Effect of technological practices on individual betalains and antioxidant activity of Columbian betalain-rich raw materials

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RUNNING TITLE

Technology on betalain and biological activity

KEYWORDS

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SUMMARY

The effect of different technological practices on ulluco (*ullucus tuberosus*) and *Opuntia dillenii* has been scrutinized. Their stability at different pHs and temperatures of storage or processing over time was monitored. Our attention was focused on the determination of individual betalains and antioxidant activity, not previously conducted in conjunction in these raw materials. On the basis of the results, it could be asserted that the *ullucus tuberosus* and *Opuntia dillenii* were more suitable for being added to low-acidic foods (pH 5 and 6), in the light of the higher values of betalains and antioxidant capacity (1.3-fold higher compared to pH 4). With regard to the temperature, cold storage conditions (4 ºC) were optimal to increase or maintain as possible the initial content of betalains and antioxidant capacity. After cooking (80 ºC), the identified betalains completely disappeared, but the low-acidic conditions (pH 6) favoured the greater antioxidant activity when that temperature was underwent.

INTRODUCTION

Nowadays, consumers are becoming more conscious about the importance of the consumption of natural foods, because of their many beneficial properties (Benavente-García *et al*., 1997). There are several species rich in pigments (anthocyanins, chlorophylls and carotenoids) responsible for valuable healthy properties, but there is a group of phenolic coloured compounds (Cai *et al*,. 2003, Stintzing *et al*., 2005) still poorly investigated, the so-called betalains.

Betalains are water-soluble compounds presented in a restricted number of families of the plant order Caryophyllales and of the genus *Amarita* of the Basidiomycetes (Waterman 2007). Betalamic acid is the common moiety structure, and the typology of betalains stems from the nature of the additional residues: red/purple betacyanins (condensation of betalamic acid and *cyclo*-Dopa, considering hydroxycinnamic acid

derivatives or sugars as residue) and yellow betaxanthins (imino condensation products between betalamic acid and amines or amino acids residues) (Herbach *et al*., 2006) (Figure 1).

Betalains are widely distributed in a great variety of products, being red beet (*Beta vulgaris* L.) the most studied source of betalains par excellence (De Azeredo *et al*., 2009, Nemzer *et al*., 2011). However, betalains have been also analysed in other sources, such as fruits of cactus pear (*Opuntia* sp.) or yellow beet (Stintzing *et al*., 2002a, Stintzing *et al*., 2002b, Castellanos-Santiago and Yahia 2008, Cejudo-Bastante *et al*., 2014a), flowers (Kugler *et al*., 2007) and vegetables (Kugler *et al*., 2004).

From a biological point of view, betalain-rich foods (red beet, *amaranthus*, cactus pear, among others) have an important free radical-scavenging activity (Escribano 1998; Kanner *et al*., 2001; Butera *et al*., 2002; Cai *et al*., 2003; Dantas *et al*., 2015). Concretely, red beet and red pitaya have been established as two of the most powerful vegetables with respect to their antioxidant capacity (Vinson *et al*., 1998, Vaillant *et al*., 2005), directly related with the presence and content of betacyanins. However, up to the present, very scarce studies about antioxidant activity of other betalain-rich products have been reported. In that sense, Campos *et al*. (2006) measured the antioxidant capacity and total betaxanthins of several Peruvian ulluco tubers and Ghazi *et al*. (2015) and Kumar *et al*. (2014) obtained the antioxidant activity of *Opuntia dillenii* extracts from Marocco and India, respectively. However, any scientific study about the relationship of individual betalains and antioxidant activity on these raw materials.

Therefore, this work was performed on Colombian ulluco (*ullucus tuberosus*) and cactus pear (*Opuntia dillenii*) and the interest was focused on the individual betalain characterization and antioxidant activity, not previously conducted in conjunction in those raw materials. Furthermore, not only a betalain characterization is needed, but

also even to spotlight the behaviour in terms of betalains and antioxidant activity when these raw materials underwent different commercial technological practices and are added as natural colorant to foodstuffs.

