Food Chemistry 174 (2015) 163–172

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A NMR-based, non-targeted multistep metabolic profiling revealed L-rhamnitol as a metabolite that characterised apples from different geographic origins

Satoru Tomita [a], Tadashi Nemoto [b], Yosuke Matsuo [a], Toshihiko Shoji [c], Fukuyo Tanaka [d], Hiroyuki Nakagawa [a], Hiroshi Ono [a], Jun Kikuchi [e,f,g], Mayumi Ohnishi-Kameyama [a], Yasuyo Sekiyama [a,e,*]

[a] National Food Research Institute, NARO, 2-1-12 Kannondai, Tsukuba 305-8642, Japan
[b] National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba 305-8566, Japan
[c] NARO Institute of Fruit Tree Science, 2-1 Fujimoto, Tsukuba 305-8605, Japan
[d] NARO Agricultural Research Center, 3-1-1 Kannondai, Tsukuba 305-8666, Japan
[e] RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 235-0045, Japan
[f] Graduate School of Medical Life Science, Yokohama City University, 1-7-29 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan
[g] Graduate School of Bioagricultural Sciences and School of Agricultural Sciences, Nagoya University, 1 Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

ARTICLE INFO

Article history:
Received 31 July 2014
Received in revised form 27 October 2014
Accepted 3 November 2014
Available online 8 November 2014

Chemical compounds studied in this article:
L-Rhamnitol (PubChem CID: 445052)

Keywords:
NMR-based metabolic profiling
Apple
Geographic origin
L-Rhamnitol
PCA
PLS-DA

ABSTRACT

This study utilises ^{1}H NMR-based metabolic profiling to characterise apples of five cultivars grown either in Japan (Fuji, Orin, and Jonagold) or New Zealand (Fuji, Jazz, and Envy). Principal component analysis (PCA) showed a clear separation between the Fuji–Orin–Jonagold class and the Jazz–Envy class, primarily corresponding to the differences in sugar signals, such as sucrose, glucose, and fructose. Multistep PCA removed the influence of dominant sugars and highlighted minor metabolites such as aspartic acid, 2-methylmalate, and an unidentified compound. These minor metabolites separated the apples into two classes according to different geographical areas. Subsequent partial least squares discriminant analysis (PLS-DA) indicated the importance of the unidentified metabolite. This metabolite was isolated using charcoal chromatography, and was identified as L-rhamnitol by 2D NMR and LC/MS analyses. The remarkable contribution of L-rhamnitol to geographic discrimination suggests that apples may be characterised according to various factors, including storage duration, cultivation method, and climate.

1. Introduction

Apples are an important global fruit crop. A number of studies have examined the factors that drive the economics and breeding of apples, such as the relationships between quality and consumer attitude, and development of methods for quality assessment and control (Harker, Gunson, & Jaeger, 2003; Lopuch, Syrett, Conrad, & Steenhuis, 2011; Monakhova et al., 2013; Troggio et al., 2012). Metabolomics is a field of omics research concerned with the high-throughput identification and quantification of metabolites. To establish measures for assessing quality, such as screening out apples with internal browning, as well as discriminating between apples of different cultivars and geographic origins, several metabolomic studies using gas chromatography/mass spectrometry (GC/MS) (Aprea et al., 2011; Capitani et al., 2013; Lee, Mattheis,

Abbreviations: Ala, alanine; Asn, asparagine; Asp, aspartic acid; BuOH, 1-butanol; CA, citric acid; ChA, chlorogenic acid; D_2O, deuterium oxide; DQF-COSY, double quantum filtered correlated spectroscopy; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt; EtOH, ethanol; FA, formic acid; Fru, fructose; Fuc, fucose; GC/MS, gas chromatography/mass spectrometry; Glc, glucose; HILIC, hydrophilic interaction liquid chromatography; HMBC, heteronuclear multiple-bond connectivity; HOAc, acetic acid; HR-MAS, high resolution magic angle spinning; HSQC, heteronuclear single quantum coherence; LA, lactic acid; LC/MS, liquid chromatography/mass spectrometry; MA, malic acid; MeMA, citramalic acid (2-methylmalic acid); MeOH, methanol; *t*-BuOH, *tert*-butanol; PA, pivalic acid; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; PrOH, 1-propanol; QA, quinic acid; Rha, rhamnose; Suc, sucrose; TMS, trimethylsilyl; TOCSY, totally correlated spectroscopy; Xyl, xylose.

