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Variation in Lycopene and Lycopenoates, Antioxidant Capacity, and Fruit Quality of Buffaloberry (*Shepherdia argentea* [Pursh]Nutt.)

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Abstract

Buffaloberry (*Shepherdia argentea* [Pursh] Nutt.) has historically been used as an important food source by North American indigenous peoples, but its commercial production has been limited. These plants produce fruits rich in carotenoid and phenolic antioxidants, which may have health benefits that may make buffaloberry commercially valuable. Here, we examined these constituents in the fruit of 7 Dakota-grown buffaloberry selections. Primary carotenoids were determined by liquid chromatography-mass spectral analysis and by nuclear magnetic resonance spectroscopy to be lycopene (0.27 ± 0.02 g/kg FW) and methyl apo-6'-lycopenoate (MA6L; 0.32 ± 0.03 /kg FW). MA6L comprised the greatest proportion (55%) of carotenoid antioxidants, but its role in human nutrition is still to be evaluated. The fruit contained high total phenolics concentrations (9.06 ± 0.71 g gallic acid equivalents/kg FW). Hydrophilic antioxidant capacity among the 7 selections averaged 49.0 ± 6.6 mmol trolox equivalents/kg FW, respectively, as measured by ferric reducing ability of plasma assay. The soluble solids and titratable acids concentrations were 21% and 2.2%, respectively. This species is adapted to poor soils and can tolerate drier climates. In the Dakotas,

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Author Contributions Ken Riedl conducted the MS analyses and wrote parts of the manuscript. Krunal Choksi was responsible for the initial carotenoid and phenolic extractions, and measurement of phenolic contents. Faith Wyzgoski conducted the NMR studies and NMR data evaluation, and wrote parts of the manuscript. Joseph Scheerens contributed to the design of the research and was responsible for purification of carotenoids for NMR analysis. Steven Schwartz provided the HPLC-MS facilities and interpretation of the MS data. Neil Reese initiated the research, directed the undergraduate research, and was the primary author of the manuscript.

buffaloberry flourishes on the American Indian Tribal Reservations, yielding copious amounts of health-beneficial fruit for fresh and processing markets, making it a potentially valuable new crop for marginal lands.

Keywords

fruit; HPLC-MS; lycopene; NMR; *Shepherdia argentea*

Introduction

Buffaloberry (*Shepherdia argentea* [Pursh] Nutt.) is a native, North American member of Elaeagnaceae. This dioecious shrub produces edible drupaceous berries (Figure 1) that have traditionally been an important component of the diets of American Indian peoples (Gilmore 1919; Remlinger and St.-Pierre 1995; Burns Kraft and others 2008). Buffaloberries were first cultivated in 1818 and were first brought into commercial production in Wyoming in 1890 (Remlinger and St.-Pierre 1995). Buffaloberries are currently being used in windbreak and wildlife production plantings. They grow in a wide variety of habitats from stream bank to dry upland grasslands (Hladek 1971). Commercial production methods have been published (Grubb 2007) and successful plantings have been made in sandy to clay soils in areas having 13 or more inches of rainfall annually (USDA-NRCS 2006).

The fruit have a tart flavor as a consequence of their acid and phenolic contents. Their red color results from carotenoid pigmentation, as has been shown for 2 closely related species: soapberry (*Shepherdia canadensis*) and autumnberry (*Elaeagnus umbelatum*). Fruit of both latter species have been shown to contain significant amounts of lycopene (Kjoesen and Liaaen-Jensen 1969; Fordham and others 2001). Additionally, soapberry has been shown to contain a second major carotenoid identified as the methyl ester of apo-6' lycopenoate (Kjoesen and Liaaen-Jensen 1969).

The carotenoid profile of buffaloberry has not been reported previously. In buffaloberry extracts, we found by high-performance liquid chromatography (HPLC) 2 peaks of similar intensity, one clearly matching behavior of an (all-E)-lycopene standard. Our goal was to identify the second carotenoid (conditionally designated as a lycopene derivative) using liquid chromatography-mass spectral analysis (LCMS-MS) and proton nuclear magnetic resonance spectroscopy (NMR). Additionally, we quantified the phenolic content, the antioxidant capacity, the soluble solid (SS) content, and acidity as an initial assessment of the quality of the native buffaloberry fruit found growing in the Dakotas.

