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Authentication of Indian citrus fruit/fruit juices by untargeted and targeted metabolomics

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ABSTRACT

Citrus fruits are one of the most important horticultural crops grown India, and a food commodity that is often targeted for mislabeling worldwide. Authentic citrus fruit samples (Kinnow mandrain (*citrus nobilis x Citrus deliciosa*), Jaffa and Mosambi orange (*Citrus sinensis*), and Red blush grapefruit (*Citrus paradisi*)) were obtained from the Indian Agriculture Research Institute and analysed by an untargeted method using ultra performance liquid chromatography-quadrupole-time of flight mass spectrometry to identify characteristic markers that could potentially be used to control citrus fruit authenticity. The most influential markers identified were: didymin, rhoifolin, isorhoifolin, neohesperidin, hesperidin, naringin, narirutin, limonin glucoside, and vicenin-2. A targeted liquid chromatography-tandem mass spectrometry method was then optimised for the analysis of these markers. Ratios of limonin glucoside to hesperidin, narirutin, and didymin; narirutin to hesperidin and vicenin-2; didymin to hesperidin and narirutin; and vicenin-2 to didymin, have the potential to be used to test for authenticity of Indian citrus fruits/fruit juices and to detect adulteration down to 2%. In addition, using an untargeted qualitative approach and applying PCA, it was possible to discriminate between authentic and adulterated samples (down to 1%), by generation of Cooman's plots.

1. Introduction

Citrus fruits are one of the most important horticultural crops grown India, mainly in Assam, Karnataka, Madhya Pradesh, Maharashtra, Meghalaya, Mizoram, Nagaland, Rajasthan, Tamil Nadu and West Bengal states. They are of particular interest because of their high nutritional value and high content of vitamin C and phenolics. India ranks sixth in the production of citrus fruit in the world. In terms of area under cultivation, citrus is the third largest fruit industry after banana and mango (Sankar, Gopi, Deepa, & Gopal, 2014). Over the last 30 years, the area and production under citrus cultivation has increased at the rate of 11 and 9%, respectively, which shows that the expansion of citrus industry was quite sustainable. Of the various types of citrus fruits grown in India, mandarin (Kinnow, Nagpur, Coorg, and Khasi), sweet orange (Mosambi, Jaffa, Malta, and Satgudi) and lime/lemon are of commercial importance. Sweet orange is commercially important for the production of palatable juice.

Food quality, including food safety, is a major concern facing the food industry today. Current food labelling and traceability systems cannot guarantee that the food we eat is authentic, of good quality and safe (Aung and Chang, 2014). Globalization in food trade and the increased complexity of food supply chains has increased the need for effective food control systems to protect consumers from impure, contaminated and fraudulently presented food. Citrus fruits and fruit juices play an important role in the human diet in India and citrus fruit industry is very large and profitable. Their economic value makes citrus fruits a target for misrepresentation and their juices a target for adulteration. This has a negative impact on both industry and consumers, since high quality authentic products have to compete with less expensive adulterated ones. The value of citrus fruit commodities is variable in international trade and consequently there is a risk of finding undeclared mixes on the market, in which cheaper fruit juices (like mandarin) are used to dilute juices stated as 100% orange juice (Moreau and Canivenc, 2008).

In recent years adulteration of orange juice (various varieties of *Citrus sinensis*) by addition of juice from *Citrus reticulata* (mandarins

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and tangerines), *Citrus aurantium* (sour orange) and tangors or hybrids of sweet orange and tangerine has been studied (Liu and Shyu, 2006; Ooghe and Detavernier, 1997; Ooghe, Ooghe, Detavernier, & Huyghebaert, 1994 a,b; Rouseff, 1988, p. 49; Stander, Kühn, & Hiten, 2013). It was found that the addition of low percentages of juice from *Citrus paradisi* and *Citrus aurantium* to *Citrus sinensis* juice may be detected by the presence of some specific flavonoids (Ooghe et al., 1994a; Rouseff, 1988, p. 49), while the detection of addition of 10% *Citrus reticulate* and hybrids thereof and of 5% *Citrus aurantium* was based on the determination of flavanone glycosides and the polymethoxylated flavones (Ooghe and Detavernier, 1997). DNA markers have also been used for the characterisation of different sweet orange varieties grown in India (Le Gall, Puaud, & Colquhoun, 2001; Malik et al., 2012; Sankar et al., 2014).

According to the European Commission (2009), the addition of non-*Citrus sinensis* to *Citrus sinensis* juice is not allowed in the European Union countries. *Codex Alimentarius* guidelines state that *Citrus sinensis* juice may contain up to 10% *Citrus reticulata* juice (Codex Alimentarius Commision, 1992), while the Food and Drug Administration (FDA) permits the addition of 10% *Citrus reticulata* to pasteurized and canned orange juice, and up to 5% of *Citrus aurantium* to frozen concentrated orange juice.

