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Metabolomics for organic food authentication: Results from a long-term field study in carrots

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ABSTRACT

Increasing demand for organic products and their premium prices make them an attractive target for fraudulent malpractices. In this study, a large-scale comparative metabolomics approach was applied to investigate the effect of the agronomic production system on the metabolite composition of carrots and to build statistical models for prediction purposes. Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) was applied successfully to predict the origin of the agricultural system of the harvested carrots on the basis of features determined by liquid chromatography–mass spectrometry. When the training set used to build the OPLS-DA models contained samples representative of each harvest year, the models were able to classify unknown samples correctly (100% correct classification). If a harvest year was left out of the training sets and used for predictions, the correct classification rates achieved ranged from 76% to 100%. The results therefore highlight the potential of metabolomic fingerprinting for organic food authentication purposes.

1. Introduction

Improved animal welfare, environmental protection and potential beneficial effects on human health are some of the reasons for the increasing demand for organic products. These products have a premium price over conventional products, which explain the appearance of fraud cases. Due to the lack of sound analytical methodology to distinguish organically and conventionally grown crops and in response to EU legislation (Regulation (EC) No 834/2007), analytical methods that can distinguish between both agricultural systems are needed.

Organic agricultural systems rely, among other characteristics, on the use of organic manures and biological pest controls and maintain the fertility of the soil by multiannual crop rotation including legumes and other green manure crops. In conventional agricultural systems plants are protected with chemical plant protection products. Therefore, organic production systems may increase environmental stress in plants; hence, resulting in accumulation of inducible protective secondary metabolites such as phenolic acids (Malik et al., 2009; Young et al., 2005; Zuchowski, Jonczyk, Pecio, & Oleszek, 2008) and flavonoids (Mitchell et al., 2007).

Metabolomics allows the study of multiple metabolites in a cell, a tissue or an organism. Over the past decade, which saw new developments in analytical approaches such as mass spectrometry (MS) and chemometrics, the application of metabolomics to food authentication issues has gained increasing interest. To date, metabolomics based methods have not yet been taken up by the regulatory agencies for use in food authentication issues, although in some cases they have been proved to be efficient and shown clear benefits over traditional methods. The main advantage of the use of metabolomics in food authentication is its untargeted nature. Unexpected changes in the metabolite profile, i.e. by the addition of new adulterants, may be detected without the need of an *a priori* hypothesis which offers a great advantage ahead of the fraud performers (Cubero-Leon, Peñalver, & Maquet, 2014).

The application of fingerprinting for food authentication issues is still at an early stage and more studies with larger datasets are required to draw valid conclusions. Indeed, although some of these procedures have shown to be promising, more studies are needed that account for pedo-climatic factors, agricultural productions systems, genetics, processing, etc., to obtain models with wide applicability (for reviews see Cevallos-Cevallos, Reyes De Corcuera, Etxeberria, Danyluk, & Rodrick, 2009; Cubero-Leon et al., 2014). For example, for the control of the European wine market, Commission Regulation (EC) No 555/2008 (European Commission, 2008) requires the EU Member States to collect every year grapes from selected growing areas, produce micro-vinified

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wines and subject them to isotopic analysis; the obtained data support the fight against fraud in the wine sector (mainly sugaring and watering).

Recently, metabolite fingerprinting applications have been used to study differences in organic *vs*. conventional production systems in wheat (Bonte et al., 2014; Kessler et al., 2015), maize (Röhlig & Engel, 2010), tomatoes and tomato-derived products and peppers (Novotná et al., 2012; Vallverdú-Queralt, Medina-Remón, Casals-Ribes, Amat, & Lamuela-Raventós, 2011), grapefruits (Chen & Harnly, 2010), potatoes (Shepherd et al., 2014) and white cabbage (Mie et al., 2014). The results, however, are contradictory. In wheat small differences were found between the two production systems, which tended to disappear in mature grains whereas in tomatoes, maize, grapefruits and potatoes significant differences were found. Organic and conventional production systems in some cases could only be distinguished for a given year or a given cultivar due to the massive influence of these factors on the metabolite profiles (Bonte et al., 2014; Kessler et al., 2015). One of the main limitations of these studies is that they are based on relatively small samples sizes and/or other sources of variation such as cultivated varieties, geographical location or fertilisation practice are not considered. The lack of external validation sets using samples that are not part of the statistical models built for prediction is a major limitation in most of the studies published. The authenticity of the samples also needs to be assured. In this respect, samples from controlled experimental fields/studies should not be considered as authentic as they might not reflect the natural variation obtained from real agricultural practices.

The carrot (*Daucus carota* L.), a plant of the *Apiaceae* family, is one of the most economically important vegetables worldwide (FAO, 2016) and they can be consumed raw or in a wide variety of processed products. The objective of this study was to perform a comprehensive biochemical analysis based on untargeted liquid chromatography–mass spectrometry (LC–MS) metabolomics of carrots coming from different agronomic environments. The combined molecular features extracted were then used to build prediction models and classify the origin of new samples according to their agricultural practice using external validation sets. To the best of our knowledge, this is the first time that a metabolomics approach is used for organic food authentication purposes in a long-term (four years) field study and by using external validation sample sets to predict the origin of unknown samples.