Materials and Methods

Plant materials

Buffaloberries were collected in North and South Dakota from wild plants in September and frozen within 24 h of harvest at -20°C . Field replicates of the berries were collected, with separate aliquots being freeze dried and/or placed at -80°C until being analyzed.

Carotenoid preparation

Freeze-dried berries (100 g) were homogenized in a blender. Residual moisture in the powdered material was removed by adding methanol followed by centrifugation. To the pellet, a 1:1 (v/v) mixture of acetone and hexane was applied (100 mL) to extract the lipophilic pigments. This was repeated 3 times to ensure complete extraction of pigments. The pooled acetone/hexane extracts were brought to dryness under a stream of nitrogen. The residue was dissolved in 1:1 methyl *tert*-butyl ether (MtBE)/methanol for HPLC-MS/MS injection.

HPLC-PDA-MS/MS—Carotenoid separations were conducted with an Alliance 2695 HPLC system with a 996 photodiode array (Waters Corp., Milford, Mass., U.S.A.) using a C₃₀ (4.6 × 150 mm, 5 μm) column maintained at 35 °C. A gradient of 0.1% formic acid/methanol/MtBE at 1.5 mL/min was used to resolve the major carotenoid species. The gradient started at 20/80/0 and progressed linearly through 2/20/78 over 15 min with a 5-min re-equilibration. UV-vis spectra were collected from 230 to 600 nm at a rate of 5 per s. Lycopene and the unidentified lycopene derivative in the buffaloberry extracts were calculated as lycopene equivalents at A₄₇₁ nm, using the published extinction coefficient (Fish and others 2002).

The eluate from the PDA was interfaced with a QToF-Premier (Micromass, Beverly, Mass., U.S.A.) using an atmospheric chemical ionization source operated in negative ion mode. The MS instrumental parameters included: cone 35 V, corona current 30 μA, cone gas 50 L/h, desolvation gas 400 L/h, probe temperature 450 °C, and ToF in V mode (approximately 8000 mass resolution). The QT of mass accuracy was calibrated with sodium formate (series of adduct masses) for the mass range 50 to 1000 amu. Within a given LCMS run, leucine enkephalin was used as a lockspray reference ion and was acquired every 20 s to correct the mass calibration for minor temperature fluctuations.

Milligram quantities of the unidentified lycopene derivative were isolated by semipreparative chromatography (C30 10× 250 mm, 5 μm, NIST Lane Sander) with a 6-min isocratic run of water/methanol/mtbe (x/y/z) at 6 mL/min and ambient temperature (23 °C) monitoring eluate at 470 nm. Extract dissolved in 1:1 MtBE/methanol (1 mL) was injected. Collected fractions from 10 runs were pooled, dried under nitrogen, and held under vacuum to remove residual methanol before NMR analysis.

NMR spectroscopy—Immediately prior to proton NMR experiments, the derivative was dissolved in deuterated chloroform (CDCl₃). One-dimensional ¹H spectra (0 to 8 ppm) were collected on a 400-MHz NMR instrument (Bruker DXP 400, Rheinstetten, Germany) combining 128 transients of 2.7 s each. Tetramethylsilane was used to reference the spectrum. Topspin 1.3 software (Bruker) was used to transform and integrate the NMR spectrum.

Phenolics, antioxidants, and fruit quality

A single extraction procedure designed to assay phenols (Singleton and others 1999) was used to determine total soluble phenolic content (TP) and aqueous antioxidant capacity of all

samples. Frozen field replicates were homogenized in a blender in duplicate. From each, a 3-g aliquot of pureed fruit was transferred to a polypropylene tube and extracted with 40 mL of extraction buffer containing acetone, water, and acetic acid (70:29.5:0.5 v/v) for 1 h. After filtration, acetone was removed by rotary evaporation and then the concentrated samples were brought to a final volume of 40 mL with distilled, de-ionized water.