MATERIAL AND METHODS

Samples

Magenta ulluco samples, commonly known as Ulluco "Chincheño", and red prickly pear samples of *Opuntia dillenii* were collected in the village of Catambuco and Remolinos, respectively, located in Nariño (Colombia). Both areas are located at approximately 8 km south of the city of San Juan de Pasto (Nariño, Colombia) and at 2820 m above sea level, with an average temperature at around 12 ºC. Ulluco is a tuber with a white pulp and a thin surface portion of magenta skin. *Opuntia dillenii* has a red-purple skin with cladode spines, and a red-purple pulp with several prickles.

Samples were carefully washed. Subsequently, *ullucus tuberosus* were manually peeled to separate the white pulp from the magenta peel, and spines and prickles of *Opuntia dillenii* were manually removed. The peelings of ulluco and the peel and pulp of *Opuntia dillenii* were cut into small pieces (1 cm^2) and 1 L of methanol of methanol:water (60:40) was used for extraction for 24 h at 10 ºC (maceration), according to the method proposed by Cejudo-Bastante *et al*. (2014b). After filtration, the organic solvent was evaporated at 35 ºC using a rotary evaporator (Heidolph, Schwabach, Germany) and the re-dissolved with distilled water was lyophilized (Labconco, MO, USA) and stored at 4 ºC until their analysis.

Preparation of extracts

Lyophilized samples (3 g) were added to 9.5 mL of a mixture of methanol:water (50:50), containing 50 mM of sodium ascorbate in order to avoid possible oxidations. Subsequently, samples were centrifugated at 12000 x *g* at 10 ºC for 5 min, according the

method described by Cejudo-Bastante *et al.* (2014b). Supernatants were recovered and the procedure was developed once more with the extraction solution and finally with 100 % methanol in order to a complete discoloration of the plant tissue. Later, the extract obtained was concentrated in vacuum (30 ºC) and re-suspended until 15 mL with purified water. All experiences were carried out in duplicate.

Technological treatments of the extracts

Extracts were adjusted to different acidities (pH 4, 5 and 6) using hydrochloride acid 0.1 and 1 M. Subsequently, each pH-sample were submitted to three different temperatures: 4 ºC, and 20º and 80 ºC, putting samples into a refrigerator and an oven JP Selecta, S.A. (Barcelona, Spain), respectively. Samples were analysed at the beginning of the treatment (t_0) and after a period of storage (t_f), which was 12 days when samples were kept at 4 ºC and 20 ºC, and 1 day when they underwent 80 ºC.

HPLC-DAD analysis

The Agilent 1200 chromatographic system equipped with a quaternary pump, an UV-vis diode-array detector, an automatic injector, and ChemStation software (Palo Alto, CA, USA) were used to the HPLC separation, identification and quantification of betalains. Prior direct injection, the samples were filtered through a 0.45 µm nylon filter (E0034, Análisis Vínicos, Spain). All analyses were made in duplicate.

Betalains were separated in a Zorbax C18 column (250 x 4.6 mm, 5 µm particle size) maintained at 25 ºC at a flow rate of 1 mL/min. The eluents were 1% formic acid in water (v/v, eluent A) and a mixture 80:20 of acetonitrile:water (eluent B). The injection of the volume for all extracts was 20 µL. The elution profile of betalains was different depending on the sample: ulluco, 0 min, 5% B; 10 min, 10% B; 20 min 10% B; 25 min 20% B; 30 min 30% B; 35 min 100% B; 37 min 100% B; *Opuntia dillenii*, 0 min, 5% B; 10 min, 12% B; 20 min 14% B; 25 min 20% B; 30 min 30% B; 35 min 100% B; 37

126 min 100% B. In order to re-establish the initial conditions, a linear gradient from 100%
127 B to 5% B was used during 2 min, and maintained for 2 min more. For all samples,
128 UV–Vis spectra were recorded from 200 to 800 nm with a bandwidth of 1.0 nm.
129 Betacyanins and betaxanthins were monitored at 535 and 482 nm, respectively. The
130 identification of each chromatographic peak were tentative assigned by their visible
131 spectral characteristics in comparison with retention times. Semi-quantification was
132 done on the basis of the mean areas of individual betalains.

