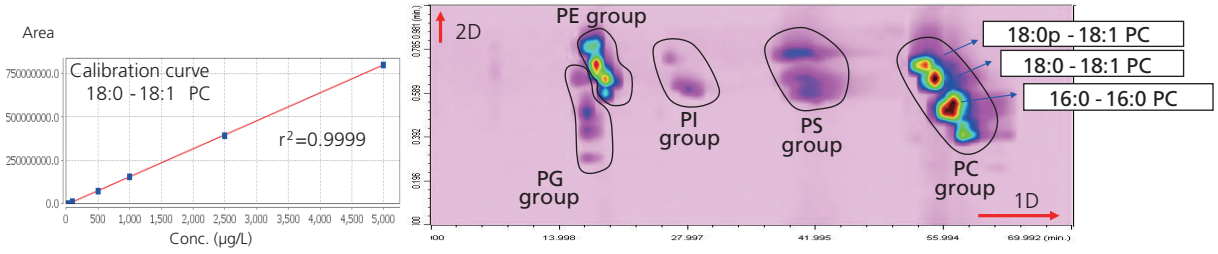
Glycerophospholipids: NP×RP-MS/MS (Whole MRM plots)

Quantitative analysis of phosphatidylcholines



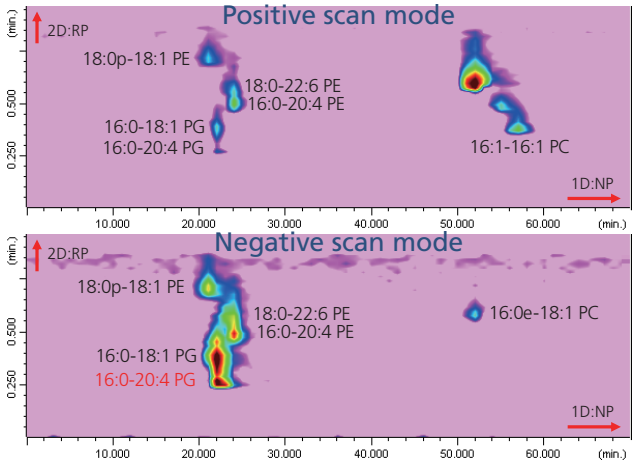
Compound	MRM transition	Total retention time	Retention time (2D)	Blob Area
16:0-16:0 PC	m/z 734.6 > 184.1	0.007	0.9	6.8
18:0-18:1 PC	m/z 788.6 > 184.1	0.013	1.1	8.9
18:0p-18:1 PC	m/z 772.6 > 184.1	0.013	1.2	6.4

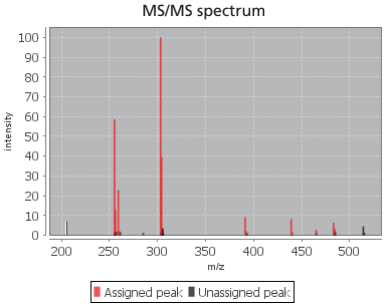
Fig.5 Comprehensive separation of phospholipids, linearity of 50-5000 μg/L and repeatability of 5 replicated analyses of 500 μg/L STD solution in %RSD

Glycerophospholipids: NP×RP IT-TOF (Scan)

Qualitative analysis with IT-TOF based on accurate *m/z*

The GPLs mixture was comprised of 50 mg/L of each of PG, PE and PC. The contour plot of ESI- positive/negative scan (*m/z* 700-800) by LCMS-IT-TOF showed the separation of these compounds. PC compounds were detected more sensitively in ESI positive, whereas PE and PG compounds in negative mode. Then MS^2 analysis to identify 16:0-20:4 PG was performed. Obtained data from both MS and MS/MS were contributed to automatic lipid identification using the data-base matching software "Lipid search" that compares the experimental and theoretical *m/z* values of both parent and fragment ions and it successfully indicated 16:0-20:4 PG as the most expecting candidate. Additionally, MS2 fragment peak assignations supported the identification of 16:0-20:4 PG.





Assigned fragment peaks in MS/MS spectrum

DataId	LipidIon	LipidGroup	Scan	ObsMz	CalcMz	Delta(Da)
1056	PG(16:0/20:4)-H	PG(36:4)-H	MS2	769.4972	769.5025	-0.005

Fig.6 Contour plot of positive/negative scan mode and lipid identification analysis using the data-base matching software

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Conclusion

1. Novel comprehensive two-dimensional LC "Nexera-e" was successfully applied to quantitative, qualitative and differential analyses of complex samples.
2. PDA, MS/MS and IT-TOF detection system could go with comprehensive two-dimensional separation with satisfactory sensitivity and ruggedness.
3. Combination use of different separation modes afforded sufficiently orthogonal separation of Chinese crude drug and phospholipids.
4. Excellent repeatability, linearity of blob area as well as repeatability of total retention time by "Nexera-e"-MS/MS or PDA setup were confirmed.
5. Reliable identification of target lipid in 2D contour plot was achieved by data-base matching analysis based on high resolution IT-TOF data.

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