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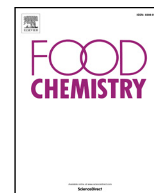

Profiling the impact of thermal processing on black raspberry phytochemicals using untargeted metabolomics

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ABSTRACT

Clinical and laboratory studies have implicated black raspberries (BRBs) and their associated phytochemicals in the modulation of several chronic diseases. Most research on the health benefits of BRBs is conducted using freeze-dried or otherwise minimally processed products, yet BRBs are typically consumed as thermally processed goods like jams and syrups. The objective of this work was to profile the chemical changes that result from thermal processing of BRB powder into a nectar beverage. Using an untargeted UHPLC-QTOF-MS metabolomics approach, key degradation products of anthocyanins were identified along with several other proposed phenolic degradants. The effects of processing on other key BRB compound groups, including ellagitannins, are also discussed. This work demonstrates the utility of an untargeted metabolomics approach in describing the chemistry of complex food systems and provides a foundation for future research on the impact of processing on BRB product bioactivity.

1. Introduction

Black raspberries (BRBs) are heavily researched for their anti-cancer potential. *In vitro* models suggest BRBs may be active against a variety of cancer types (Seeram et al., 2006), while animal studies and clinical trials provide compelling evidence for the potential role of BRBs, and their phytochemical components, in preventing aerodigestive cancers (Bishayee et al., 2015; Kresty et al., 2001). For example, a two week treatment with BRB-based troches reduced malignant tumor levels of hallmark biomarkers of cancer in oral cancer patients (Knobloch et al., 2016). Studies such as this support further research on BRBs as part of a food-based approach for cancer prevention.

BRBs contain a wide array of phytochemicals including anthocyanins, flavonols, ellagitannins, and hydroxycinnamic acids, among others (Paudel et al., 2013). Work to understand which of these components contribute to the bioactivity of BRBs has shown that singular phytochemicals cannot explain the complete bioactivity of the fruit (Paudel et al., 2014; Wang et al., 2009). Thus, the complete phytochemical profile of the fruit is critical when conducting research on the potential health effects of BRBs.

In assessing BRB bioactivity, laboratory and clinical studies have historically used lyophilized BRB powder in treatments, though consumers do not typically encounter these berries in their fresh or freeze-driedforms. Instead, BRBs are more commonly found incorporated into thermally processed products, such as jams and syrups. Knowledge of how thermal processing affects the phytochemical profile of BRB products is limited to a few select compounds (Gu et al., 2014; Hager, Howard, Prior, & Brownmiller, 2008), despite the biological importance of the whole phytochemical profile. Untargeted metabolomics is an analytical technique that aims to provide a comprehensive chemical profile of as many small molecules in a system as possible, which allows for a more thorough and encompassing analysis of molecular composition than traditional methods.

The objective of this study is to understand how thermal processing impacts the phytochemical profile of BRBs using an untargeted metabolomics approach. The product used in this work is a BRB nectar beverage, which has been previously described (Gu et al., 2014). This nectar is an optimal product to study, as it contains the whole BRB fruit and components typically incorporated in other BRB products, such as sugar and pectin, and thus applicable to additional BRB products.

2. Materials and methods

2.1. Chemicals

All chemicals used were obtained from Fisher Scientific (Pittsburgh,

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PA).

2.2. Nectar production

Nectar was produced in 250 g batches (n = 3) according to the formulation described by Gu and colleagues, with slight modifications (Gu et al., 2014). The modified formula is as follows (% wet basis): water (89.9%), sucrose (3.0%), pectin (0.5%), corn syrup (1.0%), BRB powder (5.6%). The BRB (*Rubus occidentalis* cv. Jewel) powder used in this work was acquired from Stokes Berry Farm (Wilmington, OH) as freeze-dried product and was produced from a single lot of berries. To manufacture the nectar, all components, except the BRB powder, were combined and heated to approximately 30 °C with constant stirring. Once the pectin was dissolved, the BRB powder was added and the product was heated to 95 °C with constant stirring until a final soluble solids content of 9 °Brix was achieved (~20 min). These parameters were chosen to model the pasteurization procedure previously described for this product (Gu et al., 2014). Nectar was immediately flash frozen with liquid nitrogen, lyophilized, and stored at −20 °C until analysis. A process blank, which consisted of all nectar components without the BRB powder (i.e. water, sugar, corn syrup, pectin), was also produced in the same manner as the BRB nectars.

2.3. Determination of total monomeric anthocyanins

BRB powder and lyophilized nectar were extracted according to Hager and colleagues with slight modification (Hager et al., 2008). Briefly, 100 mg was combined with 2 mL of 60:37:3 methanol:water:formic acid and vortexed for 15 s. Following centrifugation at 4000 × *g* for 10 min, the supernatants were decanted and pellets extracted twice more. Pooled extracts were diluted to 10 mL with 0.01% aqueous HCl, and total monomeric anthocyanin content was determined as previously described (Giusti & Wrolstad, 2001). Results are expressed as mg cyanidin-3-glucoside equivalents/g powder (using ε = 26,900 and MW = 449.2 amu), and levels in the lyophilized nectar were multiplied by a factor of 1.8 to account for additional dry ingredients in the nectar formulation (BRB powder constitutes 55.4% of the dried nectar solids).

