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Application News

No.C132

Liquid Chromatography Mass Spectrometry

Comprehensive Analysis of Primary and Secondary Metabolites in Citrus Fruits Using an Automated Method Changeover UHPLC System and LC/MS/MS System [LCMS-8050]

Due to an increasing trend for healthy lifestyles, the functional properties of agricultural products and foods are being emphasized alongside their taste qualities, and the production and development of foods with high additional value is becoming increasingly important. Metabolome analysis, which is the comprehensive analysis of all metabolites present in a living organism, is now being applied in a variety of areas and not just to analyze the metabolites of living organisms. Metabolome analysis is becoming an important tool in the food sector in areas such as food processing and plant breeding.

In food analysis, it is very important to carry out comprehensive analysis of primary and secondary metabolites that contribute to the color, smell, taste, and functional properties of food. To date, there has been only a few examples of performing these analyses simultaneously.

In this article, we describe a method of performing comprehensive analysis of primary and secondary metabolites in food (major organic acids, amino acids, sugars, carotenoids, and flavonoids) using LC/MS/MS, and also present an example application of this analytical method using citrus fruits as an actual sample.

■ Sample Preparation

We used seven citrus fruits (mikan orange, ponkan, shiranui orange, amakusa orange, hassaku orange, buntan orange, and hyuganatsu) shown in Fig. 1. Each metabolite was fractionated and extracted using two solvents, as shown in Fig. 2.

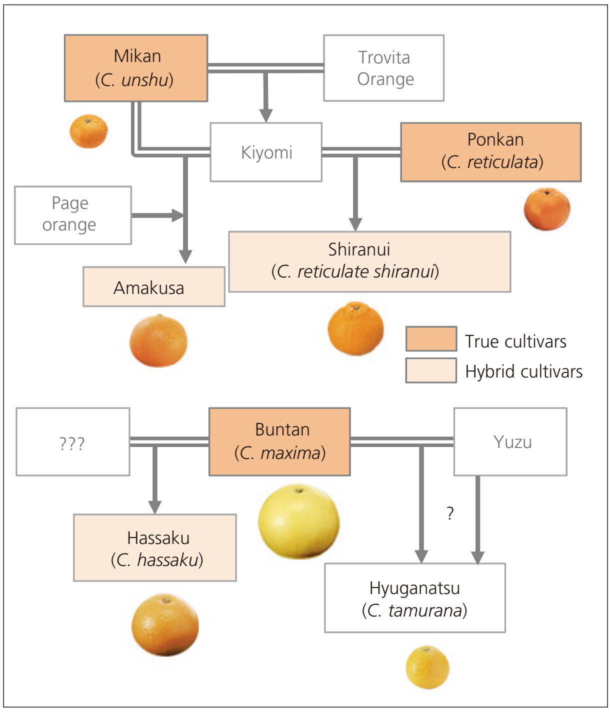

Fig. 1 Taxonomic Tree of Seven Citrus Fruits

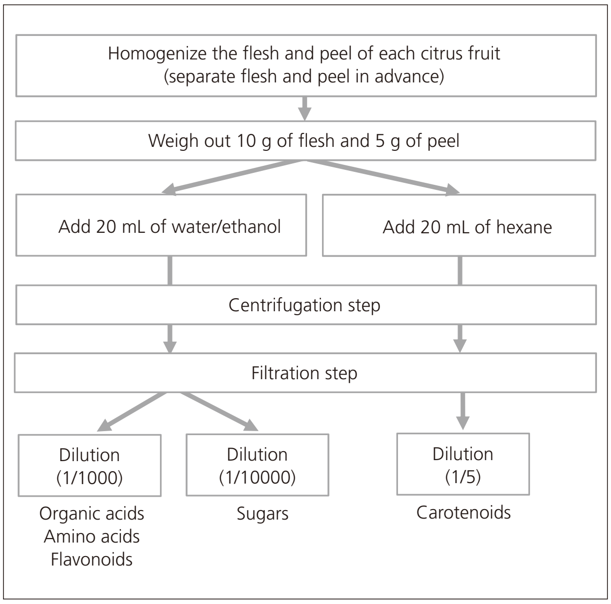

Fig. 2 Sample Pretreatment Protocol

LC/MS/MS Analysis

Due to the difficulty of analyzing the chemical properties of primary and secondary metabolites under the same chromatographic conditions, three chromatographic conditions were chosen that differed in mobile phase and column type. We used the Nexera method scouting system (comprehensive method search system) to perform measurements while automatically changing between the three analytical methods (Fig. 3). Although we used four mobile phases and three columns for this experiment, the system itself can accommodate up to eight mobile phases and six columns. The analytical conditions used are shown in Table 1.

