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Comprehensive analysis of primary & secondary metabolites in citrus using an automated method changeover UHPLC system coupled to LC/MS/MS

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Introduction

There is a demand for better tasting, healthier and safer food-stuffs which better meet the needs of both industry and the consumer. New technology has been required to monitor and improve the quality of food, metabolomics has become an important tool for food processing, plant breeding and so on. In-food science, comprehensive analysis of primary metabolites through to secondary metabolites is very important. However, there is no application which can analyze them at the same time. In this study, we developed the analytical methods using LC/MS/MS for monitoring the primary and secondary metabolites in foods, focus on the major compound's categories such as organic acids, amino acids, sugars, carotenoids and flavonoids.

Methods & Materials

Sample preparation

7 varieties of citrus fruits (Mikan, Ponkan, Shiranui, Amakusa, Hassaku, Buntan, Hyuganatsu) in citrus genus were selected and purchased from local grocery store in Kyoto, Japan.
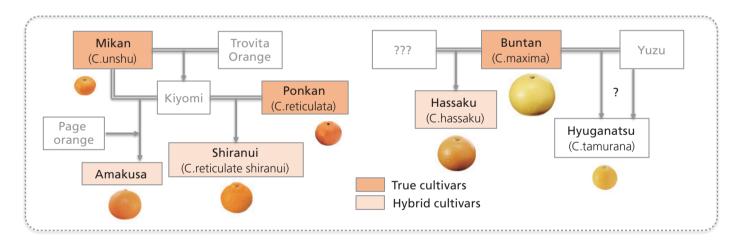

Figure 1   The breed trees of tested citrus fruits (the cultivars with grey were not surveyed in this study)
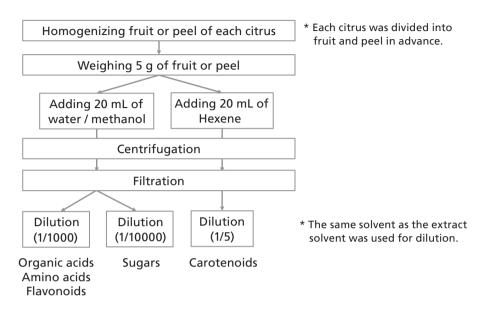
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* Each citrus was divided into fruit and peel in advance.

Homogenizing fruit or peel of each citrus
Weighing 5 g of fruit or peel
Adding 20 mL of water / methanol
Adding 20 mL of Hexene
Centrifugation
Filtration
Dilution (1/1000)
Dilution (1/10000)
Dilution (1/5)
Organic acids Amino acids Flavonoids
Sugars
Carotenoids

* The same solvent as the extract solvent was used for dilution.

Figure 2   Protocol of sample preparation

LC/MS/MS analysis

We developed 3 analytical conditions for separation of target compounds. These 3 analytical conditions, comprising combinations of 4 mobile phases and 3 columns, were automatically performed using UHPLC multi-method system in 40 minutes. This system enables multiple separations based on the combination of up to 8 different mobile phases and 6 different columns.
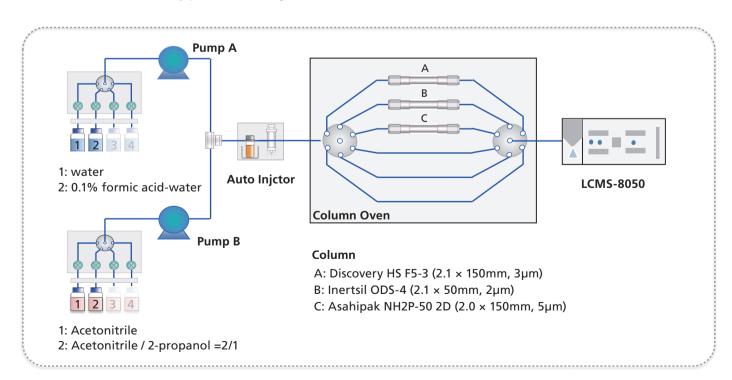

Pump A
1: water
2: 0.1% formic acid-water
Auto Injctor
Pump B
1: Acetonitrile
2: Acetonitrile / 2-propanol =2/1

Column Oven
A
B
C
LCMS-8050

Column
A: Discovery HS F5-3 (2.1 × 150mm, 3µm)
B: Inertsil ODS-4 (2.1 × 50mm, 2µm)
C: Asahipak NH2P-50 2D (2.0 × 150mm, 5µm)

Figure 3   System configuration

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Table 1 Analytical conditions

Conditions		Condition 1	Condition 2	Condition 3
HPLC	Instrument	UHPLC Nexera system (Shimadzu)		
	Target compounds	Organic acids Amino acids Flavonoids	Carotenoids	Sugars

	Column	A: Discovery HS F5-3
		  (2.1×150mm, 3μm)
		  Sigma-aldrich
			B: Inertsil ODS-4
			  (2.1×50mm, 2μm)
			  GL-science
				C: Asahipak NH2P-50 2D
				  (2.0×150mm, 5μm)
				  Shodex

	Column oven temp.	40°C		
	Mobile phase A	2: 0.1% formic acid-water	1: water	1: water
	Mobile phase B	1: Acetonitrile	2: Acetonitrile / 2-propanol =2/1	1: Acetonitrile
	Flow rate	0.25 mL/min.	0.4 mL/min.	0.4 mL/min.
	Time program	0%B (0-2min.) → 95% (10-13min.) → 0% (13.01-16min.)	60%B (0min.) → 100% (5-8min.) → 60% (8.01-10min.)	65%B (0-8min.) → 30% (8-11min.) → 65% (11.01-15min.)
	Measurement time	16 min.	10 min.	15 min.
	Total run time	41 min.		
	Injection volume	2 μL		
MS	Instrument	LCMS-8050		
	Ionization	ESI (+ / -)		
	Mode	MRM		

