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Analytical Methods

Method for the isolation of citric acid and malic acid in Japanese apricot liqueur for carbon stable isotope analysis

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ABSTRACT

A method for detecting the undeclared addition of acidic ingredients is required to control the authenticity of Japanese apricot liqueur. We developed an analytical procedure that minimizes carbon isotope discrimination for measurement of the $\delta^{13}C$ values of citric and malic acid isolated from Japanese apricot liqueur. Our results demonstrated that freeze-drying is preferable to nitrogen spray-drying, because it does not significantly affect the $\delta^{13}C$ values of citric acid and results in smaller isotope discrimination for malic acid. Both 0.1% formic acid and 0.2% phosphoric acid are acceptable HPLC mobile phases for the isolation of citric and malic acid, although the $\delta^{13}C$ values of malic acid exhibited relatively large variation compared with citric acid following isolation using either mobile phase. The developed procedure allows precise $\delta^{13}C$ measurements of citric and malic acid isolated from Japanese apricot liqueur.

1. Introduction

Japanese apricot (*Prunus mume* Siebold et Zucc.) is a traditional deciduous fruit tree (C_3 plant) in Japan and East Asia, and its fruit is mainly used for making liqueurs, pickles, and sauces (Mitani et al., 2013; Tsuchida et al., 2015). The yield in 2014 was 111,400 t in Japan, and about 70% of the harvested fruit was used in processed foods such as pickles and beverages (Ministry of Agriculture, Forestry and Fisheries (MAFF), 2015). Japan shipped 38,961,177 L of Japanese apricot liqueur in 2014, a 1.3-fold increase over a decade (Japan Spirits & Liqueurs Makers Association, 2015) despite a 30% reduction in fruit yield for liqueur production (MAFF, 2015). This increase in liqueur production despite the decrease in fruit yield might be due to increased use of low-cost acidulants such as citric acid, malic acid, and aroma chemicals. These chemicals reduce the cost of the liqueurs, and so help market penetration, but their clandestine use may jeopardize Japanese apricot orchards by replacing some of the fruit in the liqueur. Therefore, reliable and reproducible scientific methods for detecting acidulants are needed to control the liqueur's quality and authenticity.

Citric and malic acid are industrially produced, and are widely used as food acidulants. Industrially produced citric acid is mainly fermented by the filamentous fungus *Aspergillus niger*, using beet, cane, corn, or fruit sugars as carbon sources, depending on local availability (Papagianni, 2007). Malic acid is synthesized mainly from petrochemical feedstocks (Sauer, Porro, Mattanovich, & Branduardi, 2008). For identifying and quantifying individual organic acids such as citric and malic acid in beverages, many analytical techniques have been developed including gas chromatography with flame ionization and mass spectrometry detection; liquid chromatography with ultraviolet, chemiluminescent, electrochemical, refractive index, conductivity, and mass spectrometry detection; and isotope ratio mass spectrometry (Flores, Hellín, & Fenoll, 2012; Ogrinc, Košir, Spangenberg, & Kidrič, 2003). Carbon stable isotope analysis can identify the origin of a compound, because a given compound can exhibit large differences in carbon stable isotopic composition ($\delta^{13}C$) depending on the photosynthesis type (C_3 vs C_4). Although the method may have difficulty distinguishing organic matter from plants of the same photosynthesis type, other elements, such as hydrogen can be used instead of carbon in some cases (Gonzalez et al., 1998; Jamin, Martin, Santamaria-Fernandez, & Lees, 2005). Materials from C_4 plants such as corn and sugarcane have a higher $\delta^{13}C$ value (approximately −11‰) than those from C_3 plant materials such as beet (approximately −27‰). Citric acid, produced industrially from C_4 plants is usually cheaper than that from C_3 plants; therefore, methods for detecting adulteration with acidulants derived from C_4 plants in alcoholic and fruit beverages made from C_3 plants have been developed based on carbon stable isotope analysis (Gensler

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& Schmidt, 1994; Guyon et al., 2014; Raco, Dotsika, Poutoukis, Battaglini, & Chantzi, 2015; Weber, Roßmann, Schwarz, & Schmidt, 1997). However, slight carbon isotope discrimination (approximately 0.3‰) is observed during the isolation of organic acids from fruit juices (e.g., Gensler & Schmidt, 1994). Therefore, the development of a method of measuring the $\delta^{13}C$ values of organic acids with small differences in isotope ratios would also help control the authenticity of the liqueurs by allowing the accurate detection of their adulteration with acidulants.

We here examined isolation and drying methods for Japanese apricot liqueur to enable $\delta^{13}C$ measurements of citric and malic acid. These methods helped achieve a convenient minimum carbon isotope discrimination procedure. The $\delta^{13}C$ values of citric and malic acid extracted from authentic Japanese apricot liqueurs using the developed procedure were determined.