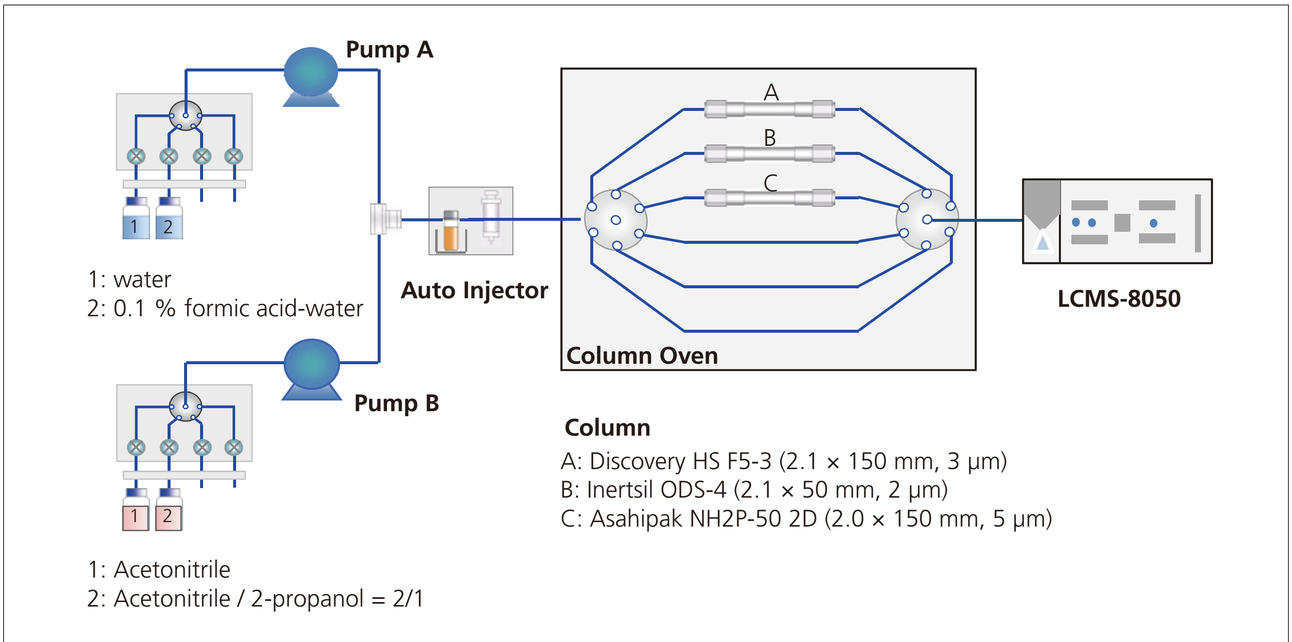

Fig. 3 System Configuration

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 (150 mm L. × 2.1 mm I.D., 3 μm) Sigma-aldrich	B: Inertsil ODS-4 (50 mm L. × 2.1 mm I.D., 2 μm) GL-science	C: Asahipak NH2P-50 2D (150 mm L. × 2.0 mm I.D., 5 μm) 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
	Flowrate	0.25 mL/min	0.4 mL/min	0.4 mL/min
	Time Program	0 %B (0-2 min.) → 95 % (10-13 min.) → 0 % (13.01-16 min.)	60 %B (0 min.) → 100 % (5-8 min.) → 60 % (8.01-10 min.)	65 %B (0-8 min.) → 30 % (8-11 min.) → 65 % (11.01-15 min.)
	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		

Multiple Reaction Monitoring (MRM) Analysis of Primary and Secondary Metabolites

The MRM chromatograms obtained for compounds analyzed under each set of analytical conditions are shown in Fig. 4. The calibration curve ranges for all compounds are also shown in Table 2.

