Nutrition

Evaluating commercial food products for presence of blueberries and for their anti-inflammatory effects

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Incorporation of blueberries in food products has increased rapidly in last decade. The purposes of this study were to evaluate the blueberry in commercial food products presence by measuring signature compounds, and to further assess their anti-inflammatory effects. Anthocyanins (ACNs) and chlorogenic acid (CGA) were selected as candidate signature compounds. In ten selected blueberry-containing commercial food products, only four contained ACNs, while eight contained CGA. Because CGA was more stable, it was considered as a better indicator of blueberry presence in processed foods. Blueberry contents in these commercial food products were then estimated based on their CGA contents. Polyphenol-enriched fractions from these food products were prepared and subjected to NF-xB inhibition assay to evaluate their anti-inflammatory effects. All fractions inhibited NF-xB activation dose-dependently. There was a weak correlation between anti-inflammatory effects and blueberry contents. The data also pointed out that ACNs in their native forms were probably the most active anti-inflammatory components in blueberries. Journal of Nature and Science, 1(4):e65, 2015

Anthocyanin | blueberry | chlorogenic acid | food products | NF-xB

1. Introduction
Blueberry is one of the few fruits native to North America. Consumption of blueberries has increased rapidly over the past decade driven by their health benefits. Blueberry is now one of the most commonly consumed berries in the US, ranks only second to strawberries in popularity. Blueberries are often known as the “super fruit” or “super food” due to their high concentration of antioxidants [1]. Recent studies have found that blueberries contain extremely high level of polyphenols and exhibit strong antioxidant activities and potential anti-inflammatory effects [2,3]. A diet rich in blueberries has been shown to prevent chronic diseases including cardiovascular diseases, certain types of cancer and osteoporosis, with anti-inflammatory as one of the major underlying mechanisms [4-8]. Blueberries have been shown to reduce pro-inflammatory cytokine production through inhibiting NF-xB activation dose-dependently. There was a weak correlation between anti-inflammatory effects and blueberry contents. The data also pointed out that ACNs in their native forms were probably the most active anti-inflammatory components in blueberries. Journal of Nature and Science, 1(4):e65, 2015

2. Materials and apparatus
2.1. Chemicals and reagents
Methanol (MeOH), dimethyl sulfoxide (DMSO), formic acid, acetic acid, phosphate-buffered saline (PBS), 5-O-caffeoylquinic acid (chlorogenic acid) were all obtained from Sigma-Aldrich (Milwaukee, WI). The 3-O-a-glucoside standards of pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin (six anthocyanin standards, HPLC grade) and cyanidin 3-glucoside (HPLC grade) were purchased from Polyphenols Laboratories (Sandnes, Norway). C-18 powder was purchased from Nacalai Tesque (Cosmosil 75C18-PREP, Nakagyo-ku, Kyoto, Japan). 60-mL cartridges were obtained from SUPELCO (Milwaukee, WI).

2.2. Blueberry samples and testing food products
Three authentic freeze-dried whole lowbush BB (Vaccinium angustifolium) powder and one authentic freeze-dried whole highbush BB (Vaccinium corymbosum) powder were kindly provided by Future Ceuticals (Momence, IL). The fresh blueberries and commercial blueberry-containing foods were purchased from the local grocery stores in Harrisburg, Pennsylvania, USA (Table 1).

Conflict of interest: The authors declare no conflict of interest.

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compartment according to a published method [2]. A 250 × 4.6 mm i.d. Zorbax SB-C18 column was used for separation. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol) as following gradient: 5% B, 0-2 min; 5-20% B, 2-10 min; 20% B, 10-15 min; 20-30% B, 15-30 min; 30% B, 30-35 min; 30-45% B, 35-50 min; 45% B, 50-55 min; 45-5% B, 55-65 min, 5% B, 65-68 min. The flow rate was 1 mL/min, and two detection wavelengths were set at 520 nm for ACNs and 650 nm for CGA. Quantification of anthocyanins was performed by the procedure reported previously by using six anthocyanin standards (glucosides of six common anthocyanidins: delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin) and to create standard curves [18]. The results were expressed as anthocyanin glucoside equivalents. Quantification of CGA was conducted based on a standard curve created with CGA standard.

2.5. NF-κB inhibitory assay

The NF-κB assay was conducted based on a recent publication [17]. Briefly, the mouse myoblast cell line C2C12 stably transfected with a NF-κB luciferase reporter (Panomics Inc., Fremont, CA, USA) was used. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and penicillin–streptomycin at 37 °C in an incubator with 5% CO2. For measurement of the NF-κB activation, 5 × 104 cells were seeded in 96-well plates and cultured overnight. The growth media was then replaced by serum-free media, and the cells (triplicate wells) were incubated for 2 h at 37 °C with 80 µL of polyphenol-rich fractions (50, 100, 200 and 400 µg/mL, in a serum-free medium). The cells were then stimulated with TNF-α (20 ng/well) for 6 h, and the luciferase activity was measured using a luciferase assay system kit (Promega, Madison, WI) on a Synergy 2 plate reader (Bio-Tek, Winooski, VT).