High Speed Mass Spectrometer
  Ultra Fast Scanning
    - 30,000 u / sec.
  Ultra Fast Polarity Switching
    - 5 msec.
Ultra Fast MRM
    - Max. 555 transitions /sec

Figure 4   LCMS-8050 triple quadrupole mass spectrometer

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Result

Standards of primary & secondary metabolites

All tested compounds (10 organic acids, 24 amino acids, 5 sugars, 5 flavonoids and 9 carotenoids) were successfully separated using 3 analytical conditions (Fig. 5). Calibration range and LOQs of all compounds were showed in Table 2.
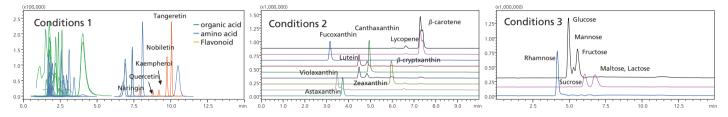

Figure 5   MRM chromatograms in each analytical conditions

Table 2 Calibration range of tested compounds

Amino acids	CAL of range
Cystine	1-100
Aspartic acid	5-100
Asparagine	5-100
Serine	5-100
4-Hydroxyproline	1-100
Glycine	5-100
Lysine	1-100
Cysteine	50-100
Threonine	5-100
Glutamic acid	1-100
Alanine	5-100
Proline	1-100
Ornitine	5-1000
Glutamine	5-100
Histidine	5-100
Arginine	5-100
GABA	5-100
Valine	1-100
Methionine	5-100
Tyrosine	5-100
Isoleucine	5-100
Leucine	10-100
Phenylalanine	1-100
Tryptophan	5-500

(µg/L)

Organic acids	CAL of range
Tartaric acid	50-10000
2-Ketoglutaric acid	10-1000
Isocitric acid	50-10000
Malic acid	10-5000
Lactic acid	50-10000
Citric acid	50-10000
Pyroglutamic acid	10-10000
Succinic acid	10-1000
Fumaric acid	500-1000
Maleic acid	50-10000

(µg/L)

Carotenoids	CAL of range
Fucoxanthin	0.1-100
Violaxanthin	1-100
Astaxanthin	0.5-100
Lutein	0.1-100
Zeaxanthin	0.5-100
Canthaxanthin	0.05-100
β-Cryptxanthin	0.05-100
Lycopene	50-100
β-carotene	0.1-100

(µg/L)

Sugars	CAL of range
Rhamnose	50-1000
Fluctose	50-5000
Glucose	50-5000
Sucrose	100-5000
Maltose, Lactose	200-2000

(µg/L)

Flavonoids	CAL of range
Naringin	10-1000
Quercetin	5-1000
Kaempferol	5-1000
Nobiletin	0.1-1000
Tangeretin	0.1-1000

(µg/L)

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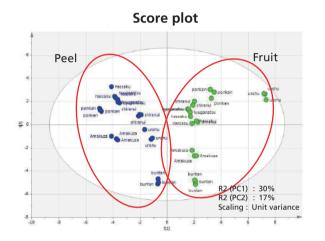
Sample analysis

■ Comparison between fruit and peel

Principal component analysis (PCA) was performed to compare fruit with peel in all tested citrus cultivars. Fruit and peel samples were tend to be separated into 2 groups in the score plot. Analysis of the loading plot reveals some compounds responsible of the separation between fruit and peel samples (sugars, flavonoids and carotenoids).

Score plot

Loading plot

Figure 6   Result of PCA in both fruit and peel samples (SIMCA-P)

• Primary metabolites (sugars)

Figure 7   Comparison of amount (mean) and composition between fruit and peel

Result of the quantification, there is no difference in total amount of sugars between fruit and peel samples. However, composition of them was obviously different.

• Secondary metabolites

In secondary metabolites, total amount was significantly different between fruit and peel.
We confirmed that fruit is more rich in carotenoids than peel but poor in flavonoids.
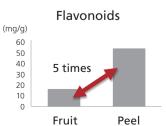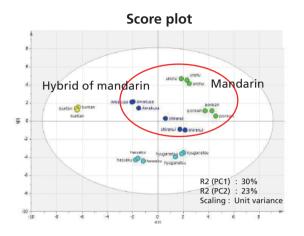

Figure 8   Comparison of amount (mean) in secondary metabolites

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■ Comparison between cultivars (fruit)
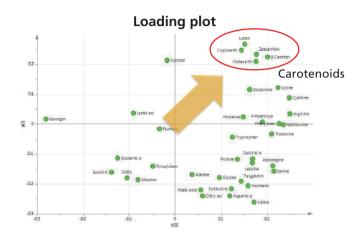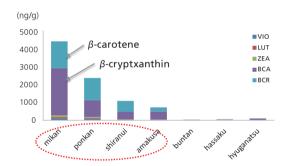
Score plot

Loading plot

Figure 9   Result of PCA in fruit samples (SIMCA-P)

The cultivars in mandarin and hybrid of mandarin, their plots tend to be located in the upper right in the score plot. The loading plot showed that carotenoids are characteristic of these cultivars. Result of the quantification, the cultivars in mandarin and hybrid of mandarin showed a high revel of carotenoids, especially β-cryptxanthin and β-carotene.

Figure 10   Comparison of amount of carotenoids between 7 citrus

Conclusions

- Primary & secondary metabolites were successfully separated using 3 analytical conditions, and UHPLC multi-method system enables contentious analysis without replacement of the columns or mobile phases.
- We confirmed that the cultivars in mandarin group showed high level of carotenoids.