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# Novel Comprehensive Two-dimensional LC and Related Application for Complex Samples

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# Novel Comprehensive Two-dimensional LC and Related Application for Complex Samples


## Abstract

A novel two-dimensional LC (2D LC) has been developed and successfully applied to phospholipids, polyphenols and medicinal compounds in biological, food and crude drug samples, respectively. A comprehensive 2D LC is recognized as very effective strategy for both quantitative and qualitative analyses, especially for natural and biological samples in complex matrices. However, the combined effect of the necessity to minimize band broadening in the 1st dimension and the very short analysis time in the 2nd dimension has been a difficulty for spreading this technology. Recent progress in the technology of solvent delivery at very low flow rate and ultra-high speed HPLC (UHPLC) analysis realizes a practical and comprehensive 2D LC. Our new, truly comprehensive 2D LC system, "Nexera-e", combines our latest UHPLC Nexera X2 product line with photo diode array and/or mass spectrometric detectors. Comprehensive 2D LC allows for highly efficient separation of a variety of samples, even in complex matrices, as well as ordinary quantitative analysis. The orthogonal separation selectivity between 1st and 2nd dimensions is a key issue for this technique but difficult to accomplish due to solvent compatibility and column selection in the 1st and 2nd dimensions. In our study for improving the limited orthogonality, it showed dramatic improvement when employing a shifted gradient profile in the 2nd dimension, where initial and final mobile phase composition increase stepwisely, to match the 1st dimension gradient profile. Raw data is mathematically manipulated by dedicated software, resulting in enhanced peak capacity and identification ability compared to those of independent 1D and 2D separations.


## Experimental

**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 3.0 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)<br>With Auto-gradient; Initial.B Conc. 5% (0 min) → 45% (0.75 min) → 5%(0.76 min) → STOP (1 min)<br>Final.B Conc. 20% (0 min) → 60% (0.75 min) → 20%(0.76 min) → STOP (1 min)<br>The initial and final B conc. has been changed by a stepwise method |
| Detector | : | SPD-M30A Photo diode array detector (standard cell 1µL, wavelength= 254 nm) |

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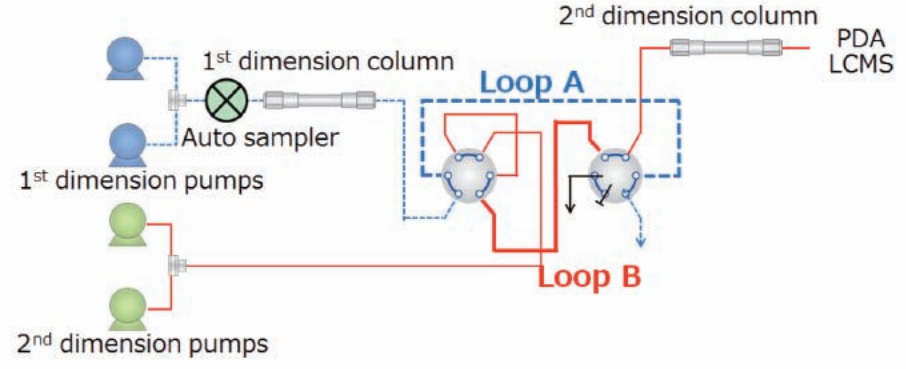
## Red Wine

1D Colum : Shim-pack XR ODSⅡ (100 mm L. X 1.5 mm I.D., 2.2 µm)
Mobile Phase : A; 10 mM (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)→5% (90.1 min) →STOP (110 min)
Column Temp. : 40°C
Injection vol. : 3µL
Loop vol. : 50 µL (Modulation time : 60 sec)
2D Column : Kinetex XB-C18 (50 mm L. X 3 mm I.D., 2.6 µm)
Mobile Phase : A; 10 mM(sodium)phosphate buffer pH= 2.6 B; acetnitrile
Flow Rate : 2 mL/min
Time Program : Auto-gradient;
Initial.B Conc. 5% (0 min) → 30% (0.75 min) → 5%(0.76 min) → STOP (1 min)
Final.B Conc. 30% (0 min) → 40% (0.75 min) → 30%(0.76 min) → STOP (1 min)
The initial and final B conc. has been changed stepwisely
Detector : SPD-M30A Photo diode array detector
(high sensitivity cell 1 µL, wave length= 270 nm, 278 nm, 354 nm)

## Glycerophospholipids

1D Column : 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 (ESI positive , MRM mode)




Flow diagram of 2D LC (Nexera-*e*)

