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Volatiles and primary metabolites profiling in two *Hibiscus sabdariffa* (roselle) *cultivars via* headspace SPME-GC-MS and chemometrics

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ABSTRACT

Hibiscus sabdariffa (roselle) is a plant of considerable commercial importance worldwide as functional food due to its organic acids, mucilage, anthocyanins, macro and micro-nutrients content. Although *Hibiscus* flowers are emerging as very competitive targets for phytochemical studies, very little is known about their volatile composition and or aroma, such knowledge can be suspected to be relevant for understanding its olfactory and taste properties. To provide insight into *Hibiscus* flower aroma composition and for its future use in food and or pharmaceutical industry, volatile constituents from 2 cultivars grown in Egypt, *viz.* Aswan and Sudan-1 were profiled using solid-phase microextraction (SPME) coupled to GCMS. A total of 104 volatiles were identified with sugar and fatty acid derived volatiles amounting for the major volatile classes. To reveal for cultivar effect on volatile composition in an untargeted manner, multivariate data analysis was applied. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) revealed for 1-octen-3-ol *versus* furfural/acetic acid enrichment in Aswan and Sudan-1 cvs., respectively. Primary metabolites contributing to roselle taste and nutritional value *viz.* sugars and organic acids were profiled using GC-MS after silylation. The impact of probiotic bacteria on roselle infusion aroma profile was further assessed and revealed for the increase in furfural production with *Lactobacillus plantarum* inoculation and without affecting its anthocyanin content. This study provides the most complete map for volatiles, sugars and organic acids distribution in *two Hibiscus* flower cultivars and its fermented product.

1. Introduction

Hibiscus sabdariffa L. (roselle; Malvaceae) is ranked among the most highly traded commercial and medicinal plants worldwide. Whether native to Africa or Asia, roselle is widely consumed in tropical countries as processed food, flavoring agent, traditional hot or cold beverage as well as a reputed herbal remedy for hypertension and hyperlipidemia (Hopkins, Lamm, Funk, & Ritenbaugh, 2013). Medicinal value of roselle has been verified by several biological studies exploiting its hepatoprotective, antioxidant, anti-inflammatory, antibacterial and cytotoxic properties (Ali, Ashraf, Biswas, Karmakar, & S., 2011; Hirunpanich *et al.*, 2006; Liu *et al.*, 2006; Lo, Huang, Lin, Chien, & Wang, 2007). The brilliantly red calyces of *Hibiscus* are rich in minerals, vitamin C and polyphenols (Ali, Wabel, & Blunden, 2005), however, it is the anthocyanin pigments that have drawn more attention in roselle owing to its antioxidant properties and health attributes (Du & Francis, 1973; Tsai, McIntosh, Pearce, Camden, & Jordan, 2002). Few studies have investigated the volatiles in roselle beverages (Chen, Huang, Ho, & Tsai, 1998; Gonzalez-Palomares, Estarrón-Espinosa, Gómez-Leyva, & Andrade-González, 2009; Jirovetz *et al.*, 1992). Furfural and 5-methyl furfural were detected in beverages prepared from dried hibiscus, while those prepared using fresh hibiscus were rich in linalool and 2-ethyl-1-hexanol. The appreciably variable aroma of roselle hot and cold extracts, comprise a total of 22 volatiles has been described as a combination of earthy, floral and fruity relevant to the extraction process (Ramírez-Rodrigues, Balaban, Marshall, & Rouseff, 2011). Nevertheless, the true aroma of roselle flower itself has yet to be reported. The appealing color and acidic sweet taste of roselle extract, provides a plausible substitute for grapes deficiency affecting the wine industry in the tropics passively (Maldonado, Rolz, & de Cabrera, 1975).

According to Food and Agriculture Organization (FAO), the world's best roselle originates from Sudan (Plotto, 1999). Egypt is also considered an important supplier, but most of its harvest is consumed domestically. The current study aims to investigate the volatile constituent profiles of two *Hibiscus* flower cultivars grown in Egypt using solid-phase microextraction (SPME) coupled to GCMS. This is the first report on roselle volatile analysis using SPME, and further unveiling differences intelligible to cultivar type *e.g.*, Aswan and Sudan-1 using chemometrics. SPME accentuates one true green revolution in volatiles sample preparation techniques gaining a broad spectrum of applications particularly in aroma profiling in herbal drugs over the past two decades (de Koning, Janssen, & Brinkman, 2009). Typical headspace SPME coupled to GC procedure implicates volatiles extraction and

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concentration on the polymeric coating of a silica fiber and the direct desorption of the loaded fiber into the GC injection port (Vas & Vékey, 2004).

Previous studies have reported the influence of incorporating roselle extracts on the biochemical and organoleptic properties of wine and yogurt (Alobo & Offonry, 2009; Iwalokun & Shittu, 2007). As a strong competing candidate in the field of functional foods, the impact of probiotic bacteria, *Lactobacillus plantarum* inoculation on the volatile profile of roselle infusion was also evaluated in this study. This study provides not only the first large scale volatiles profiling in roselle flower from 2 major cultivars in Egypt, but rather extend to determine changes impact of fermentation on *Hibiscus* infusion aroma.

2. Materials and methods

2.1. Plant material, SPME and chemicals

H. sabdariffa flower cultivars Aswan and Sudan-1 were purchased from Haraz Herbal Company (Cairo, Egypt) as dried calyx and epicalyx of *Hibiscus* flowers and devoid of other leaf and or floral parts. SPME holder and fiber coated with 50 μm/30 μm DVB–CAR–PDMS was supplied by Supelco (Oakville, ON, Canada). All other chemicals, volatile standards were provided from Sigma Aldrich (St. Louis, Mo., U.S.A.).

