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Metabolomic approaches for orange origin discrimination by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

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ABSTRACT

In this work, hybrid quadrupole time-of-flight mass spectrometer (QTOF MS) coupled to ultra high performance liquid chromatography (UHPLC) has been used for biomarkers identification for correct authentication of Valencia (Spain) oranges. Differentiation from foreign Argentinean, Brazilian and South African oranges has been carried out using XCMS application and multivariate analysis to UHPLC-(Q)TOF MS data acquired in both, positive and negative ionisation modes. Several markers have been found and corroborated by analysing two seasons samples. A seasonal independent marker was found and its structure elucidated using accurate mass data and MS^E fragmentation spectrum information. Empirical formula was searched in Reaxys database applying sub-structure filtering from the fragments obtained. Three possible structures were found and citrusin D, a compound present in sweet oranges, has been identified as the most plausible as it fits better with the product ion scan performed for this compound. As a result of data obtained in this work, citrusin D is suggested as a potential marker to distinguish the geographic origin of oranges.

1. Introduction

Food quality and traceability are issues of wide concern to costumers and markets (Arvanitoyannis & Vaitsi, 2007; Reid, O'Donnell, & Downey, 2006). This quality is often linked to their origin as, for example, French wine or Spanish ham, which in some cases increase considerably the price of the product (Luykx & van Ruth, 2008). Thus, to guarantee the traceability of these items becomes essential, not only for costumers but also for manufacturers and dealers (Reid, O'Donnell, & Downey, 2006). To this aim, analytical methodologies able to unequivocally distinguish and detect the fraud are welcome.

Although not an especially high value product, oranges are an important market in the Valencian region, located in the east coast of Spain. This "Denomination of Origin" (D.O.) is well-known for Spanish costumers and the region economy's strongly depends on it, as recently published (Malet, 2010). Furthermore, it is suspected that a fraud really exists, mainly in late maturing orange varieties in order to overcome the production limitations during the summer, when no oranges are available at Valencia latitude. Thus, it is not uncommon that oranges are imported from southern hemisphere countries during this period and sold with Valencian D.O. Unfortunately, there is a lack on reliable analytical methodologies to avoid this fraud.

Normally, food authenticity methods are focused on the determination of a few target compounds that characterise the sample, as some metals by Inductively Coupled Plasma (ICP) (Anderson & Smith, 2006; Perez, Smith, & Anderson, 2006), natural products as amino acids (Gómez-Ariza, Villegas-Portero, & Bernal-Daza, 2005) and antioxidants (Iijima, Suda, Suzuki, Aoki, & Shibata, 2008; Makris, Kallithraka, & Mamalos, 2006) or even on searching for common adulteration substances, as tartaric acids in orange juice (Saavedra, García, & Barbas, 2000). One of the most promising approaches to solve these problems is metabolomics, or more precisely the metabolic fingerprinting (Lu et al., 2008), defined as "*the unbiased, global screening approach to classify samples based on metabolite patterns or "fingerprints" that change in response to disease, environmental or genetic perturbations with the ultimate goal to identify discriminating metabolites*" (Dettmer, Aronov, & Hammock, 2007). Although typically focussed on human (or animal) tissues (liver, lung, kidney, etc.) or biological fluids (mainly urine and plasma) to evaluate the effect of drugs (Nicholson, Connelly, Lindon, & Holmes, 2002) as well as to understand and diagnose

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diseases (Pendyala, Want, Webb, Siuzdak, & Fox, 2007), metabolomic approaches have been recently applied to food characterisation for coffee (Choi, Choi, Park, Lim, & Kwon, 2010; Risticevic, Carasek, & Pawliszyn, 2008), wine (Arvanitoyannis, Katsota, Psarra, Soufleros, & Kallithraka, 1999; Cuadros-Inostroza et al., 2010), tomato (Arvanitoyannis & Vaitsi, 2007; Consonni, Cagliani, Stoccchero, & Porretta, 2009) or fruit juices (Vaclavik, Schreiber, Lacina, Cajka, & Hajslova, 2011) among others.

There are a wide range of chromatographic modes and detector combinations (Arvanitoyannis & Vaitsi, 2007) available to achieve this global screening, like UV–VIS or NIR spectroscopy, etc. (Cozzolino, Smyth, & Gishen, 2003), although two main tools are used NMR spectroscopy and gas/liquid chromatography coupled to mass spectrometry (GC–MS and LC–MS) (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009). NMR has been the technique of choice during the last years due to its universality and versatility as well as the stability and large structure information obtained. In the last years, MS hyphenated to chromatography is appearing as a less expensive and more sensitive alternative approach (Dunn & Ellis, 2005), very powerful when modern analyzers are employed.