# Results

## Kakkonto (Chinese Crude Medicine): RP×RP -PDA

### Effect of Auto Gradient Function and Determination of Glycyrrhzic Acid

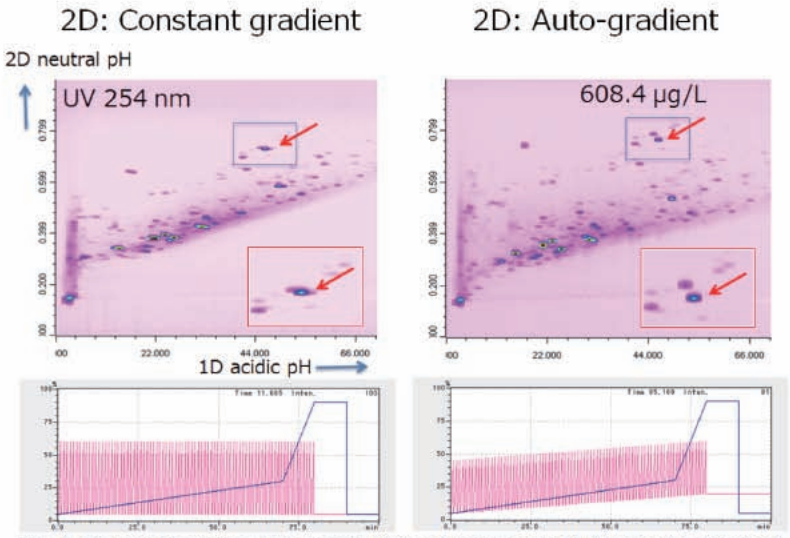
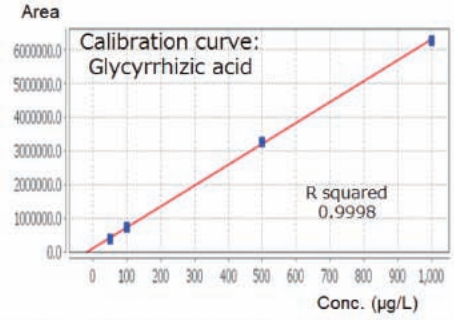



Fig.1 Comprehensive-2D separation of commercial Kakkonto product with/without "Auto gradient program" function




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

Fig.2 Repeatability (in %RSD) of 5 replicated analyses and Linearity of 50-1000 µg/L