2. Experimental

2.1. Liqueur model samples

The isolation procedure for carbon stable isotope analysis was verified using five liqueur model samples prepared by mixing standard organic acids (citric and DL-malic acid), amino acids (L-alanine, L-arginine, L-asparagine, L-aspartic acid, γ-aminobutyric acid, glycine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine) and sugars (D(+)-glucose and D(−)-fructose) with ethanol:water (20:80, v/v) based on preliminary measurements of authentic Japanese apricot liqueurs, described below (see Table 1). The standard organic acids, ethanol, sugars, and amino acids were analytical reagent grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Ultrapure water was produced using a Millipore water purification system (Merck KGaA, Darmstadt, Germany).

2.2. Authentic Japanese apricot liqueur

A total of 10 Japanese apricot liqueurs were prepared as authentic samples without adulteration by citric and malic acid. For each liqueur, 1 kg Japanese apricot fruit (cultivar *Nanko*, *Prunus mume* Siebold et Zucc.) was harvested at a Japanese apricot laboratory (33°82′N, 135°35′E, Minabe, Wakayama Prefecture) in June 2014, and soaked in 1.8 L distilled spirit (Nakano BC, Wakayama, Japan) (35% ethanol (v/v)) containing 0.8 kg crystal sugar (Nissin Sugar Manufacturing, Tokyo, Japan) in the dark for six months. Each supernatant was filtered (0.45 μm), and subjected to the isolation procedure for carbon stable isotope analysis.

Table 1
Chemical components and their weight concentrations in 1 L 20% ethanol solution used as a model of Japanese apricot liqueur.

Component	Weight (mg/L)
D(+)-glucose	200,000
D(−)-Fructose	200,000
Citric acid	10,000
DL-malic acid	2,500
L-alanine	500
L-arginine	30
L-asparagine	700
L-aspartic acid	500
γ-aminobutyric acid	350
Glycine	30
L-isoleucine	50
L-leucine	70
L-lysine	40
L-phenylalanine	40
L-proline	100
L-serine	90
L-threonine	60
L-tyrosine	30
L-valine	80

2.3. Extraction

The extraction of pure citric acid and malic acid from the model ($n = 5$) and authentic Japanese apricot liqueur samples ($n = 10$) comprised two clean-up steps using cation- and anion-exchange solid phase extraction (SPE) columns. The procedure is outlined in Fig. 1. The cation-exchange SPE column was conditioned successively with 10 mL methanol, 10 mL 0.1 mol/L HCl- methanol (1:1, v/v), and 10 mL 0.1 mol/L HCl. The anion-exchange SPE column was conditioned successively with 5 mL methanol and 5 mL ultra-pure water. In the first clean-up step, 5 mL sample diluted with water (1:4 for model liqueurs and 1:8 for authentic liqueurs) adjusted to pH 1.5 with 1 mol/L HCl was applied to a cation-exchange SPE column (InertSep MC-1, 500 mg/6 mL, GL Science, Tokyo, Japan) to remove amino acids and other cationic compounds, eluted with 2 mL ultra-pure water, and the eluate was adjusted to pH 9.0 with 1 mol/L NaOH. In the second step, the pH-adjusted eluate (about 8 mL) was loaded onto an anion-exchange SPE column (InertSep SAX, 500 mg/6 mL, GL Science, Tokyo, Japan) to adsorb citric and malic acid, followed by a wash with 6 mL ultra-pure water, and then the organic acids were eluted with 3 mL 1 mol/L HCl. This latter eluate was dried by rotary evaporation (50 °C, 25 kPa, 15 min). The residue was dissolved in either 0.1% formic acid or 0.2% phosphoric acid mobile phase (see below) to a final volume of 400 μL, filtered through a 0.45 μm membrane, and injected onto a high-performance liquid chromatography (HPLC) system.

2.4. Isolation

Citric and malic acid were isolated from the eluate using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan)

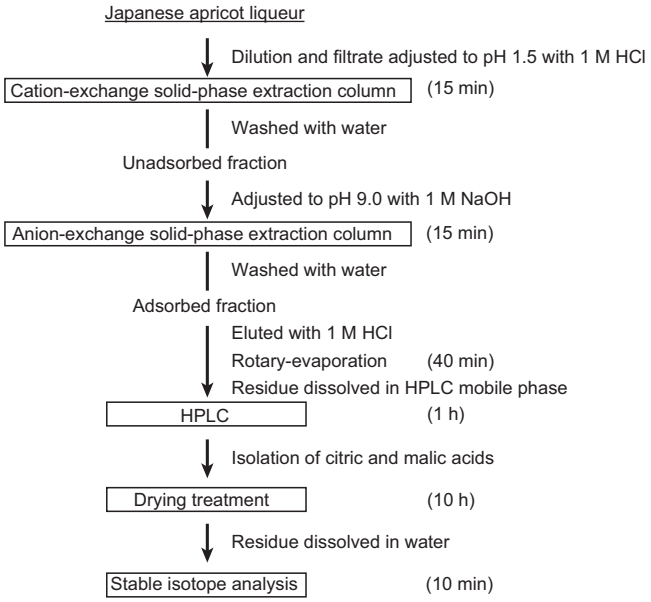

Fig. 1. The proposed analytical procedure for carbon stable isotope analysis of citric and malic acid from Japanese apricot liqueurs. Numbers in parentheses indicate the amount of time required for each process.