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Discrimination of conventional and organic rice using untargeted LC-MS-based metabolomics

Ran Xiao[a], Ying Ma[a,*], Dongjie Zhang[b], Lili Qian[b]

[a] School of Chemical Engineering and Technology, Harbin Institute of Technology, Harbin, Heilongjiang 150090, China
[b] Department of Food Science, Heilongjiang August First Agriculture Land Reclamation University, Daqing, Heilongjiang 163319, China

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ABSTRACT

A metabolite profiling method was developed for rice samples under conventional and organic agricultural practices. In this study, an ultra-high performance liquid chromatography combined with quadrupole time-of-flight MS based (UHPLC-QTOF based) metabolite approach in combination with multivariate statistical analyses, including principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) and hierarchical clustering analysis (HCA), was applied to determine metabolite patterns among rice samples. In addition, an orthogonal partial least squares-discriminant analysis (OPLS-DA) was applied to identify key constituents to efficiently distinguish between cultivation methods. In total, 30 discriminant components were chosen from these two kinds of rice samples, in which 8 secondary metabolites could be considered to be potential biomarkers for the discrimination of organic and conventional rice. These results suggest that a metabolomics approach could be a reliable, precise, and effective method for the identification of rice under different cultivation practices.

1. Introduction

Rice (*Oryza sativa L.*) is considered to be the world's principal cereal crop from both production and consumption views. Organic farming is a quality-stable and environmentally sustainable agricultural production system. In the specific case of organic cereal production, the use of synthetic fertilizers, insecticides and herbicides are restricted by current legislation. Instead, green manures (sometimes combined with organic waste) and biological pest control should be applied to farming practices (Barbosa et al., 2016). Due to the principles above, organic plants have been regarded as purer, healthier, and even better-tasting products when compared with conventionally produced crop plants (Karlund et al., 2015).

Nevertheless, from the analytical point of view, the discrimination of food products from organic agricultural system is still a controversial issue. Numerous analytical methods have been applied to distinguish the chemical compositions of organic and conventional products (Capuano et al., 2013). The physicochemical and sensory characteristics have been tested to value differences between kiwi fruits from organic, integrated and conventional farming systems(Nunes-Damaceno et al., 2013). Similar methods have been applied to banana, orange and other fruits (Caussiol and Joyce, 2015). In an attempt to authenticate between conventional and organic cultivation, stable isotopes from food matrices (including wheat, barley, fava bean, potato, tomato and lettuce) were applied in multivariate analysis, and the findings suggested that systematic differences were highlighted in the concentrations of certain elements(Laursen et al., 2013). Nutrient and contaminant intake through the consumption of vegetables have also been used as an index of food products cultivated under organic versus conventional regimes (Hoefkens et al., 2010). Roasted and ground coffee with different cultivation techniques were distinguished using FT-MIRPAS, and chemometric analysis results indicated that conventional and organic cultivation was a more important factor than plant variety that influenced the recorded spectra (Gordillo-Delgado et al., 2012). For rice samples, comparative studies of conventional and organic rice are relatively scarce. Analyses of physicochemical properties (including yield, hulling and milling quality, protein, mineral, phytic acid and phosphorus contents) reveal that organic nutrient sources have better performance for the chemical, physicochemical properties, and even comparatively preferable cooking quality than rice from inorganic sources(Saha et al., 2007). The $\delta^{13}C$ and $\delta^{15}N$ examination has been successfully proven to authenticate environmentally friendly rice from conventional rice in Korea (Chung et al., 2017). A photoacoustic spectroscopy study also demonstrated crucial differences between samples from different agricultural practices (Delgado and Morales, 2012). Although there is evidence that organic food products can be

* Corresponding author.
E-mail address: maying@hit.edu.cn (Y. Ma).

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differentiated from their conventional counterparts, there is no consistent conclusion can be made through the analytical methods mentioned above.

Metabolomics, also known as metabonomics (Nicholson et al., 1999) or metabolic profiling (Niwa, 1986), is a relatively new frontier in 'omics' research that focuses on the high-resolution identification and high-throughput quantification of small molecule (< 1500 Da) compounds in the metabolome (German et al., 2005). Metabolomic profiling has generally been divided into targeted and untargeted analyses. The aim of targeted analyses is a particular group of metabolites. The majority of cases require the identification or quantification of the intended metabolites in different samples (Ramautar et al., 2006). In contrast, untargeted metabolomics, also known as comprehensive metabolomics, aims to detect as many groups of metabolites as possible to explore the fingerprints or patterns of biological processes. Neither identification nor quantification of a specific compound is necessary for these studies(Monton and Soga, 2007). Recently, technical innovations in the separation and identification of small molecules have made metabolic profiling much easier and more precise. Capillary electrophoresis (CE) (Maria et al., 2012) and ultra-high-pressure liquid chromatography (UPLC) (Kim et al., 2014) have served as the foundations for rapid and high-throughput separation systems for diverse compounds. Nuclear magnetic resonance (NMR) spectrometers (Monakhova et al., 2014) and mass spectrometry (MS) make it possible for robust and high-resolution determination. In addition, electronic databases of constituent chemicals providing both spectral and descriptive information, such as MassBank, METLIN, KEGG, NCBI, ChemSpider, and PubChem, have been gradually improved, which is equally important to the identification of potential biomarkers. Within last decade, metabolite profiling has already been applied to authenticate the geographical origins of foods (Kim et al., 2014; Bondia-Pons et al., 2014). Moreover, metabolomics analysis, especially LC-MS methods, have been used for discriminating food products produced using different agricultural practices. A systematic influence pointing to cultivation methods was revealed through 1600 compounds, which were obtained from an LC-MS based untargeted metabolomic profiling of white cabbage grown both conventionally and organically (Mie et al., 2014). Another untargeted metabolite approach using LC-QTOF-MS was applied to three strawberry cultivars from organic and conventional agricultural systems, and chemometric results revealed that the accumulation of several secondary metabolites in specific strawberry genotypes might be enhanced under organic farming practices (Karlund et al., 2015). Different ketchup products were subjected to LC-QTOF-MS based untargeted metabolomic profiling analysis, and interestingly, the results indicated that higher contents of antioxidant micro-constituents were found in the tomatoes and tomato-derived products under organic cultivation (Vallverdu-Queralt et al., 2011). HPLC and flow-injection mass spectrometric (FIMS) fingerprinting techniques combined with chemometrics were used to differentiate organic and conventional sweet basil leaf samples. The results indicated that organic basil samples contained greater concentrations of almost all major compounds to their conventional counterparts on a per botanical weight basis(Lu et al., 2014). Thus, metabolite profiling may be a new angle for differentiating food matrices under various agricultural practices.