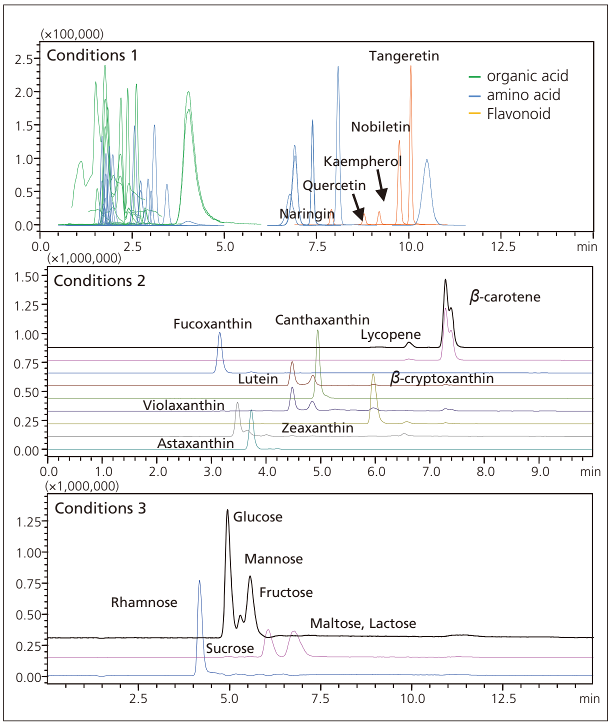

Fig. 4 Example Chromatograms Obtained Under Three Analytical Conditions

Table 2 Calibration Curve Ranges of Target Compounds

Amino acids	Range	Organic acids	Range
Cystine	1-100	Tartaric acid	50-10000
Aspartic acid	5-100	2-Ketoglutaric acid	10-1000
Asparagine	5-100	Isocitric acid	50-10000
Serine	5-100	Malic acid	10-5000
4-Hydroxyproline	1-100	Lactic acid	50-10000
Glycine	5-100	Citric acid	50-10000
Lysine	1-100	Pyroglutamic acid	10-10000
Cysteine	50-100	Succinic acid	10-1000
Threonine	5-100	Fumaric acid	500-1000
Glutamic acid	1-100	Maleic acid	50-10000
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		

Sugars	Range
Rhamnose	50-1000
Fluctose	50-5000
Glucose	50-5000
Sucrose	100-5000
Maltose, Lactose	200-2000

Carotenoids	Range	Flavonoids	Range
Fucoxanthin	0.1-100	Naringin	10-1000
Violaxanthin	1-100	Quercetin	5-1000
Astaxanthin	0.5-100	Kaempferol	5-1000
Lutein	0.1-100	Nobiletin	0.1-1000
Zeaxanthin	0.5-100	Tangeretin	0.1-1000
Canthaxanthin	0.05-100		
β-Cryptoxanthin	0.05-100		
Lycopene	50-100		
β-carotene	0.1-100		(μg/L)

Comparison of Flesh and Peel Constituents

Constituents of the flesh and peel of the seven citrus fruits were compared by performing principal component analysis. Flesh and peel constituents tended to separate into two groups on a score plot. The results of the loading plot indicate that the carotenoids, flavonoids, and sugars content of flesh and peel separates these sample types.

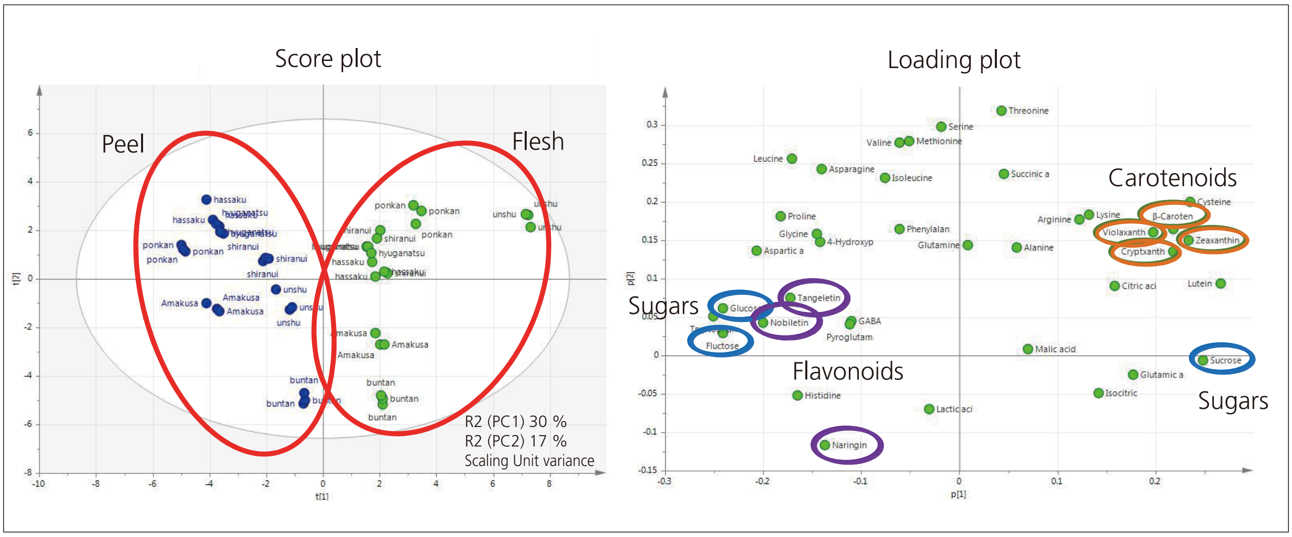

Fig. 5 Principal Component Analysis (PCA) of Flesh and Peel

Comparison of Sugars (Flesh and Peel)

The results of a quantitative comparison of sugars in flesh and peel are shown in Fig. 6. Although there is no difference in the total quantity of sugars present in flesh and peel, the sugar composition of flesh and peel differs substantially.