To determine levels of TP in sample extracts, 1 mL of each duplicate was combined with Folin-Ciocalteu's phenol reagent and water 1:1:20 (v/v/v) and incubated for 8 min followed by the addition of 10 mL of 7% (w/v) sodium carbonate. After 2 h, the absorbance of each duplicate was measured at 750 nm on a DU650 spectrophotometer (Beckman-Coulter Fullerton, Calif., U.S.A.). Values of TP were determined using a standard response curve generated with gallic acid (Sigma-Aldrich, St. Louis, Mo., U.S.A.).

The total antioxidant capacity of the extracts was measured using the ferric reducing ability of plasma (FRAP) assay (Benzie and Strain 1996). Briefly, aqueous stock solutions containing 0.1 mol/L acetate buffer (pH 3.6), 10 mmol/L 2,4,6-tris(2-pyridyl)-1,3,5-triazine (Sigma-Aldrich) acidified with concentrated hydrochloric acid, and 20 mmol/L ferric chloride (1000:3:3 v/v/v). These solutions were prepared and stored in the dark under refrigeration. Stock solutions were combined (10:1:1 v/v/v) to form the FRAP reagent just prior to analysis. For each of the assays, 2.97 mL of FRAP reagent and 30 μ L of sample extract were combined and vortexed. After 30 min, the absorbance of the reaction mixture was determined at 593 nm. The concentration of each sample was determined by comparison to a standard curve (10 to 100 μ mol/L) prepared with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich) and expressed as trolox equivalents (TE).

Buffaloberry purees were also assayed for SS contents by refractometry and for acidity (titratable acids) by titration (Perkins-Veazie and Collins 2004).

Means, standard errors, and regressions were calculated using Microsoft Excel (Microsoft Intl., Redmond, Wash., U.S.A.) spreadsheet functions.

Results and Discussion

Buffaloberry appears to have potential as a valuable food crop species, being well adapted to moderately poor soils and drier climates (Remlinger and St. Pierre 1995). Historically, Native American peoples consumed buffaloberry fruits in food preparations specific to their cultures (Gilmore 1919; Remlinger and St.-Pierre 1995). In addition, buffaloberry wines have recently become commercially available in the United States. The potential for growing, consuming, and marketing buffaloberry fruit on and around Midwestern Native American Reservations provides both an economic and nutritional opportunity that should be exploited.

Carotenoids

Buffaloberry carotenoids were analyzed using extracts from fruit collected at 5 locations in North Dakota and 2 locations in South Dakota (Table 1). HPLC-PDA areas and MS

intensities are shown in Figure 2A–D. HPLC-PDA separation showed that there are only trace amounts of other carotenoid constituents (Figure 2A). The concentrations of these minor constituents were too limited for complete confirmation of their identities. The almost complete lack of cyclized carotenoids suggests that this species lacks the crucial enzymes for cyclization of lycopene, β -cyclase, and ϵ -cyclase, which allow for carotene formation (Benvenuti and others 2004). The lack of expression of these genes during ripening of *Elaeagnus umbellata* fruit has recently been shown (Benvenuti and others 2004) and may be a characteristic of this plant family.

An HPLC chromatogram of the crude acetone/hexane extract is shown in Figure 2A. The trace shows the extracted wavelength chromatogram at 470 nm. Figure 2B shows the HPLC-MS chromatogram as the sum of radical anion signals for methyl-apo-6'-lycopenoate (m/z 472.3) and lycopene (m/z 536.4), which together account for the UV-vis absorption in Figure 2A. Figure 2C shows the lycopene HPLC-MS trace at m/z 536.4 demonstrating presence of (Z)-lycopene isomers (peaks before 22.68 min) with the last peak being all-(E) lycopene, and Figure 2D shows the methyl-apo-6'-lycopenoate HPLC-MS trace at m/z 472.3 with minor peaks, presumed to be the (Z)-isomers, eluting prior to main (all-E)-lycopene peak at 20.22 min. The λ_{max} was 472 nm and the base MS peak at 472.334 m/z .

