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Development and validation of UHPLC-HRMS methodology for the determination of flavonoids, amino acids and organosulfur compounds in black onion, a novel derived product from fresh shallot onions (*Allium* cepa var. *aggregatum*)

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ABSTRACT

Black onion, a new derived product from fresh onion, has been developed by processing (aging) fresh shallot onion in a temperature- and humidity-controlled room without using any artificial additives. The aim of this study was to adapt, optimize and validate two ultra-high-performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) methodologies for the determination of flavonoids, amino acids and organosulfur compounds in black onion. UHPLC-HRMS methods involving RP-C18 and HILIC columns were adapted and validated in terms of specificity, linearity, limit of detection (LD) and quantification (LQ), precision inter- and intra-day, recovery and matrix effect. Linearity ranged from 0.012 to 12.5 ngμL^{-1} and from 0.1 to 75 ngμL^{-1} for flavonoid and amino acids and organosulfur compounds, respectively. LD varied from 0.004 to 0.06 ngμL^{-1} and LQ from 0.012 to 0.2 ngμL^{-1}. The intra-day and inter-day precision for all compounds were less than 15% and the recovery ranged from 69 to 106%. The matrix effect ranged from 80 to 114% for flavonoids, amino acids and organosulfur compounds. The described methods were successfully applied for the correct separation and determination of 53 compounds in black onion. These results establish the value of these new two UHPLC-HRMS protocols in providing detailed compound profiles of black onion, highlighting their potential applicability to similar vegetables.

1. Introduction

Onion (*Allium cepa* L.) is one of the most important bulb crops and is commonly used as food, spice and medicinal plant almost worldwide. With an estimated global production rate of 5.7 Mt per year (http; http://faostat.fao.org, 2016), onion is the first most produced bulb crops in Spain, and together with garlic, is the most consumed bulb vegetable either fresh or after processing into various (cooked) products. Epidemiological and clinical studies have reported that the consumption of *Allium* vegetables such as onion, garlic and leek protects against the development of metabolic diseases such as diabetes (Akash, Rehman, & Chen, 2014) or cardiovascular diseases (Bahadoran, Mirmiran, Momenan, & Azizi, 2017). Besides, its consumption is associated with a reduce risk of developing diverse types of cancers including stomach, colorectal (Nicastro, Ross, & Milner, 2015) and breast cancer (Pourzand et al., 2016), playing a significant role in human nutrition. From a nutritional point of view, free proteogenic amino acids account for 5–7% dry weight of an onion bulb with arginine and glutamine being the most abundant ones. In addition, onion is characterized by its high levels of health-promoting constituents comprising flavonoids and a huge variety of sulfur-containing compounds which accounted to its well-known nutritional properties (Böttcher, Krähmer, Stürtz, Widder & Schulz, 2017).

Nowadays, the food industry is looking for new foodstuffs with added value and functionality. Recently, a derived product from onion, black onion, is gaining great popularity among the Spanish consumers. This new product is made by processing (aging) fresh shallot onion in a temperature- and humidity-controlled room without using any artificial

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additives. This manufacturing process led to colour changes from white onion to black onion, and to improve organoleptic properties, also increasing the fruit-like sweetness of the final product. These changes are linked to substantial changes on chemical composition during the heating process of onion. However, the scientific data in relation to this issue is scare. Previous studies on an analogous product called "black garlic" showed that the profiles of bioactives compounds in that product increased after the heating process of the raw garlic (Jung et al., 2014). Therefore, this new product not only has a great commercial value by its culinary use, but it could also be used as functional food.

To date, the analysis of the primary and secondary metabolites in foods is challenging due to their different structure, distributions and concentrations in plants vary greatly and the limitation of commercially available reference standards. Several techniques based on high-performance liquid chromatography coupled to mass spectrometry (LC-MS) offer versatile tools for addressing the identification and quantification of a wide number of compounds in onion samples. For instance, Soininen et al. (2014) analysed by LC-MS and NMR the composition of flavonols, free amino acids and organic acids in different Allium species. Other authors characterized the flavonol profile of several onion varieties by LC-DAD-ESI-MS-MS analysis (Bonaccorsi, Caristi, Gargiulli, & Leuzzi, 2008) and identified a complete profiling of polar and semi-polar onion metabolites including fructooligosaccharides, proteinogenic amino acids, peptides, S-substituted cysteine conjugates, flavonoids and saponins by LC-ESI-QTOFMS (Böttcher, Krahmer, Stürt, Widder, & Schulz, 2017). Moreover, an entire set of sulfur-containing onion metabolites in onion has been determined by RP-LC-ESI-Fourier transform ion cyclotron resonance mass spectrometry in conjunction with ^{13}C labelling (Nakabayashi et al., 2013).

To the best of our knowledge, no data on the fully characterization on primary (amino acids) and secondary metabolites (phenolic compounds and sulfur-containing compounds) of black onion have been reported. This study aims to identify and quantify these compounds in black onion, a new product derived from fresh shallot onion, by the optimization and validation of the extraction procedure as well as two rapid analytical UHPLC-HRMS methodologies.