2.2. SPME volatiles isolation

Headspace volatiles analysis using SPME was adopted from (Farag & Wessjohann, 2012) with few modifications. Briefly, flowers were ground, and 3 g was placed inside 20 mL clear glass vials. (*Z*)-3-Hexenyl acetate absent from flower sample VOCs was used as an internal standard (IS), dissolved in water and added to each vial at a concentration of 1 μg/vial. Vials were then immediately capped and placed on a temperature controlled tray for 30 min at 50 °C with the SPME fiber inserted into the headspace above the flower sample. Adsorption was timed for 30 min. A system blank containing no plant material was run as a control.

2.3. GC-MS volatile analysis

SPME fibers were desorbed at 210 °C for 1 min in the injection port of a Shimadzu Model GC-17A gas chromatograph interfaced with a Shimadzu model QP-5000 mass spectrometer (Japan). Volatiles were separated on a DB5-MS column (30 m length, 0.25 mm inner diameter, and 0.25 μm film (J&W Scientific, Santa Clara, CA, USA). Injections were made in the splitless mode for 30 s. The gas chromatograph was operated under the following conditions: injector 220 °C, column oven 38 °C for 3 min, then programmed at a rate of 12 °C/min to 180 °C, kept at 180 °C for 5 min, and finally ramped at a rate of 40 °C min^{-1} to 220 °C and kept for 2 min, He carrier gas at 1 mL min^{-1}. The transfer line and ion–source temperatures were adjusted at 230 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV. The scan range was set at *m/z* 40–500. Volatile components were identified using the procedure described in (Farag & Wessjohann, 2012) and peaks were first deconvoluted using AMDIS software (www.amdis.net) and identified by its retention indices (RI) relative to n-alkanes (C6-C20), mass spectrum matching to NIST, WILEY library database and with authentic standards when available.

2.4. GC-MS analysis of silylated primary metabolites

For analysis of primary metabolites (*viz*. amino acids, organic acids, and sugars), 100 μL of 70% aqueous extract (prepared by extracting 100 mg of dried flowers in 5 mL 50% MeOH with sonication for 30 min followed by centrifugation to remove plant debris) was evaporated under nitrogen till dryness. For derivatization, 150 μL of N-methyl- N-(trimethylsilyl)-trifluoroacetamide (MSTFA) was then added and incubated at 60 °C for 45 min. The samples were equilibrated at 28 °C and subsequently analyzed using GCMS. Silylated derivatives were separated on a Rtx-5MS (30 m length, 0.25 mm inner diameter, and 0.25 μm film) column. Injections were made in a (1:15) split mode and the GC was operated under the following conditions: injector 280 °C, column oven 80 °C for 2 min, then programmed at a rate of 5 °C/min to 315 °C, kept at 315 °C for 12 min. He carrier gas at 1 mL min^{-1}. The transfer line and ion–source temperatures were adjusted at 280 and 180 °C, respectively. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV. The scan range was set at 50–650 *m/z*. Silylated compounds were identified as previously described under GC-MS volatile analysis, and their contents were determined based on peak areas relative to summed peak areas of identified metabolites.

2.5. Probiotic bacterial inoculation

A fresh plate was cultured from −70 °C freezer glycerol stock on MRS agar. A fresh *L. plantarum* colony was used to inoculate overnight in MRS broth until the OD600 was adjusted to give 10^9 CFU/mL. 2 mL of adjusted culture was centrifuged, washed twice, re suspended in PBS buffer pH 7.4 and used to inoculate roselle infusion (Infusion was prepared by adding 100 mL hot water containing 7% sucrose to 5 g of dried roselle powder and kept for 30 min at room temperature followed by filtration to remove plant debris). The initial pH for roselle infusion was adjusted to 4.8 ± 0.2 using $CaCO_3$ prior to sterilization. Sterilization was done in a 250 mL cotton plugged flask at 110 °C for 3 min, followed by rapid cooling in cold room. Samples were aliquoted in glass fitted tubes at 0, 24, 48 and 96 h for pH measurement, volatiles analysis and viable counts (CFU/mL) measurements. A 20 μL aliquot was taken at each time interval and serially diluted in 180 μL peptone saline diluents. 10 μL from each dilution was spotted on MRS agar plate and incubated for 48 h with bacterial count expressed as CFU/mL. Volatile analyses in fermented infusion was performed by placing 5 mL in 20 mL clear glass vials as described in (Sections 2.2 and 2.3) for volatile analysis from dried flowers. Volatiles were collected from two independent bacterial inoculated roselle juice cultures.

2.6. Multivariate data analyses

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed on the MS-data using custom script under the R 2.9.2 environment. Partial least squares-discriminant analysis (OPLS-DA) was performed with the program SIMCA-P Version 13.0 (Umetrics, Umeå, Sweden). Markers were subsequently identified by analyzing the S-plot, which was declared with covariance (p) and correlation (pcor). All variables were mean centered and scaled to Pareto variance. Distance to the model (DModX) test was used to verify the presence of outliers and to evaluate whether a submitted sample fell within the model applicability domain.

2.7. Anthocyanins quantification

For relative determination of anthocyanins in hibiscus fermented infusion at 0, 24, 48 and 96 h time points, 200 μL were aliquoted, and diluted with 200 μL of milliQ water. The mixture was vortexed vigorously for 1 min, centrifuged at 3000 g for 2 min and measured using UV spectrophotometer at 525 nm.

3. Results and discussion

3.1. Volatiles analysis

The goal of this study was to investigate the diversity of volatile profiles within 2 *Hibiscus* cultivars grown in Egypt, *viz*. Aswan and Sudan-1 using GCMS and chemometrics. To assess for biological variance, 3