In the study presented here, the main objective was to explore the feasibility of using untargeted (by ultra-performance liquid chromatography – quadrupole time of flight mass spectrometry, UPLC-QToF MS) and targeted (by high-performance liquid chromatography-mass spectrometry LC-MS/MS) analysis to discriminate authentic and adulterated citrus fruits/fruit juices, as well as to explore the capability of this approach for the verification of the authenticity of specific varieties of Indian citrus fruits or juices prepared from them.

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure water was obtained from a Milli-Q purification system (Millipore, Molsheim, France). LC-MS grade acetonitrile, ammonium acetate (≥99%), hesperidin, neohesperidin, didymin, naringin, methanol, 2-propanol, and formic acid were supplied by Sigma–Aldrich (St. Louis, MO, USA). Limonin glucoside was obtained from LKT Laboratories (St. Paul, MN, USA), vicenin-2 from Pharma solutions (Ruelsheim, Germany) and, rhoifolin, isorhoifolin and narirutin from Extrasynthese (Genay Cedex, France).

2.2. Instrumental conditions

For UPLC-QToF MS analysis, experiments were performed on a Waters ACQUITY™ UPLC™ system connected to Xevo G2 Q-ToF MS equipped with an electrospray ionization source (Waters, Milford, MA, USA). The chromatographic and instrumental conditions (UPLC-QToF MS) used were as described by Jandrić et al. (2014).

For LC-MS/MS analysis, experiments were performed on a Waters Alliance HPLC 2695 connected to a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Chromatography was performed using an Atlantis T3 C18 column (100 × 2.1 mm I.D., 3 μm particle size) including an Atlantis T3 prep guard cartridge, (10 mm × 10 mm I.D., 5 μm particle size, 100 Å) (Waters, Milford, MA, USA). The elution was achieved using mobile phase composed of formic acid (0.3%) in (A) water, (B) acetonitrile, and (C) 2-propanol with a linear elution gradient (Table 1). The flow rate of the mobile phase was 0.35 mL/min, and the column temperature was maintained at 20 °C. The autosampler temperature was kept at 20 °C and injection volume was 10 μL.

Table 1
Mobile phase gradient conditions used in LC-MS/MS analysis.

Time (min)	A (%)	B (%)	C (%)
0.0	83	9	8
2.8	83	9	8
7.0	22	70	8
8.5	83	9	8
14.0	83	9	8

MS/MS detection was performed in positive electrospray ionisation mode using multiple reaction monitoring (MRM) acquisition mode with inter-scan and inter-channel delays set at 0.1 and 0.02 s, respectively. Nitrogen was used as a desolvation and cone gas (flow rates of 550 L/h and 50 L/h, respectively) and argon as collision gas at 0.6 bar.

2.3. Sample preparation

The authentic citrus fruits (Kinnow mandrain (*Citrus nobilis x Citrus deliciosa*), Jaffa and Mosambi orange (*Citrus sinensis*), and Red blush grapefruit (*Citrus paradisi*) were obtained from the Indian Agriculture Research Institute (IARI, Delhi, India). All these citrus varieties are grown in a controlled area (IARI research field) and they are free from pesticides and chemical fertilizers. The authentic samples of the same variety were collected from a single tree. In the orchard, different varieties are grown in separate rows, and the different citrus fruit varieties for this study were collected from the same orchard. The distance between the trees in a row is approximately 200 m and distance between rows is between 200 and 300 m.

Fruit juice samples were prepared in the laboratory by pressing fresh fruits (2 kg, approximately 10 fruits) of each of the varieties above, after careful removal of the peel, to avoid the contamination of other components contained in the flavedo. The juice samples were stored at −20 °C until needed for analysis. Aliquots of fruit juice were centrifuged first at low speed (7334 g, 20 °C, 10 min) to remove large particles and an aliquot of the supernatant was subsequently centrifuged at high speed (36670 g, 20 °C, 10 min) to further clarify. The samples were filtered through 0.2 μm PTFE membrane filters before injection. An aliquot of the supernatant was diluted with methanol (1:1) for LC-MS/MS analysis, while pure filtered fruit juice was directly injected into the UPLC-QToF MS ESI^-. Mixtures of citrus fruits juice adulterated with each other were prepared at 1, 2, 5 and 10% (three individual admixtures for each ratio were prepared and two repeat analyses were performed).

2.4. Preparation of standard solutions

Stock standard solutions (1 g/L) of limonin glucoside, narirutin, and naringin were prepared individually in methanol, rhoifolin, isorhoifolin, vicenin-2 and didymin in DMSO/MeOH, while poncirin, hesperidin and neohesperidin were prepared in water. From these individual stock solutions, an intermediate standard mixture (10 ng/μL) and working standard solution (1 ng/μL) were prepared in methanol. All standard solutions were kept in a refrigerator (at 5 °C).

2.5. Data acquisition and processing

The data were acquired by MassLynx v4.1 and pre-processed (peak detection, data mining, alignment and normalisation, removing of isotope masses) using MarkerLynx XS v4.1 software (both from Waters, Milford, MA, USA). The following parameters were applied for data acquisition: initial retention time 1 min, final