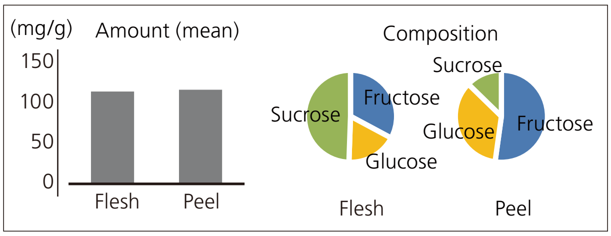

Fig. 6 Comparison of Sugar Quantity and Sugar Composition in Flesh and Peel

Comparison of Secondary Metabolites (Flesh and Peel)

The results of a quantitative comparison of secondary metabolites in flesh and peel are shown in Fig. 7. Flesh contained ≥ 30 times the quantity of carotenoids compared to peel. Meanwhile, peel contained around five times the quantity of flavonoids compared to flesh. These results showed a substantial difference in the secondary metabolites of flesh and peel.

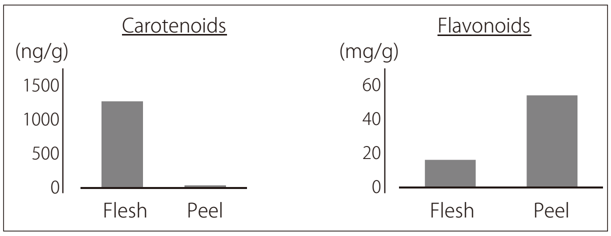

Fig. 7 Comparison of Mean Secondary Metabolites

Comparison of Citrus Fruits (Flesh)

Mikan orange and its hybrid varieties tended to appear on the upper right of the score plot. Since we confirmed that carotenoids appear in the upper right of the loading plot (Fig. 8), we also performed a quantitative analysis of carotenoids. This quantitative analysis confirmed mikan orange and its hybrid varieties have a high carotenoid content, and in particular contain a high quantity of β-cryptoxanthin and β-carotene (Fig. 9).

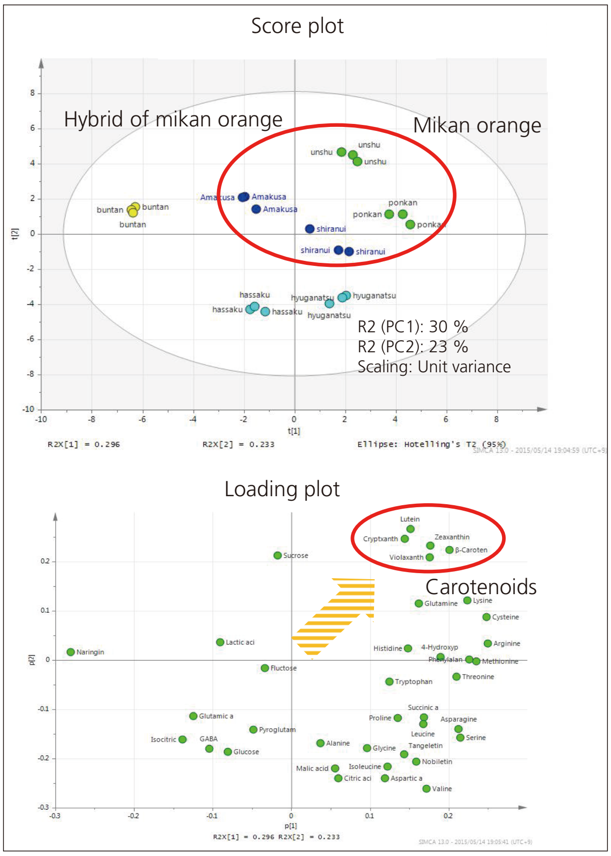

Fig. 8 Principal Component Analysis (PCA) of Flesh

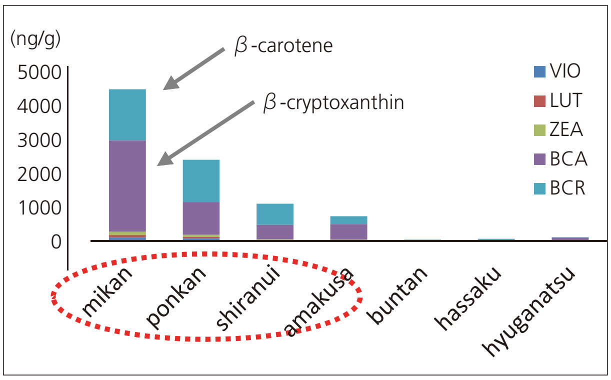

Fig. 9 Comparison of Carotenoid Content of Seven Citrus Fruits

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