Daughter ion of 403 was observed after collision-induced dissociation (CID) corresponding to M-isoprenyl. After saponifying the extract with methanolic KOH, an earlier eluting peak with m/z of 458 was detected which matches loss of a methyl group. CID of this peak (Figure 3) afforded a daughter ion of 413 m/z which agrees with loss of HCO_2 . Together these UV-vis and MS features suggested a methyl ester of a carboxylic acid formed after cleavage of lycopene at the apo-6' site. Minor UV-vis peaks in the PDA chromatogram (less than 5% the intensity of the main peaks), eluting before the major species, had corresponding m/z of 472 and 536 m/z matching that of the supposed methyl apo-6'-lycopenoate (MA6L) and lycopene suggesting they are (Z)-isomers of these 2 major forms possibly formed during extraction and handling.

The 1D proton NMR spectrum was also consistent with MA6L (Figure 4). For example, the singlet at 3.761 ppm is characteristic of the 6'' methyl carbon of the methoxy group of the 6' ester group in methyl-apo-6'-lycopenoates. The integrated values, chemical shifts and coupling constants for H-7' (1H, d, 5.87, $J = 15.5$); H-8' (1H, d, 7.39, $J = 15.5$), as well as the data for H-19' (3H, s, 1.941) are in close agreement with those reported for methyl-(all E)-apo-6'-lycopenoate (Collins and others 2006; Carlsen and others 2010). Resonances characteristic of methylapo-6'-lycopenoates for isoprenoid methyl side groups, conjugated polyene methylenes, and ethylenes were also observed for MA6L. Because of numerous spin couplings and the presence of a minor compound (indicated by an overlapping methoxy resonance at 3.749 ppm), the 1D spectrum shown in Figure 4 is complex; employment of multidimensional NMR techniques could lead to an unambiguous assignment of configuration.

The second major peak was confirmed to be (all-E)-lycopene. The lycopene standard co-eluted (27.76 min in Figure 2A) and had a matching UV-vis spectrum and base MS peak at

536.439 m/z. It also produced an M-69 daughter ion characteristic of compounds with a terminal isoprenyl group.

The extinction of MA6L should be nearly identical to that of lycopene since the conjugated polyene chain is of equal length and structure. If one compares the chromatogram areas for the 2 peaks the relative abundance of MA6L and lycopene is almost 1 to 1 (Table 1).

MA6L has been reported as a minor chemical species in *Bixa orellana* fruit and as a major component of soapberry fruit, a species closely related to buffaloberry (Collins and others 2006; Carlsen and others 2010). However, soapberry is practically inedible due its high saponin and low sugar content. The considerable abundance of MA6L in buffaloberry may have practical marketing and potential health impacts. It could be valuable as a natural colorant since the free acid of MA6L is more polar, permitting unique applications compared to conventional orange–red colorants. In addition, Native Americans already consuming buffaloberry products may benefit from MA6L activities which remain uncharacterized.

It appears that the majority of the MA6L in buffaloberry is the (all-E)-form (Figure 5), but some atoms of the structure may be in (Z)-configuration. Typically, (all-E)-lycopene predominates in *Shepherdia* (Kjoesen and Liaaen-Jensen 1969) but other isomers could be present. For example, Mercadante and others (1996, 1997) have identified, in the seed coat of *Bixa orellana* fruit, both methyl (9'Z)-apo-6'-lycopenoate (approximately 1% of total carotenoid) and methyl (7Z,9Z,9'Z)-apo-6'-lycopenoate. However, the resonances of our ¹H NMR spectra of MA6L are not an exact match for either of these isomers as reported in the literature.

Lycopene has been shown to be a very strong antioxidant involved in singlet oxygen quenching (Di Mascio and other 1989). The lycopene content of buffaloberries tends to be high in comparison to tomatoes and other commercially available fruit (Gallander 1987; Collins and others 2006). These data strongly suggest that addition of these fruits to the human diet may provide protection from many diseases by providing an important source of hydrophobic antioxidants (Guo and others 2009).