2. Material and methods

2.1. Chemicals

Formic acid (FA), LC-MS grade acetonitrile, LC-MS grade methanol, ammonium acetate, ammonium formate, the reference compounds quercetin (95%), rutin (94%), isorhamnetin (99%), quercetin-3-*O*-glucoside (98%), kaempferol-3-*O*-rutinoside (98%), luteolin (98%), apigenin (95%) and the amino acids leucine (98%), isoleucine (98%), phenylalanine (98%), tryptophan (98%), methionine (98%), valine (98%), proline (99%), tyrosine (98%), alanine (98%), threonine (98%), glycine (99%), glutamic acid (99%), glutamine (99%), serine (99%), asparagine (98%), lysine (98%), histidine (99%), ornithine (99%), aspartic acid (98%), arginine (98%) and gamma-aminobutyric acid (GABA) (98%) and the organosulfur compounds alliin (95%) and s-allyl-L-cysteine (SAC) (95%) were purchased from Sigma-Aldrich (Madrid, Spain). All the standards used were not further purified. Deionized water was used throughout the analytical analyses.

2.2. Materials and sample preparation

Two kg of fresh shallot onions and black onions were obtained from a local supplier which provided authenticated fresh and black onion for the study. Both fresh and black onions were randomized and 0.5 kg was first frozen under liquid nitrogen to avoid enzymatic activity, then lyophilized and grinded afterwards to a final particle size of 10 μm using a mixer mill equipment (Retsch GmbH, Haan, Germany) and stored at −80 °C until analysis.

2.3. Extraction method

The optimization of the extraction of fresh and black onions were performed using three different solvents: deionized water:methanol (20:80, v/v) acidified with 1% formic acid (A), deionized water:-methanol (50:50, v/v) acidified with 1% formic acid (B) and deionized water:acetonitrile (20:80, v/v) acidified with 1% formic acid (C). The extraction method was as follow: 0.5 g of fresh or black onion lyophilized and grinded was mixed with 5 mL of solvent (A), (B) or (C) for 2 min at room temperature and the mixture was sonicated for 15 min and then centrifuged at 4900 rpm for 15 min. The supernatant was collected and residues were re-extracted twice using 5 mL of the same solvent by following the same protocol described previously. All the supernatants were pooled and frozen at −80 °C until UHPLC-HRMS analysis.

2.4. UHPLC-HRMS analysis

Identification and quantification of flavonoids, amino acids and organosulfur compounds in fresh and black onion extracts were carried out using an UHPLC-PDA-MS mass spectrometer system (Thermo Scientific, San José, CA, USA) comprising of a UHPLC pump, a PDA detector scanning from 200 to 600 nm, and an autosampler operating at 4 °C (Dionex Ultimate 3000 RS, Thermo Corporation).

2.4.1. Analysis of flavonoids

Separation of flavonoids was performed on a 100 × 2.1 mm i. d. 1.8 μm Zorbax SB-C18 RRHD column (Agilent, Santa Clara, CA) preceded by a guard pre-column of the same stationary phase and maintained at 40 °C. The mobile phases, A: acidified water 1% formic acid and B: acetonitrile, were pumped at a flow rate of 0.15 mL min^{-1} with a 33 min gradient starting in 3% B and maintained during 1 min, then rising 60% B in 24 min, maintained during 3 min and then rising 70% B in 5 min. After that, the column was equilibrated to the previous conditions within 5 min.

After passing through the flow cell of the PDA detector the column eluate went directly to an Exactive Orbitrap mass spectrometer (Thermo Scientific, San José, CA) fitted with a Heated Electrospray Ionization Probe (HESI) operating in negative ionization mode for the determination of flavonoids. Full scans were recorder in *m/z* range from 100 to 1000 with a resolution of 50.000 Hz and with a full AGC target of 100000 charges, using 2 microscans. Analyses were also based on scans with in-source collision-induced dissociation (CID) at 25.0 eV. MS experiment condition with HESI in negative ionization mode was: (i) capillary temperature was 275 °C, the heater temperature was 100 °C, the sheath gas was 19 units, the auxiliary gas was 15 units, and the spray voltage was 4.0 kv.

Quality control samples (QC) were applied to assess and ensure the analytical process. The QC samples, consisting of a pool of all fresh or black onion samples, were injected regularly throughout the run. Data acquisition and processing were carried out using Xcalibur 3.0 software (Thermo Scientific, San José, CA).

2.4.2. Analysis of amino acids and organosulfur compounds

Separations of amino acids and organosulfur compounds in fresh and black onion extracts were based on a 2.1 × 150 mm ACQUITY UPLC 1.7 μm BEH amide column (equipped with a ACQUITY UPLC BEH amide 1.7 μm van-guard pre-column) (Waters, Spain) which was maintained at 35 °C and eluted using two mobile phases: A: deionized water with 5 mM of ammonium acetate, 5 mM ammonium formate and 1% formic acid and B: acetonitrile, over the course of 20 min at 0.4 mL min^{-1}. The gradient started with 5% of A rising 10% A in 0.5 min, then rising 30% A in 8 min following 46% of A after 4.5 min and finally return to 5% A in 3 min and maintained during 4 min to equilibrate the column to the initial conditions.

After passing through the flow cell of the PDA detector the column