### Differential Analysis Between Two Kakkonto Products

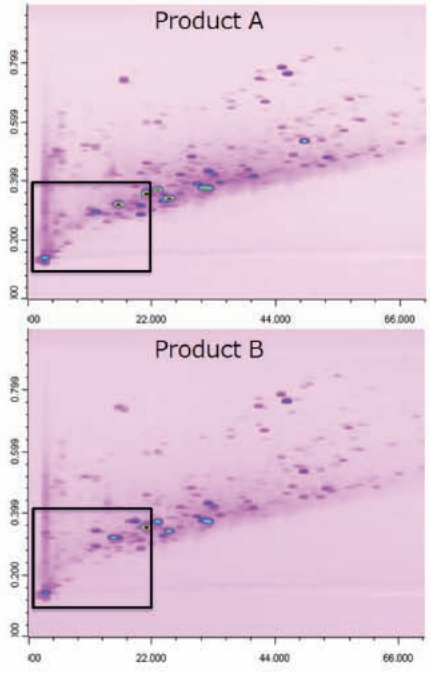



Fig.3 Comparison of comprehensive 2D plots of two Kakkonto products

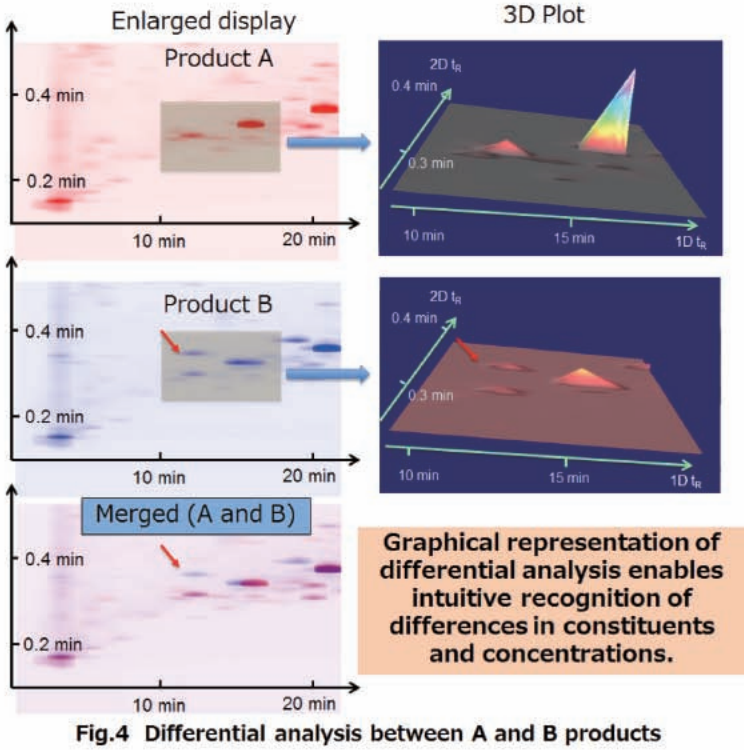



Fig.4 Differential analysis between A and B products

Graphical representation of differential analysis enables intuitive recognition of differences in constituents and concentrations.

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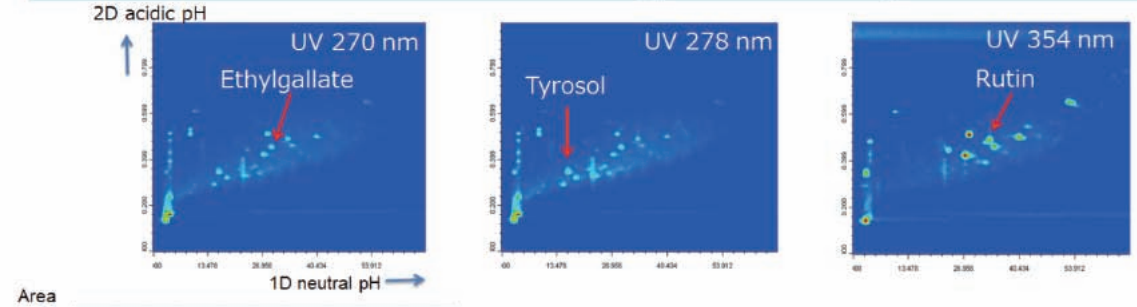
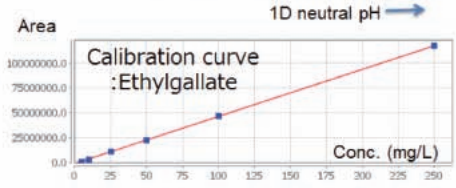
## Red Wine: RP×RP -PDA

### Determination of Polyphenolic Compounds



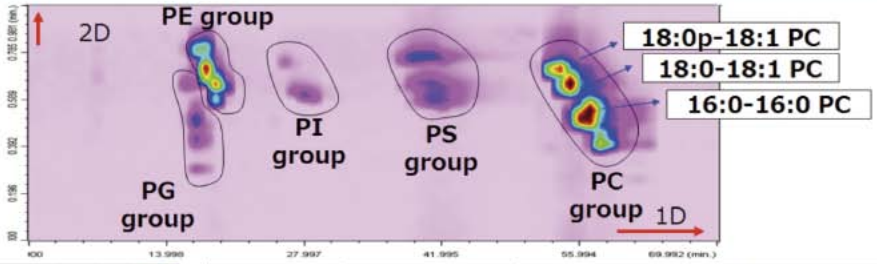



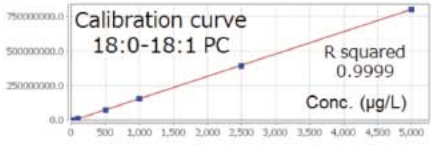

| Compound | Total retention time | Retention time(2D) | Area | R squared | Concentration(mg/L) |
|---|---|---|---|---|---|
| Tyrosol | 0.007 | 0.16 | 2.7 | 0.9998 | 101.5 |
| Ethylgallate | 0.007 | 0.49 | 3.8 | 0.9999 | 15.1 |
| Rutin | 0.007 | 0.52 | 4.1 | 0.9988 | 14.2 |

**Fig.5 Comprehensive separation of polyphenols in red wine with appropriate wavelength, repeatability for retention time and blob area (%RSD, n=5), R2 value of calibration curve, and concentration**

## Glycerophospholipids: NP×RP -LCMS-8050 (Whole MRM Plots)

### Detection with Triple Quadrupole Mass Spectrometer




| 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.6 Comprehensive separation of phospholipids, linearity of 50-5000 µg/L and repeatability (%RSD, n=5)**

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## Conclusion

1. Novel comprehensive 2D LC Nexera-e was successively applied to quantitative, qualitative and differential analyses of complicated natural samples.
2. Both PDA and MS/MS detection system are used for comprehensive 2D separation with satisfactory sensitivity and robustness.
3. Limited orthogonality between 1D and 2D separation can be improved by chromatographic technique such as shifted gradient profile.

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