133 **Antioxidant activity**

134 The antioxidant capacity was measured *in vitro* on the basis of the ability to scavenge
135 the $ABTS^{\bullet+}$ radical (Jara-Palacios *et al.*, 2014). The $ABTS^{\bullet+}$ radical was produced by
136 the oxidation of 7 mM ABTS with potassium persulfate (2.45 mM) in water. After
137 storage under dark conditions and room temperature for 16 h before its use, $ABTS^{\bullet+}$
138 solution was diluted with phosphate-buffered saline (PBS) at pH 7.4, until reaching an
139 absorbance of 0.70 ±0.02 at 734 nm. Then, 50 µL of each sample was mixed with 2 mL
140 of the $ABTS^{\bullet+}$ diluted solution and vortexed for 10 s, and the absorbance was measured
141 at 734 nm after 3 min of reaction at 30 °C. The results were achieved with the help of a
142 calibration curve made with Trolox (30-1000 µM). Three replicates were performed for
143 each sample, and the results were expressed as Trolox equivalent antioxidant activity
144 (TEAC; µmol of Trolox equivalent (TE) with the same antioxidant capacity of 1 L of
145 sample).

146 **Statistical analysis**

147 The complete set of analytical data was submitted to analysis of variance (ANOVA),
148 Principal Component Analysis (PCA) and cluster analysis (CA) using the statistical
149 computer packages Statistica version 8.0 software (Statistica 2007).

150 **RESULTS AND DISCUSSION**

Betalain profile

In this research, several betalain compounds were identified and semi-quantified in both raw materials: betanin and isobetanin (R_1, H; R_2, β-glucoside), as betacyanins, and indicaxanthin (R_3, proline), as betaxanthins (Fig. 1). The mean areas are exposed in Table 1. Moreover, Table S1 show the ANOVA analysis on the basis of the three factors studied: pH, temperature and time.

At the beginning of the treatment (t_0), the content of betanin was significantly (ANOVA; $p < 0.05$) higher than of the isobetanin in ulluco samples for all pHs (Table 1), whereas the ratio betanin/isobetanin was nearly 1 in *Opuntia dillenii* samples. Similar results were observed by Svenson *et al*. (2008) in red ulluco samples, but any reference about individual betalains of *Opuntia dillenii* has been found in bibliography.

In order to statistically highlight the differences on the betalain content among *ullucus tuberosus* and *Opuntia dillenii*, the complete set of data were submitted to ANOVA, considering the effect of three factors: temperature, pH and time (Table S1). Cooked samples (80 ºC) are not included in the statistical analysis due to the obvious significant differences in comparison with the rest of temperatures. Results revealed that significant ($p < 0.05$) differences according "temperature" and "time" factors were observed in both raw materials, whereas "pH" did not displayed remarkable differences.

In an attempt to deepen on the effect of the three factors on individual betalains, a more detailed statistical analysis have been developed. Thus, at the beginning of the treatment (t_0), the significant (ANOVA; $p < 0.05$) highest content of betalains was found at low pH-values (pH 4) in *Opuntia dillenii*, although without significant differences among pHs in *ullucus tuberosus* (Table 1). The storage made the content of betalains significantly (ANOVA; $p < 0.05$) decrease in both ulluco and *Opuntia dillenii* samples, although the behaviour over time was greatly influenced by the temperature.

176 Thus, under refrigeration conditions, the highest values of betacyanins of ulluco
177 correspond to pH 5 (Table 1), with a percentage of diminution of betanin around 10 %
178 and negligible of isobetanin. The rest of pHs (pH 4 and 6) suffered an important
179 decrease (around 40 and 25%, respectively). In the case of *Opuntia dillenii*, the highest
180 values of betacyanins and betaxanthins (and a consequence the best conditions of
181 acidity at 4 ºC) corresponded to low-acidic samples (pH 6), with the lowest percentage
182 of diminution over time. It is also highlighted that the percentage of diminution of
183 indicaxanthin at cold storage was lessened as pH increased.
184 The panorama changed when samples were kept under room temperature conditions (20
185 ºC). The content of betalains decreased as pH increased in ulluco samples, and the
186 highest values of betalains (and, subsequently, the best conditions of conservation) were
187 found when they were adjusted to pH 4. Contrary results exhibited *Opuntia dillenii*
188 samples, which the highest values of betalain content correspond to samples adjusted to
189 pH 6. Same value of pH were ascertained at maximum HPLC area in several species of
190 *Opuntia* (Castellar *et al.*, 2003).
191 Besides, it is highlighted that the behaviour of the samples submitted to 80 ºC greatly
192 differed from the rest of temperatures, and the individual betalains completely
193 disappeared in both kind of samples, maybe for the formation of derivative compounds
194 as a consequence of the thermal process (Stintzing and Carle 2007).
195 In brief, if *Opuntia dillenii* was added to foods with pH value over 4 (for example,
196 vegetables), higher quantities might be added to foodstuffs to counteract its lower
197 betalain content. These results were in concordance with those obtained by Cejudo-
198 Bastante *et al*. (2015) in a colorimetric study carried out on *Opuntia dillenii*,
199 demonstrating the lower chromaticity provided at that pH value. On that matter, ulluco
200 could be suitable for being added to acid or basic foodstuffs (vegetables or meats)