2.6. Statistical analysis

The data were presented as mean ± SD (n = 3). Significant differences were determined by one-way analysis of variance followed by Tukey’s test. Differences were considered significant at p < 0.05. Statistical analyses were performed using SigmaStat statistical software (SigmaStat 3.5) (Jandel Scientific Software, San Rafael, CA).

3. Results

3.1. ACNs and CGA in authentic blueberry samples

ACNs in the two blueberry species showed similar profiles but different contents. By comparing retention times with previous report [15], twenty-two major ACNs peaks were identified (Figure 2A1 and 2B1). CGA was the major peak under 320 nm (Figure 2A2 and B2), which agreed to previous report [16]. Total ACNs (the sum of individual ACNs) and CGA were quantified using external standards. The results were expressed as fresh weight (FW) after conversion from dry weight (Table 2) by using the moisture information provided by the vendor. In general, lowbush blueberries contained higher ACNs but similar CGA comparing to highbush blueberries. In addition, fresh blueberry sample was also analyzed and quantified (Figure 2C1 and C2). It was shown to be highbush blueberries based on its ACN profile.

Table 1. Commercial food products and their abbreviations used in this study

<table>
<thead>
<tr>
<th>Product category</th>
<th>Product name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh fruit</td>
<td>Fresh blueberries</td>
<td>FB</td>
</tr>
<tr>
<td>Dried fruit</td>
<td>Dried Blueberries</td>
<td>DB</td>
</tr>
<tr>
<td>Bagel</td>
<td>Deluxe Blueberry Bagel</td>
<td>Bagel</td>
</tr>
<tr>
<td>Jam</td>
<td>Spreadable Blueberry Fruit Jam</td>
<td>Jam</td>
</tr>
<tr>
<td>Syrup</td>
<td>Blueberries in Light Syrup</td>
<td>Syrup</td>
</tr>
<tr>
<td>Energy drink</td>
<td>Energy Drink – Blueberry</td>
<td>Energy</td>
</tr>
<tr>
<td>Bar</td>
<td>Blueberry Fruit &amp; Brain Bar</td>
<td>Bar</td>
</tr>
<tr>
<td>Chip</td>
<td>Pastry Chips in Blueberry</td>
<td>Chip</td>
</tr>
<tr>
<td>Pouch</td>
<td>Fruit Burst Squeezers - Blueberry</td>
<td>Pouch</td>
</tr>
<tr>
<td>Yogurt</td>
<td>Blueberry Greek Yogurt</td>
<td>Yogurt-1</td>
</tr>
<tr>
<td></td>
<td>Original Blueberry Yogurt</td>
<td>Yogurt-2</td>
</tr>
</tbody>
</table>

Figure 1. Extraction and purification procedure used in this study.

2.3. Sample preparation

About 2 – 10 g of sample (depending on the type of food product) was weighed and put into a 50 mL screw-cap tube and 25 mL of extraction solvent (methanol/water/formic acid, 85:15:0.5; v/v) were added and vortexed for 30 s followed by sonication for 5 min. The tube was then kept at room temperature for 10 min, being vortexed for 30 s after 5 min. Subsequently, the tube was centrifuged at 4550 g for 10 min and the supernate was collected. The residue was extracted one more time with 20 mL of extraction solvent using the same procedure, and the supernatants were combined. The combined supernatant was transferred and brought to 50 mL with extraction solvent. A small portion of extract was vortexed for 30 s after 5 min. The tube was then kept at room temperature for 10 min, being vortexed for 30 s after 5 min. Subsequently, the tube was centrifuged at 4550 g for 10 min and the supernate was collected. The residue was extracted one more time with 20 mL of extraction solvent using the same procedure, and the supernatants were combined. The combined supernatant was transferred and brought to 50 mL with extraction solvent. A small portion of extract was diluted appropriately and filtered using a 0.22 µm Teflon syringe filter (Cameo 25F, Micron Separations, Westboro, MA) for HPLC analysis.

To prepare polyphenol-rich fractions, the above prepared crude extracts were further purified by solid phase extraction (SPE). In brief, the organic solvent was evaporated in a Buchi R-215 rotavapor (Büchi, Germany) under vacuum to obtain the gel-like extract. The residue was extracted one more time with 20 mL of extraction solvent using the same procedure, and the supernatants were combined. The combined supernatant was transferred and brought to 50 mL with extraction solvent. A small portion of extract was diluted appropriately and filtered using a 0.22 µm Teflon syringe filter (Cameo 25F, Micron Separations, Westboro, MA) for HPLC analysis.