Phenolics

In this study, total phenolic contents of acetone extracts of buffaloberry fruits (Table 2) showed that the buffaloberries contained higher phenolic concentrations than found in the closely related autumnberries (Hou and others 2004) or in other common fruits (Hubbard and others 2004). The tartness caused by these compounds has been noted (Remlinger and St.-Pierre 1995) and most traditional users of these fruits wait until the fruit have experienced a heavy frost before picking them, as the frost appears to reduce the astringent taste of ripening fruit.

Water-soluble (predominantly phenolic compounds) antioxidant capacity was determined spectrophotometrically using the FRAP assay. This assay was utilized as it is a commonly available procedure and there are a large number of published reports of fruits to which the results can be compared (Johansson and others 2002). The antioxidant values measured for

these water-soluble compounds showed that the fruit of buffaloberry contain levels of antioxidants greater than the 90th percentile of other berries and berry products as measured by FRAP (Johansson and others 2002) and compare favorably to raspberries, strawberries, elderberries, and other fruits (Hubbard and others 2004; Ozgen and others 2006; Kader 2008).

Phenolic compounds assuredly act as antioxidants in the human diet (Seeram 2008a,b; Tsao 2010), and may reduce chronic inflammation (that is, prolonged leucocyte activity, increased mediator levels) leading to cellular damage, plaque formation, fibrosis, angiogenesis, and/or damaged cell survival (lack of apoptosis) associated with common degenerative immune-response-based diseases (Seeram and others 2001; Johansson and others 2002; Wang and Mazza 2002; Hubbard and others 2004). Moreover, these effects likely result from the interaction of several compounds or compound classes rather than a single antioxidant (Liu, 2003, 2004; Seeram 2008b), leading to the possibility of additive or synergistic benefits of buffaloberry fruit lycopene, M6AL, and phenolic compounds for human health.

Fruit quality

The palatability of fruits and wines made from them depend upon their acidity and SS (for example, sugars, organic acids, and so on) content (Kader 2008). A low pH with titratable acidity of 0.6% to 0.9% and a Brix of >8 are generally preferred by fresh fruit consumers and wine makers (Gallander 1987; Kader 2008). Fruit quality among the 7 buffaloberry selections was evaluated by measurement of SS and titratable acids (Table 2). The data show that buffaloberry fruits contain significant levels of SS and that their content is high enough to make them desirable as fresh fruit and for wine production. Buffaloberry is very high in sugars (⁰Brix 21%). Dried buffaloberries are very hygroscopic and remain soft, having the consistency of raisins. Dry matter in these fruits makes up $28.1 \pm 4.4\%$ of the fresh weight.

The acidity of buffaloberry (2.2%) as well as its high phenolic content (Table 2) counters the high sugar content and has made it a favorite fruit for the nascent wine industry in South Dakota. The impact of frost and the effects of postharvest storage on fruit quality remain unclear and require further study to demonstrate the full potential of this species.

Conclusions

Buffaloberries, which flourish on some of the harshest sites found on the American Indian Tribal Reservations, produce fruits that contain significant amounts of lycopene and MA6L. Additionally, they are a good source of phenolic antioxidants. The sugar and acidity of the fruit make it very desirable as a fresh or dried fruit and of much interest to the regions nascent wine industry. The presence of lycopenoates may also provide a marketable new food colorant. Commercial production of these fruits is currently very limited, but appears to have potential in a region in need of economic development.

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Practical Application

Buffaloberry, which grows on marginal lands on Indian Reservations in the Dakotas, produces fruits that contain principally lycopene and methyl apo-6'-lycopenoate (an acidic derivative) that may provide health benefits and marketable produce for consumption and sale. The fruit are a traditional food of the indigenous peoples of the region and have found favor with several commercial wine producers. The acidic lycopene derivative may become a valuable natural food colorant.

Figure 1.
Shepherdia argentea leaves and fruit.

