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Authenticity assessment of garlic using a metabolomic approach based on high resolution mass spectrometry

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ABSTRACT

Depending on conditions in a growing locality and several other factors, marketed garlics (*Allium sativum* L.) may largely differ in content of flavour significant compounds and other biologically active components. To enable verification of tradeŕs declarations on the geographic origin, a new analytical, metabolomic fingerprinting, was employed for analysis of 47 samples of garlic with the designated country of origin Czech Republic, Spain and China. Non-target screening of metabolome components occurring in garlic extracts was performed employing following three instrumental platforms based on high resolution mass spectrometry (HRMS): (i) ambient mass spectrometry utilizing direct analysis in real time ionization (DART) ion source coupled to HRMS; (ii) direct infusion (DI) of sample into electrospray ion source (ESI) coupled to HRMS; (iii) high performance liquid chromatography (HPLC) – ESI – HRMS. Statistical models (Orthogonal Partial Least Squares-Discriminant Analysis, OPLS-DA) models were constructed on generated data with the aim to identify the best HRMS technique enabling a reliable differentiation of a country of origin. The best prediction ability, up to 100%, was obtained by processing the data generated by HPLC-HRMS. Alliin, phosphatidylcholine (16:0/18:2), arginine, dehydroalanine, phosphatidylethanolamine (16:0/22:6), L-γ-Glutamyl-*S*-allyl-L-cysteine and choline glycerolphosphate, were identified as compounds most contributing to a correct classification of the samples.

1. Introduction

Garlic (*Allium sativum* L.) is a food ingredient widely used in gastronomy around the world. Besides its culinary applications, garlic bulbs have been used for thousands of years in folk medicine for both protective and curative purposes (Amagase, 2006; Corzo-Martínez et al., 2007; Griffiths et al., 2002). Garlic belongs to the family of *Alliaceae* (or other division in the *Liliaceae* family) and genus *Allium*, with more than 600 known species (Block et al., 1993; Rahman, 2003) differing in taste, shape and color, nevertheless, close in biochemical, phytochemical and nutraceutical content (Benkeblia, 2005). Until now, more than two hundred compounds representing various chemical classes have been found in garlic (Goncagul and Ayaz, 2010) and other new compounds are still being discovered (Zhou et al., 2014). The major components of garlic by dry weight (approx. 35%, w/w) are fructans and other carbohydrates, followed by other biopolymers such as fiber and proteins (Rahman, 2003). The latter group is also accompanied by relatively high amounts of common free amino acids, with dominating arginine followed by aspartic and glutamic acid, methionine and threonine (Sendl, 1995). Vitamins, trace elements, flavonoids and enzymes represent other biologically active compounds occurring in garlic, nevertheless, regarding its medical functions and characteristic pungent flavour, the dominating role is played by organo-sulphur compounds (Rahman, 2007; Rana et al., 2011; Tapiero et al., 2004). Their concentrations and pattern are characteristic for particular varieties, however, these quality parameters also fairly depend on the growing and storage conditions (Hornickova et al., 2010, 2011). The key sulphur-containing secondary metabolites occurring in garlic bulbs are S-alk(en)yl-L-cysteine sulphoxides (Kiss et al., 2013; Kubec and Dadakova, 2009), such as alliin, and γ-glutamylcysteines (Griffiths et al., 2002; Hornickova et al., 2010). It is worth noting that disintegration of garlic tissue results in a release of aliinase (EC 4.4.1.4, alliin alkenyl-sulfenate lyase, or alliin C-S lyase), the enzyme specific for the *Allium* genus. Alliinase catalyses the rapid conversion of alliin to pyruvate, ammonia, alk(en)ylsulfenic acid and subsequently produces dialkyl(en)ylthiosulfinates (Rose et al., 2005). The latter sulphur-containing group of compounds is responsible for the characteristic aroma of freshly chopped garlic. However, dialkyl(en)ylthiosulfinates are unstable, depending on the ambient conditions (e.g. temperature), they are converted into a variety of breakdown products (thiosulfonates,

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disulfides, trisulfides, etc.) associated with aroma of culinary processed garlic. As far as the primary garlic metabolites are to be analyzed, various inhibitors are typically added to avoid this allinase reaction (Lundegardh et al., 2008; Ovesna et al., 2015; Rose et al., 2005).

More than 60,000 tons of garlic are annually imported into the European Union (EU), mainly from China, Argentina and other countries (e.g. Russia or Egypt). Recently, the growing demand for high-quality garlic, has prompted EU farmerś interest in increasing production of this crop; the same trend has taken place also in the Czech Republic. It should be noted that Czech consumers strongly prefer, in spite of a rather higher cost, domestically produced garlic varieties. This is mainly because of their more intense and richer flavour compared to garlics of other geographic origin such as those from China, which is one of the major imports to the Czech market. Unfortunately, under these conditions, fraudulent practices including intentional substitution of traditional Czech garlić by cheaper brands has been encountered. On this account, not surprisingly, the demand for authentication of garlic varieties/geographic origin has become very urgent. Of course, molecular biology based tests aimed at garlic DNA analysis represent a conceivable solution (Lin et al., 2015; Ovesna et al., 2015) for variety identification, nevertheless, PCR (polymerase chain reaction) is not available in common food control laboratories. Several studies concerned with chemical methods enabling characterization of various pre-selected garlic quality parameters (e.g. content of alliin, profile of volatiles, minerals) have been published (Camargo et al., 2010; Grégrová et al., 2013; Smith, 2005; Vadalà et al., 2016). However, such approaches, may omit some features of tested samples because they are targeted. Recently, an alternative strategy represented by metabolomics, i.e. non-target screening of low molecular weight metabolites, has been demonstrated to be an effective tool in various research areas, including food authentication (Rubert et al., 2015; Cubero-Leon et al., 2014; Simó et al., 2014). Metabolomics allows to obtain a unique chemical fingerprints characterizing specific cellular processes in particular crops (Castro-Puyana and Herrero, 2013; Wishart, 2008).