because it provides similar betalain contents. In order to preserve as possible the content of betalains in both raw materials, the best temperature of conservation were under refrigeration conditions. However, the choice of the best acidity conditions was dependent of the raw material, pH 5 for ulluco and pH 6 for *Opuntia dillenii*. These results complement those obtained by Cejudo-Bastante *et al*. (2014b) who affirmed that *ullucus tuberosus* could be more suitable for being added to low-acidity foodstuffs (meat industry) because it provides higher values of chroma, but assuming that possible yellow tonalities could confer over time.

Antioxidant activity

Table 1 show the values of antioxidant capacity of both raw materials (*Ullucus tuberosus* and *Opuntia dillenii),* and Table S1 the results of the ANOVA analysis on the basis of "pH", "temperature" and "time" factors.

As it can be seen, *ullucus tuberosus* manifested a significantly higher capacity in comparison with *Opuntia dillenii*, and regardless acidity conditions. Even so, both *Opuntia dillenii* and ulluco extracts were important sources of antioxidant activity, as other authors affirmed (Kumar *et al*., 2014; Ghazi *et al*., 2015).

When ANOVA was applied to understand if significant differences occurred in antioxidant capacity according temperature, pH and time for all set of data, it is highlighted that only pH was significant (ANOVA; $p < 0.05$) in both samples (Table S1), with remarkably higher values of antioxidant activity in low-acidic samples (Table 1). Escribano et al. (1998) also demonstrated that antiradical activity was srongly influenced by the pH of *Beta vulgaris* roots. Also "time" was a significant (ANOVA; $p < 0.05$) factor in *Opuntia dillenii*, mainly due the behaviour of the low-acidic samples (pH 6).

225 At the beginning of the treatment (t_0), although the effect of pH on antioxidant capacity
226 was similar for both ulluco and *Opuntia dillenii* (i.e., significant (ANOVA; $p < 0.05$)
227 higher values in samples adjusted to pH 6), the behaviour after storage greatly depended
228 on each raw material (Table 1).
229 In general, antioxidant capacity experimented a significant (ANOVA; $p < 0.05$)
230 attenuation after storage. Although highly-acidic (pH 4) extracts of *ullucus tuberosus*
231 and *Opuntia dillenii* (pH 4) showed scarce variations of antioxidant activity regardless
232 of temperature, they were the samples with the lowest values of antioxidant capacity.
233 That fact was in concordance with the behaviour of the content of individual betalains,
234 which showed a significantly (ANOVA; $p < 0.05$) lower amount under those acidity
235 conditions. With regard to the intermediate- (pH 5) and low-acidic (pH 6) extracts,
236 temperature played an important role in the evolution of the antioxidant capacity. Thus,
237 when pH 5-extracts were submitted to cold conditions (4 ºC), a remarkable decreased of
238 antioxidant potential was observed, above all in ulluco extracts (around 40 %). That fact
239 demonstrated that it could be more appropriate to conserve these samples at room
240 temperature (20 ºC) or be cooked (80 ºC).
241 In ulluco, the values of antioxidant activity significantly decreased when extracts was
242 submitted to room temperature and cooked, but significantly ($p < 0.05$) increased when
243 cold storage was experimented. However, in *Opuntia dillenii* samples, 4 ºC and 80 ºC
244 made antioxidant capacity increase, keeping constant when room temperature was
245 underwent. It is remarkable that any detailed study concerning antioxidant activity of
246 these raw materials have been previously performed up.
247 In any case, if *ullucus tuberosus* and *Opuntia dillenii* were added to high-acidic
248 foodstuffs (pH 4), it would provide significant lower antioxidant capacity, regardless of
249 the temperature of storage. These raw materials could be also used in foods with