2.4. HPLC analyses of anthocyanins and chlorogenic acid

Analyses were performed on an HP 1100 series HPLC (Hewlett-Packard, Palo Alto, CA) equipped with a diode array detector, a binary pump, an autosampler and a thermostat column compartment according to a published method [18]. A 250 × 4.6 mm i.d. Zorbax SB-C18 column was used for separation. Elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol) as following gradient: 5%
Table 2. ACN and CGA contents in freeze-dried blueberry samples and in commercial food product, and the estimation of blueberry content (based on fresh weight) in food products.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACN (mg/g)</th>
<th>CGA (mg/g)</th>
<th>ACN/CGA Ratio</th>
<th>BB content (g/g FW)</th>
<th>Serving (g)</th>
<th>BB/serving (g/serving)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB BB 1</td>
<td>1.46 ± 0.04</td>
<td>0.27 ± 0.02</td>
<td>7.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB BB 2</td>
<td>1.79 ± 0.03</td>
<td>0.23 ± 0.01</td>
<td>7.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB BB 3</td>
<td>1.88 ± 0.04</td>
<td>0.24 ± 0.01</td>
<td>8.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB BB 1</td>
<td>2.48 ± 0.04</td>
<td>0.30 ± 0.02</td>
<td>8.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FB</td>
<td>1.37 ± 0.03</td>
<td>0.25 ± 0.01</td>
<td>5.4</td>
<td>1</td>
<td>145</td>
<td>145.0</td>
</tr>
<tr>
<td>Yogurt-1</td>
<td>0.08 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>4.1</td>
<td>0.07</td>
<td>170</td>
<td>12.5</td>
</tr>
<tr>
<td>Yogurt-2</td>
<td>0.07 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>3.5</td>
<td>0.08</td>
<td>170</td>
<td>12.8</td>
</tr>
<tr>
<td>Jam</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>1.0</td>
<td>0.25</td>
<td>40</td>
<td>10.1</td>
</tr>
<tr>
<td>DB</td>
<td>0.01 ± 0.00</td>
<td>0.33 ± 0.02</td>
<td>0.03</td>
<td>1.28</td>
<td>40</td>
<td>51.3</td>
</tr>
<tr>
<td>Pouch</td>
<td>0</td>
<td>0.08 ± 0.00</td>
<td>0</td>
<td>0.30</td>
<td>90</td>
<td>27.3</td>
</tr>
<tr>
<td>Chip</td>
<td>0</td>
<td>0.01 ± 0.00</td>
<td>0</td>
<td>0.01</td>
<td>25</td>
<td>0.3</td>
</tr>
<tr>
<td>Bar</td>
<td>0</td>
<td>0.01 ± 0.00</td>
<td>0</td>
<td>0.03</td>
<td>39</td>
<td>1.1</td>
</tr>
<tr>
<td>Syrup</td>
<td>0.22 ± 0.01</td>
<td>0.13 ± 0.00</td>
<td>1.6</td>
<td>0.51</td>
<td>140</td>
<td>72.0</td>
</tr>
<tr>
<td>Bagel</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>Energy</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.25</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Data was expressed as fresh weight basis for freeze-dried powders (LB and HB) and “as is” for the rest samples (FB and all commercial food products); BB: blueberries; FW: fresh weight; serving size was from the label of product;
3.5. Anti-inflammatory of polyphenol-rich extracts from blueberry-containing food products

Polyphenol-rich fractions were prepared from the crude extracts of fresh blueberries as well as eight BB-containing food products. The fractions were used in NF-κB inhibitory assay. All of these samples displayed dose-dependence anti-inflammatory effects (Figure 5). Polyphenol-rich fraction of fresh blueberries showed the highest inhibitory effects.

3.6. Correlation between anti-inflammatory effects and blueberry contents

The correlation between NF-κB inhibition and blueberry contents of these samples were calculated (Figure 6). The correlation coefficient was calculated as 0.5577 with all nine sample, suggesting that there was a weak correlation between the anti-inflammatory effects with their actual blueberry contents. For comparative purpose, DB, Yogurt-1 and Yogurt-2 were removed, and the correlation coefficient was dramatically improved to 0.8658.