Figure 2.

HPLC-MS chromatograms of main carotenoids in buffaloberry extract. (A) HPLC-UV-Vis trace at 470 nm, (B) HPLC-MS chromatogram as sum of radical anion signals for methyl-apo-6'-lycopenoate (m/z 472.3) and lycopene (m/z 536.4), which together account for the UV-vis absorption in A, (C) lycopene HPLC-MS trace at m/z 536.4 demonstrating presence of (Z)-lycopene isomers while the last peak is (all-E)-lycopene, and (D) methyl-apo-6'-lycopenoate HPLC-MS trace at m/z 472.3 with minor presumed (Z)-isomers eluting prior to main (all-E)-peak at 20.22 min.

Figure 3.

Me-apo-6'-lycopenoate after saponifying with methanolic KOH. Peak eluting with m/z of 458 was detected which matches loss of a methyl group. CID of this peak afforded a daughter ion of 413 m/z which agrees with loss of HCO₂.

Figure 4. One-dimensional ^1H spectra collected on a 400 MHz NMR. (A) Upfield region: 0 to 4 ppm, (B) downfield region: 4 to 8 ppm.

Figure 5.
Proposed structure of Me-apo-6'lycopenoate.

Table 1

S. argentea fruit carotenoid composition calculated using HPLC peak areas.

Buffaloberry selections	Lycopene content g/kg FW	% Lycopene	Methyl-6'-lycopenoate g/kg FW	% Methyl-6'-lycopenoate
Bismarck, N.Dak.	0.213 ± 0.009	40.2 ± 2.9	0.317 ± 0.015	59.8 ± 2.9
Fort Berthold, N.Dak.	0.299 ± 0.009	42.0 ± 1.0	0.413 ± 0.007	58.0 ± 1.0
Garrison, N.Dak.	0.182 ± 0.003	39.5 ± 1.2	0.279 ± 0.006	60.5 ± 1.2
Newtown, N.Dak.	0.360 ± 0.015	49.3 ± 2.1	0.37 ± 0.015	50.7 ± 2.1
Sanish Bay, N.Dak.	0.325 ± 0.003	44.0 ± 1.7	0.414 ± 0.013	56.0 ± 1.7
Cascade, S.Dak.	0.242 ± 0.004	47.9 ± 0.6	0.263 ± 0.003	52.1 ± 0.6
Sisseton, S.Dak.	0.245 ± 0.009	54.0 ± 0.4	0.209 ± 0.002	46.0 ± 0.4
Mean	0.267 ± 0.023	45.3 ± 5.6	0.322 ± 0.033	54.7 ± 5.6

Table 2 Phenolic content, antioxidant capacity, percent soluble solids, and titratable acidity of *S. argentea* fruit.

Buffaloberry selections	Total phenolics g GAE/kg FW	FRAP mmol TE/kg FW	Soluble solids (%)	Titratable acidity (%)
Bismarck, N.Dak.	7.84 ± 0.33	31.6 ± 5.4	23.4 ± 0.0	2.18 ± 0.01
Fort Berthold, N.Dak.	10.17 ± 0.11	57.1 ± 1.7	20.9 ± 0.1	2.99 ± 0.03
Garrison, N.Dak.	6.75 ± 0.42	24.0 ± 1.5	24.7 ± 0.0	1.35 ± 0.06
New Town, N.Dak.	10.82 ± 0.49	62.7 ± 3.5	24.0 ± 0.0	3.40 ± 0.07
Sanish Bay, N.Dak.	8.61 ± 0.84	53.9 ± 2.1	20.2 ± 0.1	2.26 ± 0.04
Cascade Falls, S.Dak.	11.76 ± 0.55	72.5 ± 7.5	16.4 ± 0.1	1.41 ± 0.03
Sisseton, S.Dak.	7.49 ± 0.72	41.8 ± 2.2	22.0 ± 0.0	1.73 ± 0.05
Mean	9.06 ± 0.71	49.0 ± 6.6	21.1 ± 1.1	2.19 ± 0.29