* Corresponding author at: National Food Research Institute, NARO, 2-1-12 Kannondai, Tsukuba 305-8642, Japan. Tel.: +81 (0)29 838 8033; fax: +81 (0)29 838 7996.
E-mail address: sekiyama@affrc.go.jp (Y. Sekiyama).

http://dx.doi.org/10.1016/j.foodchem.2014.11.028
0308-8146/

& Rudell, 2012; Lee, Rudell, Davies, & Watkins, 2012), liquid chromatography/mass spectrometry (LC/MS) (Guo, Yue, Yuan, & Wang, 2013; Kristensen, Engelsen, & Dragsted, 2012; Lee, Mattheis, et al., 2012), and nuclear magnetic resonance (NMR) spectroscopy (Belton et al., 1998; del Campo, Santos, Berregi, & Munduate, 2005; del Campo, Santos, Iturriza, Berregi, & Munduate, 2006; Vandendriessche et al., 2013; Vermathen, Marzorati, Baumgartner, Good, & Vermathen, 2011) have been carried out. Despite its low sensitivity, NMR-based metabolomics has several advantages, including its rapidity, reproducibility, that it is non-destructive, and that it can be used for high-throughput characterisation (Spraul et al., 2009). The greatest advantage of NMR is that it provides structural information regarding metabolites when identification by publicly available databases is not possible (Fan & Lane, 2008).

Whilst online databases established for NMR metabolomics can be used to identify certain metabolites, many minor metabolites remain unidentified. Additionally, an issue with use of NMR in the metabolomic analysis of fruits is that dominant sugars, such as sucrose (Suc), glucose (Glc), and fructose (Fru), produce intense signals in ^{1}H NMR spectra and cause to decrease the sensitivity for minor metabolites. These intense signals can also dominate the principal component analysis (PCA), masking the importance of minor metabolites. To determine the role and importance of these minor metabolites, it is necessary to be able to detect and identify this class of compounds if they are to be used as markers for characterisation and quality control purposes.

In this study, we used NMR-based metabolomics to profile metabolites in commercially available apples produced in Japan (JPN) and New Zealand (NZ). A non-targeted multistep PCA, which excludes dominant metabolites from dataset, was employed to quantify the contributions of minor metabolites that characterise the apples. A subsequent partial least squares discriminant analysis (PLS-DA) highlighted a previously-unidentified minor metabolite, which was identified as L-rhamnitol. We describe the isolation and identification of rhamnitol, as well as its contribution as a latent metabolite towards the characterisation of apple cultivars and their geographic origins.

2. Materials and methods

2.1. Materials and sample preparation

Apples were purchased in August 2013 at several local supermarkets in Tsukuba, Japan. Five cultivars (Fuji, Orin, Jonagold, Jazz, and Envy) were commercially available at that point. Two geographic origins were indicated on their labels; specifically, the Jonagold and Orin cultivars were produced in the Aomori prefecture in Japan, whereas the Jazz and Envy cultivars were grown in New Zealand. Fuji apples were available from both Japan and New Zealand (Fuji-JPN and Fuji-NZ, respectively), enabling us to compare the characteristics of apples of different geographic origins. Apples produced in Japan (Fuji-JPN, Jonagold, and Orin) were likely stored for at least six months, as these cultivars are normally harvested from the middle of October to the beginning of November. The apples grown in New Zealand (Fuji-NZ, Jazz, and Envy) were likely stored for a few months prior to purchase. However, no further information, such as cultivation methods, storage conditions, or storage durations was available. Regarding the genetic background of the cultivars used, the seed and pollen parents were: Fuji, Ralls Janet × Delicious; Orin, Golden Delicious × Indo; Jonagold, Golden Delicious × Jonathan; Jazz, Braeburn × Royal Gala; and Envy, Royal Gala × Braeburn (Broothaerts, Van Nerum, & Keulemans, 2004; Brown & Maloney, 2009).