4. Discussion

Numerous studies have been done on the bioactive components and health benefits of blueberries. Many of them looked at how food processing and storage affecting bioactivities of blueberries and blueberry products in the model systems [19-25]. To our knowledge, no studies ever reported on detecting blueberries in the commercial food products. This study attempted to, for the first time, address the questions on how to detect blueberries in commercial food products, how much they contain, and if they exhibit similar health benefits as blueberries do.
One of the common approaches to detect the blueberries in commercial food products was to measure the signature compounds of blueberries. In general, ideal signature compound(s) of a given plant food would be 1) unique to this plant; 2) abundant, so can be detected even existing in low amount; 3) easy to be detected with less interfering compounds; 4) bioactive compounds. In case of this study, since the focus was on the commercial food products, the fifth requirement would be stable during food processing. Based on these criteria, dietary phytochemicals were ideal candidate signature compounds. Major bioactive phytochemicals in blueberries are polyphenols, including ACNs, proanthocyanidins, phenolic acids, stilbenes and flavonols [26,27,16,28]. Among them, stilbenes and flavonols are minor component with low concentrations. Proanthocyanidins are not unique in terms of their profile. Besides, they are structurally complicated and cannot be easily characterized unless utilizing advanced instrumentation such as MALDI-TOF MS. ACNs existed at high concentrations and were widely considered as major bioactive compounds in blueberries [8,29,30]. HPLC profile of blueberry ACNs under 520 nm is very unique. It can be used to easily distinguish blueberries from other foods [15]. CGA, though not as unique as ACN profile, is one of the most abundant single bioactive phytochemicals in blueberries. It appeared as a major peak in the HPLC chromatogram under 320 nm [16]. Hence, ACNs and CGA were selected as candidate signature compounds.

Of ten commercial food products, only four (Yogurt-1, Yogurt-2, Jam and Syrup) showed similar HPLC ACNs profiles as that of authentic blueberries, indicated they contained real blueberries. Surprisingly, DB, despite it is dried blueberries and showed dark bluish color, contained barely detectable ACN peaks. What is causing almost complete loss of ACNs in DB is out of the scope of this study; one possible reason might be the polymerization of anthocyanins. No ACN peaks were detected in Bagel, Pouch and Bar. Energy and Chip were found to contain the coloring dye Red 40. In all four authentic blueberry samples and in fresh blueberries, CGA was found to be the major peak detected under 320 nm (Figure 2). Analyses of the ten food products revealed that 8 of them showed CGA peak, with the exceptions of Bagel and Energy. Naturally, it is also found in coffee bean and a number of fruits and vegetables [31]. However, by checking the ingredient lists, nine of ten food products in study did not contain ingredients other than blueberries that contained CGA. Therefore, CGA detected in these products were considered as solely from blueberries. The only
Blueberry contents in ten food products were estimated by comparing their CGA concentrations with that of blueberry samples. The values appeared to be more reasonable by using CGA than ACNs. For instance, if calculated by ACNs contents, the blueberry content of DB would be almost zero due to its extremely low level of ACNs. This is obviously not reasonable since DB is dried blueberries. When calculated with CGA, the value was 1.28 g/g FW. For foods that containing other CGA containing ingredients, such as Pouch, it would be difficult to estimate the content of blueberry due to lack of information of product formulation. Thus the value for Pouch was likely to be overestimated. Another interesting observation is that color did not necessarily reflect blueberry contents. Energy and Chip both overestimated. Another interesting observation is that color did not necessarily reflect blueberry contents. Energy and Chip both overestimated.

Blueberries still show similar health benefits as fresh blueberries do. Therefore, the ingredient list before using this approach. Blueberry-containing foods showed anti-inflammatory effects at different levels, but all lower than fresh blueberries. ACNs in their native forms seemed to be the strong anti-inflammatory components in blueberries. Thus to maintain the anti-inflammatory effects of blueberries, it is recommended to retain the native forms of ACNs as much as possible by improving food processing. Finally, it is worth mentioning that only a limited number of commercial foods on the market were selected in this study. For the future studies, a wider survey of blueberries in commercial foods would be recommended.

5. Conclusions

This study attempted to estimated blueberry contents in selected blueberry-containing food products by measuring the blueberry signature compounds. ACNs and CGA were selected for assessment and the later one appeared to be the better indicator due to its stability during food processing. However, since CGA is not unique and specific to blueberries, it is not a perfect indicator. One must be cautious to use it and it is highly recommended to check the ingredient list before using this approach. Blueberry-containing foods showed anti-inflammatory effects at different levels, but all lower than fresh blueberries. ACNs in their native forms seemed to be the strong anti-inflammatory components in blueberries. Thus to maintain the anti-inflammatory effects of blueberries, it is recommended to retain the native forms of ACNs as much as possible by improving food processing. Finally, it is worth mentioning that only a limited number of commercial foods on the market were selected in this study. For the future studies, a wider survey of blueberries in commercial foods would be recommended.

More importantly, CGA has been shown to be a good indicator, but not a perfect indicator for detecting blueberries in commercial foods. More specific indicator(s) need to be developed for more accurate estimation.