The predominant objective of this study was to develop a UHPLC-Q-TOF MS method by using untargeted metabolite profiling combined with advanced chemometrics to reveal the influence of different cultural managements on the overall chemical composition of rice samples. To the best of our knowledge, only a few papers on metabolites of different rice samples have been published. This paper reports the first study investigating the effects of organic versus conventional agricultural practices on the rice metabolome.

2. Materials and methods

2.1. Samples

All rice samples were obtained from selected experimental trials in Heilongjiang Province, China during two consecutive years (2014 and 2015). Twenty conventional samples (C01-C20) were obtained under a traditional cropping system, which relied on pesticides and synthetic fertilizers. In contrast, 20 organic rice samples (O1-O20) were harvested using organic strategies in a successive 4-year crop rotation managed in compliance with the Chinese National Standard "Organic Food" (GB/T 19630-2011) and "Rice" (GB/T 1354–2009). The organic system relied on the import of green manure or a limited amount of animal manure. At maturity, all rice samples were harvested on the same day. Every sample was taken randomly from each plot. Further information in reference to field trial characteristics such as variety, harvest dates, origin and geographical location can be found in Table S1 of the supporting material.

Samples were stored in cold air conditioning (15% RH, at −18 °C) before milling. The rough rice was dehulled and then milled through a laboratory rice huller machine (Satake model SKD, Satake Corporation, Hiroshima, Japan) following a standard protocol (Champagne et al., 1999). A rice grader was applied to separate the broken kernels from whole grains (Satake model RG-06A, Satake Corporation, Hiroshima, Japan). To obtain fine homogeneous flour, rice samples were ground in liquid nitrogen and sieved with 0.18-mm mesh. Samples were stored properly under dry-cool conditions (15% RH, at 4 °C) and protected from direct sunlight until metabolite extraction.

2.2. Sample extraction

The extraction protocol was as in Bondia-Pons et al. (Bondia-Pons et al., 2014) with slight modifications. In brief, 600 mg of rice powder was weighed into a 5-mL tube, and 3 mL of methanol/water (7:3, v/v) was added. The rice powder was extracted for 30 min in an ultrasonic bath at 23 °C and then agitated with a vortex mixer for 10 s. Then, the mixture was centrifuged for 20 min at 8500 × g at 4 °C (3–30K, Sigma, Germany). The clear liquid was transferred into a sample vial after filtering through a 0.22-μm PTFE membrane filter (Pall Corporation, USA) and stored at −80 °C until analysis.

2.3. UHPLC-Q-TOF MS analysis

Rice extract was analysed using an Agilent UHPLC-QTOF-ESI-MS system, including a 1290 UHPLC system, and a 6540 UHD accurate-mass QTOF spectrometer equipped with a Jetstream electrospray ionization (ESI) source (Agilent Technologies, Waldbronn, Karlsruhe, Germany).

Chromatographic conditions were optimized based on pilot study (data not shown). The samples were subjected to reversed phase (RP) chromatographic separation, randomly injected onto an Acquity BEH C18 column (2.1 id × 150 mm, particle size 1.7 μm) (Acquity, Waters, Milford, MA, USA) in order to avoid an instrumental drift effect in the results. During analysis, the sample tray was kept at 4 °C. The injection volume was 5 μL, and the column was kept at 36 °C. Quality control (QC) samples were included every 5 injections for analysis control purposes.

The flow rate for the mobile phase was set at 0.3 mL/min throughout the gradient. Acidified water and acetonitrile, both containing 0.1% (v/v) of formic acid (Sigma-Aldrich), were used as eluent A and eluent B. The following gradient profile was employed: 0–1.5 min, 15% B; 1.5–5.0 min, 15–55% B; 5.0–17.0 min, 55–70% B; 17.0–20.0 min, 70–90% B; 20.0–21.0 min, 90-15% B.

A dual ESI source was operated in positive ionization mode. The detailed MS conditions were as follows: drying gas temperature, 325 °C, and flow, 9 L/min; nebulizer pressure, 45 psi; capillary voltage, 4000 V;