To prepare samples, each fruit was cut into two parts lengthwise. One half was lyophilised and powdered to obtain a pulp sample, and the other half was cored, processed, and squeezed to collect fresh juice. To prevent oxidative deterioration, 10% potassium sulphite was added to the juice to a final concentration of 300 mg/L. Prepared samples were stored at −20 °C in the dark until analysis. Five fruits from each cultivar were processed and analysed (30 fruits total) as replicate experiments.

2.2. NMR spectroscopy

Juices prepared as described above were gently thawed to room temperature, and 140 μl of the sample were added to 560 μl of 125 mM potassium phosphate buffer (pH or pD 7.4) in deuterium oxide (D_2O, 99.9%, Cambridge Isotope Laboratories, Andover, MA) containing 1.25 mM of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS, Sigma–Aldrich, St. Louis, MI). For pulp samples, 10 mg of the powder were suspended in 700 μl of 100 mM potassium phosphate buffer (pH or pD 7.0) in D_2O containing 1 mM DSS. The suspensions were heated for 5 min at 90 °C whilst shaking at 1400 rpm using a Thermomixer Comfort (Eppendorf, Hamburg, Germany). The juice solutions and pulp extracts were subsequently centrifuged at 21,500×*g* for 5 min at room temperature, and the supernatants were transferred into 5.0 mm O.D. × 103.5 mm NMR tubes (Norell, Landisville, NJ) through a simple surgical cotton filter. Prepared samples were promptly used for NMR measurement.

NMR spectra were recorded on an Avance-500 spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a CryoProbe that fits 5 mm diameter NMR tubes (CPDUL, Bruker BioSpin), and an automatic sample transfer unit (SampleJet, Bruker BioSpin). Samples were recorded using the automated software IconNMR (Bruker BioSpin). NMR spectra were acquired at 298 K, operating at frequencies of 500.23 MHz for ^{1}H and 125.80 MHz for ^{13}C. For multivariate analysis, ^{1}H NMR spectra were collected using the Bruker pulse program zgpr, which uses solvent pre-saturation to remove the residual water signal. The following acquisition parameters were used: spectral width, 20 ppm; acquisition mode, sequential quadrature detection; offset frequency, 4.7 ppm; the proton 90° pulse, 20 μs; relaxation delay, 4 s; number of scans, 128. These parameters gave a total experiment time of 7 h 30 m for a set of 30 samples of the pulp extracts or juices. ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) spectra were collected using echo/antiecho gradient selection (the hsqcetgpsisp pulse program in the Bruker library) with the following parameters: 90° pulse values, 20 μs (proton) and 15 μs (carbon); relaxation delay, 2 s; spectral width, 130 ppm (f1) and 12 ppm (f2); data points, 256 increments of 2 k; scans, 64. The other 2D NMR spectra were acquired with 512 increments of 2 k data points for 10 ppm spectral width (f1 and f2) and 4 scans for double quantum filtered correlated spectroscopy (DQF-COSY), 256 of 2 k for 10 ppm spectral width (f1 and f2) and 16 scans for total correlation spectroscopy (TOCSY), and 256 of 2 k for 220 ppm (f1) and 10 ppm (f2) spectral width and 64 scans for ^{1}H–^{13}C heteronuclear multiple-bond connectivity (HMBC). The chemical shifts were referenced to the methyl group of a DSS internal standard (0.00 ppm for ^{1}H and ^{13}C).

Metabolite signals were annotated using a SpinAssign program through the PRIMe web service (Platform for RIKEN Metabolomics: http://prime.psc.riken.jp/) (Akiyama et al., 2008; Chikayama et al., 2010). To generate a peak table to compare with the chemical shift database of SpinAssign, ^{1}H–^{13}C HSQC spectra were processed with NMRPipe and analysed using NMRDraw (Delaglio et al., 1995) as described previously (Sekiyama, Chikayama, & Kikuchi, 2011). Other public databases, such as the Human Metabolomics Database (http://www.hmdb.ca/) (Wishart et al., 2013) and the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/)