2. Material and methods

The standards developed by the Metabolomics Standards Initiative (MSI; http://msi-workgroups.sourceforge.net/) were followed as much as possible.

2.1. Field samples

Carrot samples (*Daucus carota* L.) of Nerac and Namur varieties were obtained from the Walloon region of Belgium in four consecutive years (2005, 2006, 2007 and 2008). Carrots 'Namur' are an early cultivated variety (cultivar or cv.) suitable for spring harvesting whereas 'Nerac' carrots are a summer hybrid. Fields using conventional growth strategies were compared to fields using certified organic practices. The geographical location of all the fields sampled in this study is provided in Fig. S1 (Supplementary material). For information regarding dates of sowing and harvest, crop protection (pesticides), fertility management (fertilisers) and crop rotation details of the organic and conventional systems see Table S1 (Supplementary material).

In each location two paired fields were selected (maximum distance of 5 km), one with a conventional growing system and one with organic husbandry. Within each field, three parcels were selected (10 m × 10 m each), and the distance between parcels was 15 m. One sample was taken from every corner of each parcel. A fifth sample was taken in the centre of the parcel. From each field 15 carrots of similar size (biological replicates) were collected. Samples from each field were labelled and packed independently and sent the same day on ice to the laboratory. The fields were characterised by loamy soils. More information on the soil properties is detailed in Table S2 (Supplementary material). In every location carrots from both fields (organic and conventional) were harvested within less than two weeks except in 2005.

After delivery to the laboratory the carrots were kept at 4 °C for a maximum of 48 h. They were then washed and the top and bottom of the carrots (0.5 cm) removed, cut into slices and lyophilized during 72 h using a Christ Freeze dryer system/lyophilizer (Martin Christ GmbH, Osterode am Harz, Germany), after which they were homogenized with an ultracentrifugal grinder equipped with a 0.5 mm sieve (ZM 200, Retsch, Düsseldorf, Germany). The mean water content of the lyophilised carrots determined by Karl Fisher titration was 5.5%. Sample preparation was performed in a randomized way over 14 days. Samples were stored in sample bags at −80 °C freezer before analysis.

2.2. Reagents and chemicals

Water was purified using a Milli-Q Integral water purification system (Millipore, Bedford, MA, USA). Methanol (HPLC grade) used for the extraction was purchased from VWR (Leuven, Belgium), and the methanol used for chromatography (LC–MS grade) was supplied by Merck (Germany). Chloroform (purity ≥98%), formic acid (purity ~98%), sodium formate (purity ≥99.9%) and isopropanol (≥99.9%) were purchased from Sigma-Aldrich (St. Louis, USA).

Vitamins: pyridoxine ($C_8H_{11}NO_3$) and nicotinic acid ($C_6H_5NO_2$); flavonoids: (−)-epigallocatechin ($C_{15}H_{14}O_7$), (−)-epicatechin ($C_{15}H_{14}O_6$), (+)-catechin ($C_{15}H_{14}O_6$), rutin ($C_{27}H_{30}O_{16}$); and phenolic acids: ferulic acid ($C_{10}H_{10}O_4$), benzoic acid ($C_7H_6O_2$), *trans*-cinnamic acid ($C_9H_8O_2$), *p*-coumaric acid ($C_9H_8O_3$), 4-hydroxybenzoic acid ($C_7H_6O_3$) and chlorogenic acid ($C_{16}H_{18}O_9$) were purchased from Sigma-Aldrich with purity ≥97% in all cases.

2.3. Sample extraction

Different standards (STDs) representing vitamins, flavonoids, and phenolic acids (see Section 2.2) already reported to be existent in carrots were chosen to assess the performance of the extraction method based on a procedure described by Ossipov et al. (2008).

A volume of 500 μL of ice-cold chloroform was added to 0.1 g of lyophilized carrot samples, followed by 300 μL of ice-cold methanol and 200 μL of ice-cold Milli-Q water. The samples were vortexed for five seconds and mixed in a Thermomixer Comfort (Eppendorf, Hamburg, Germany) at 4 °C at maximum speed (1400 rpm) for 1 h. Then, 0.5 mL of 20% aqueous methanol was added to the extract and the resulting biphasic system was vortexed for 5 s and mixed in the Thermomixer at 4 °C for 30 min at 1250 rpm. To separate the methanol/water and chloroform fractions the samples were centrifuged for 15 min at 10,000*g* and 4 °C. The chloroform fraction of lipophilic compounds was pipetted (about 0.5 mL) and evaporated in a vacuum concentrator (Eppendorf 5301, Hamburg, Germany) and dissolved again in 200 μL of methanol. A volume of 200 μL of water was added to obtain a 1/1 (v/v) of methanol/water and the sample was then filtered through 0.2 μm nylon membrane filters (Centrifugal Filters, WVR International, PA, USA) and placed into glass vials. The methanol/water fraction (fraction of polar compounds) was also filtered as described before and placed into glass vials.