Several instrumental techniques have been employed for metabolomic fingerprinting of various plant matrices, among them those based on nuclear magnetic resonance (NMR) and mass spectrometry (MS) play a dominant, and in some respect, complementary role.

With regards to garlic, the only metabolomics-based study, aimed at discrimination of both the variety and the locality of origin within Italy, employed ^{1}H high resolution magic angle spinning-nuclear magnetic resonance (HRMAS-NMR) spectroscopy (Ritota et al., 2012).

In this study, the authors have critically assessed three mass spectrometry-based metabolomic approaches with regards to their potential to generate data enabling to characterize garlic of different origins. The following instrument platforms were used: (i) ambient mass spectrometry utilizing a direct analysis in real time (DART) ion source coupled to a mass spectrometer with an orbitrap mass analyser; (ii) electrospray ion source (ESI) – mass spectrometer with time of the flight (TOF) mass analyser, direct infusion (DI) of sample into ion source; (iii) high performance liquid chromatography (HPLC) – ESI source – mass spectrometer with a TOF mass analyser. Advanced chemometric strategies, represented by principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), were employed for interpretation of the acquired data set. The final objective of this study was to find out an optimal HRMS-based analytical strategy, that, contrary to molecular biology based approaches, would be less labour demanding, moreover, would enable obtaining required information on sample authenticity in a relatively short time, and, when considering running cost, would be cheaper.

2. Materials and methods

2.1. Samples

Altogether 47 samples of garlic (47 different batches) with the designation of the country of origin Czech Republic (19), Spain (17) and China (11) were involved in this study. These samples represented then common offer of this commodity at a Prague retail market during spring season, March – May 2014. The samples purchased in small packages (250–300 g, 3–5 bulbs) labelled not only by country of origin but also by a batch number. 3 of 19 Czech samples of garlic were obtained directly from the growers from 3 different localities: Bořitov (Middle Moravia, 49.4250361N, 16.5911861E), Olbramovice (South Moravia, 48.984804N, 16.402398E), Šlapanice (South Moravia, 49.168767N, 16.727024E). While no more detailed information was available on Chinese and Spanish samples at the labels, the set of Czech samples was composed from 6 varieties (Dukát, Lukan, Vekan, Vinar Tantal and Unikát) typically grown in the Czech Republic.

Although the number of garlic batches from each country was not too high, it should be noted, that this study provides a proof of concept. In any case, as stated in Conclusions, the database of fingerprints should be continuously updated to obtain robust classification model. The origin was verified by DNA analyses (Ovesna et al., 2015), which were done in the Crop Research Institute in Prague. Samples were stored in the dark and dry conditions at laboratory temperature (20 °C) until the time of sample preparation.

2.2. Sample preparation

Approximately 20 g of undamaged peeled garlic cloves (from different bulbs of garlic within the respective batch) were placed in a laboratory blender, then 60 mL of O-(carboxymethyl)hydroxylamine hemihydrochloride (OCMHA, 1.1 g was dissolved in 1 L of methanol:-water, 80:20 v/v) were added to inhibit enzyme alliinases. To extract metabolic components, the sample was homogenized for 1 min. The resulting slurry was transferred into a 50 mL centrifuge tube and centrifuged (5 min, 10,000 rpm, 20 °C) and then filtered through a microfilter (0.22 μm) into a glass vial and kept under refrigeration (4 °C) until analysis. The extracts prepared in this way were used for all analyses described below.

2.3. DART-HR-OrbitrapMS analysis

For the analyses based on ambient mass spectrometry, the DART ion source (DART-SVP) was fitted with a 12Dip-It™ tip scanner autosampler (IonSense, Saugus, MA, USA) coupled to an Exactive™ benchtop high resolution mass spectrometer with orbitrap mass analyzer (Thermo Fisher Scientific, Bremen, Germany). A Vapur™ interface (IonSense, Saugus, MA, USA) was employed to couple the ion source to the mass spectrometer, and low vacuum in the interface chamber was maintained by a membrane pump (Vacuubrand, Wertheim, Germany). The distance between the exit of the DART gun and the ceramic transfer tube of the Vapur was set to 10 mm, the gap between the ceramic tube and the inlet to the heated capillary of the Exactive was 2 mm.

The DART and MS instruments were operated in both positive and negative ionization modes and the optimized settings were as follows: (i) DART positive ionization: helium flow: 2.5 L min^{-1}; gas temperature: 350 °C; discharge needle voltage: −5000 V; grid electrode: +350 V; (ii) DART negative ionization: helium flow: 2.5 L min^{-1}; gas temperature: 350 °C; discharge needle voltage: −5000 V; grid electrode: −350 V; (iii) mass spectrometric detection: capillary voltage: ± 60 V; tube lens voltage: ± 120 V; capillary temperature: 250 °C. The sheath, auxiliary and sweep gases were disabled during DART–HRMS analyses.

The mass spectrometer was operated at mass resolving power 50,000 FWHM (full width at half maximum) calculated at m/z 200. Under these settings, the mass spectra acquisition rate was 2 spectra s^{-1}. Liquid samples (extracts) were delivered into the DART ionization region with the use of a 12 Dip-It tip scanner autosampler. Dip-It™ tips (IonSense, Saugus, MA, USA) were inserted into a holder and immersed into the sample extracts placed in a 96-deepwell micro-plate (Life Systems Design, Merenschwand, Switzerland). The Dip-It holder was mounted onto the body of the autosampler and the Dip-It tips were