Hybrid quadrupole time-of-flight (Q-TOF) or Orbitrap are the MS analysers recently used because of their high resolution (HR), mass accuracy and full-spectrum acquisition capabilities. As well-known, GC–MS is applied to (semi)volatile and no thermo-labile compounds and it presents a more restricted application field (Dunn & Ellis, 2005) due to the unavailability of GC systems coupled to state-of-the-art HRMS. However, the analysis of volatiles compounds by head space solid phase micro-extraction (HS-SPME) has been also successfully applied for the origin classification (Montero-Prado, Bentayeb, & Nerín, 2013). On the other hand, the less complex sample preparation typically required by LC–MS methods, together with the wider range of separation mechanism (i.e. reversed phase, HILIC, etc.) in liquid chromatography, makes this technique more suitable to develop metabolomic profiling methods and expand their applicability from medium to highly polar compounds. In addition, recent advances in chromatographic separations thanks to reducing the particle size of the stationary phase sorbent (i.e. ultra high-performance liquid, UHPLC), facilitates data processing (Moco, Vervoort, Bino, & De Vos, 2007), especially in the peak picking process where peak shape and resolution are crucial.

In this work, orange samples from Valencia Spanish region and foreign samples (Argentina, South Africa and Brazil) have been investigated using multivariate analysis from the data obtained by UHPLC-QTOF MS with the objective to discover useful biomarkers for origin differentiation. QTOF MS has been used under MS^E mode, i.e. simultaneous acquisition at low (LE) and high collision energy (HE), which provides useful information on the (de)protonated molecules (commonly at LE) and on the main fragments ions (commonly in HE) (Díaz, Ibáñez, Sancho, & Hernández, 2011). This approach provides helpful information for the speed-up identification of the discovered markers.

2. Materials and methods

2.1. Reagents and chemicals

HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), residue analysis grade acetone, sodium hydroxide >99% (NaOH), and formic acid (98–100%) were acquired from Scharlau (Barcelona, Spain). Leucine-enkephalin, used as the lock mass, and imazalil, used during mass-axis calibration, were purchased from Sigma–Aldrich and Dr. Ehrenstorfer (Augsburg, Germany), respectively.

2.2. Instrumentation

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (Q-oaTOF Premier, Waters Micromass, Manchester, UK), using an orthogonal Z-spray-ESI interface operating in positive and negative ion mode. The UHPLC separation was performed using an Acquity UPLC BEH C18 1.7 μm particle size analytical column 100 × 2.1 mm (Waters) at a 300 μL/min flow rate. The mobile phases used were A = H_2O with 0.01% HCOOH and B = MeOH with 0.01% HCOOH. The percentage of organic modifier (B) was changed as follows: 0 min, 10%; 1.5 min, 10%; 16 min, 90%; 18 min, 90%; 18.01 min, 10% in a total run time of 20 min for the positive ionisation mode and 0 min, 10%; 2.5 min, 10%; 16.5 min, 75%; 18 min, 90%; 20 min, 90%; 20.01 min, 10% in a total run time of 22 min for the negative ionisation mode. Nitrogen was used as the drying gas and nebulizing gas. The desolvatation gas flow was set at 600 L/h. TOF-MS resolution was approximately 10.000 at full width half maximum (FWHM) in V-mode at m/z 556.2771. MS data were acquired over an m/z range of 50–1000. A capillary voltage of 3.5 kV and 3.0 kV for positive and negative ion modes, respectively, and cone voltage of 25 V were used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 350 °C and the source temperature to 120 °C. The column temperature was set to 60 °C.

For MS^E experiments, two acquisition functions with different collision energies were created. The first one, the low energy function (LE), selecting a collision energy of 4 eV, and the second one, the high energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV in order to obtain a greater range of fragment ions. The LE and HE functions settings were for both a scan time of 0.2 s and an inter-scan delay of 0.05 s. The automated attenuated function was also selected to correct for possible peak saturations (extended mode).

Calibrations were conducted from m/z 50 to 1000 with a 1:1 mixture of 0.05 M NaOH:5% HCOOH diluted (1:25) with acetonitrile:water (80:20) and spiked with fungicide imazalil at a final concentration of 500 μg/L, at a flow rate of 10 μL/min. For automated accurate mass measurement, the lock-spray probe was used, using as lock mass a solution of Leucine-enkephalin (2 μg/mL) in acetonitrile:water (50:50) at 0.1% HCOOH pumped at 30 μL/min through the lock-spray needle. A cone voltage of 65 V was selected to obtain adequate signal intensity for this compound (~500 counts). The (de)protonated molecule of Leucine-enkephalin, at m/z 556.2771 in positive mode and m/z 554.2615 in negative mode, was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time. It should be noted that all the exact masses shown in this work have a deviation of 0.55 mDa from the "true" value, as the calculation performed by the MassLynx software uses the mass of hydrogen instead of a proton when calculating $[M+H]^+$ exact mass. However, because this deviation is also applied during mass axis calibration, there is not negative impact on the mass errors presented in this article. MS data were acquired in centroid mode.

2.3. Samples

Orange samples of different varieties and origin were obtained from a manufacturer company in the Valencia region. Concretely, 15 samples of Valencia Origin and 9 foreign samples were initially analysed. In a second phase, the number of samples was widened using next year oranges, collecting a total of 42 samples: 24 from Valencia and 18 from abroad. All the foreign samples were Valencia variety (VA) while the samples from Valencia ones Valencia