intermediate acidity (pH 5), although cold conditions made antioxidant activity decrease. Adding *ullucus tuberosus* to pH 5-foodstuffs and *Opuntia dillenii* to pH-6 foods could better withstand the cooking temperatures (80 ºC) because the highest values of antioxidant capacity were reached. That fact could be due to the formation of other compounds, depending on each raw material, that could provide biological function (Stintzing and Carle 2007). In any case, the best option, from an antioxidant capacity point of view, corresponded to the addition of both *Ullucus tuberosus* and *Opuntia dillenii* to low-acidic foodstuff, keeping them under cold storage.

Multivariate analysis

Principal Component Analysis (PCA), carried out on individual betalains and antioxidant capacity, was applied with the aim of establishing which of the variables were better for discriminating among samples, and carrying out an exploratory tool to uncover unknown trends in the data. Samples at the three different pHs and temperatures at the beginning and after 12 days of storage were considered. When the set of data was subjected to PCA, two main significant factors PCs arose according to Kraiser's criterion (eigenvalues > 1). Figure 2a and b show the score plot of the first two PCs for each fruit, which explained 97.33% and 99.08% of the total variance of *Opuntia dillenii* and *ullucus tuberosus*, respectively. As can be seen in Figure 2, PC 1 organized samples according to the temperature in three different groups. *Opuntia dillenii* samples were classified at the beginning of the treatment (t_0), samples stored at 4 ºC, and those kept at 20 and 80 ºC (Fig. 2a), whereas ulluco (Fig. 2b) were situated together those at the beginning of the treatment and stored at 4 ºC, coming close to those kept at 20 ºC and finally 80 ºC. It could be seen that, when temperature was increased (20 and 80 ºC), ulluco samples adjusted to pH 5 tended to separate from the rest (Fig 2b). However, scarce grouping according to pH were observed for *Opuntia dillenii*. In any case, the

content of all individual betalains identified (betanin, isobetanin and indicaxanthin) decreased with temperature (PC 1). PC2 separated *Opuntia dillenii* samples adjusted to pH 6 and stored at 4 ºC and 80 ºC (Fig. 2a), and the high-acidic *ullucus tuberosus* samples (pH 4) (Fig. 2b), with the highest values of antioxidant activity.

With the aim of highlighting the possible similarity among samples on the basis of betalains and antioxidant activity, the data matrix was subjected to a hierarchical agglomerative cluster analysis of cases. The squared Euclidean distance as the metric and the Ward method as the amalgamation rule were taken into account. The dendograms obtain for each raw material are shown in Fig. S1a and b. As can be seen, the aggrupation of the samples were similar for each raw material. The original samples (t_0) and those submitted to cold storage (4 ºC) were closely grouped according to betalains and antioxidant capacity, and differentiated from those kept at room temperature and after cooking. In the light of the results, it could be asserted that the grouping obtained responds to the temperature more than to the pH conditions.

CONCLUSIONS

Ullucus tuberosus and *Opuntia dillenii* are two sources of betalains with an important antioxidant activity. They could be added to highly-acidic foods, but higher quantities of extract must be added to counteract their lower values of betalains and antioxidant activity. For both raw materials, their optimal conditions of employment (in order to yield the highest values of betalains and antioxidant activity) would be in low-acidic foodstuffs (pH 5 and 6), as long as the conservation was under cold storage conditions. Although further researchers are needed in relation to natural colorants, this study could be a great step forward in the field of food, cosmetic and drug industries, among others.

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LEGENDS TO FIGURES

Figure 1. Chemical structures of betacyanins and betaxanthins.

Figure 2. Plot of the *Opuntia dillenii* (a) and *Ullucus tuberosus* (b) in the space defined by the first two principal components with regard to betalains and antioxidant capacity.

Figure S1. Cluster analysis. Dendrogram obtained after hierarchical agglomerative cluster analysis performed on betalain compounds and antioxidant capacity of all samples studied. OP, *Opuntia dillenii*; UL, *Ullucus tuberosus*; d, days.