2.4. Sample preparation for untargeted metabolomics

Lyophilized nectar and BRB powder were extracted using identical protocols except 180 mg of nectar was extracted, compared to 100 mg of BRB powder, to account for other ingredients used in the nectar formulation. Briefly, 1 mL of 75% methanol in water, with 0.1% formic acid, was added to each sample. Samples were sonicated in an ice bath for 15 min, centrifuged at 21,130 × *g* for 2 min, and decanted into glass vials. The resulting pellets were extracted twice more with the use of a probe sonicator (8 s, Branson Ultrasonics; Danbury, CT). Each nectar batch was extracted in triplicate (total n = 9), and an equal number of BRB powder samples were extracted (n = 9). The nectar process blank (no BRB added) was also extracted using this protocol. Extracts were centrifuged at 21,130 × *g* for 4 min and the supernatant immediately analyzed. A set of quality control (QC) samples was produced by pooling equal volume aliquots from all nectar and BRB powder extracts.

2.5. UHPLC-QTOF-MS metabolomics data acquisition

BRB and nectar samples were randomized for run order, and QC samples were positioned after every sixth injection. The use of QC samples allows for monitoring of instrument stability over the sample set. Untargeted full-scan data was acquired using a 1290 Infinity II series UHPLC (Agilent, Santa Clara, CA) coupled to an Agilent iFunnel 6550 QTOF-MS. Samples were injected (3 µL) onto a 100 × 2.1 mm Agilent SB-Aq column (1.8 µm particle size) maintained at 50 °C. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol at a flow rate of 0.6 mL/min. The mobile phase composition was as follows: 0–2 min, 0% B; 2–3 min increase to 10% B; 3–8 min, increase to 40% B; 8–14 min increase to 100% B; 14–16 min, hold at 100% B; 16–18 min, immediate switch to 0% B for a total run time of 18 min. The UHPLC was interfaced with the QTOF-MS with an ESI source operated in negative ion mode. Relevant MS parameters were as follows: gas temp 150 °C, drying gas 18 L/min, nebulizer 30 psig, sheath gas temp 350 °C, sheath gas flow 12 L/min, VCap 4000 V, nozzle voltage 2000 V, acquisition mode was 2 GHz extended dynamic range with a mass range of 50–1700 *m/z*.

2.6. Data pre-processing and analysis

Full scan UHPLC-QTOF-MS data was processed using the batch recursive feature extraction algorithm in Agilent Profinder (B.06.00). This process bins mass spectral features according to expected isotope patterns, adducts, and charge states, and then aligns them across all samples. Feature groups that appeared in at least two samples in either the nectar or BRB powder were retained and re-extracted across all samples. The recursive nature of this workflow ensures high quality data for statistical analysis. Further filtering of the data was performed in Agilent Mass Profiler Professional. First, features unique to the processing blank, which consisted of all nectar components except BRB powder, were removed from analysis. The analysis was then restricted to features with retention times between 1 and 12.5 min and a calculated neutral mass < 1200 amu. Finally, features that were present in at least 66.6% of nectar or BRB samples, and those with a CV < 25% in either of these groups were retained for statistical analyses. All data was $\log_2$ transformed and median-centered prior to analysis. Differential analysis was performed using an unpaired *t*-test ($P < 0.05$) with the Benjamini-Hochberg false discovery rate multiple testing correction applied.

2.7. Compound identification

Features that differed significantly between the BRB powder and nectar were considered for identification if their average ion abundance was $> 1.0 \times 10^5$ in either sample group. Highly abundant features (abundance $> 1 \times 10^6$) that differed < 2 times between BRB powder and nectar were also considered for identification. These thresholds were used to ensure high data quality and sufficient signal for further experimentation. Identification was achieved using a combination of MS/MS fragmentation spectra, accurate mass, isotope analysis, and database matches when available. For MS/MS fragmentation studies, extracts were injected on the previously mentioned UHPLC-QTOF-MS system operated in targeted or auto MS/MS mode using collision energies of 10, 20, and 40 eV. Fragmentation patterns were compared to literature or curated spectra in the FooDB (Wishart et al., 2013) and Metlin (metlin.scripps.edu) databases when available. CFM-ID (Allen, Pon, Wilson, Greiner, & Wishart, 2014) was used to rationalize proposed molecular structures as needed. Feature spectral data and retention times were compared to authentic standards as available, and metabolites were annotated according to the Metabolomics Standard Initiative guidelines (Sumner et al., 2007).

3. Results and discussion

In the present work, we compared the metabolomic profiles of lyophilized BRB powder and a thermally processed nectar beverage made from that same powder. This allows us a more holistic view on both phytochemicals that change, and those that do not change, with thermal treatment. A metabolomic approach has been previously used to understand chemical changes during BRB ripening (Kim et al., 2011), as well as the potential bioactivity of BRB phytochemicals (Jo et al., 2015; Paudel et al., 2014; Teegarden, Knobloch, Weghorst, Cooperstone, & Peterson, 2018). In an effort to provide context for the

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