Liquid chromatography–mass spectrometry identification of anthocyanins of isla oca (*Oxalis tuberosa*, Mol.) tubers

Cristina Alcalde-Eon[a], Gloria Saavedra[b], Sonia de Pascual-Teresa[c], Julián C. Rivas-Gonzalo[a],[*]

[a] *Unidad de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain*
[b] *Centro de tecnología agroindustrial, Facultad de Ciencias y Tecnología, Universidad Mayor de San Simón, Cochabamba, Bolivia*
[c] *Instituto del frío. CSIC, Ciudad Universitaria, 28040 Madrid, Spain*

Abstract

High-performance liquid chromatography (HPLC)–diode array detection (DAD)–mass spectrometry (MS) techniques have been successfully employed in the identification of the anthocyanins of the coloured tubers of isla oca (*Oxalis tuberosa*), the second most cultivated tuber in the Andean region. Tubers underwent a pre-treatment step in order to inhibit enzymatic reactions and to obtain a stable powder or "concentrate". This concentrate was dissolved, purified and then analysed. Eight different compounds were found. The major peaks were malvidin glucosides (malvidin 3-*O*-glucoside and 3,5-*O*-diglucoside). The rest of the peaks were 3,5-*O*-diglucosides of petunidin and peonidin, and 3-*O*-glucosides of delphinidin, petunidin and peonidin. Only malvidin 3-*O*-acetylglucoside-5-*O*-glucoside was found as an acylated anthocyanin.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Oxalis tuberosa; Isla oca tubers; Anthocyanins

1. Introduction

Colour is usually considered as a psychological factor in the acceptability of a food product and it is a criterion employed in the election by the consumers of one food product instead of another. In the food industry, the raw materials undergo different technological treatments which can provoke modifications in their natural colorants. In order to obtain the most adequate colour for each foodstuff, some colouring additives are employed in their processing.

Natural colorants, replaced in use by synthetic ones in the last two centuries, are, nowadays, recovering their importance, due to the increasing consumer concern about the safety of the latter ones. The major drawback of these natural pigments is their lack of stability. Thus, the colour of the foodstuffs to which they have been added can change or even fade over time [1]. This is the reason why these natural colorants are being widely studied. One interesting group among them are the anthocyanins. Anthocyanins are present in most of the flowering plants, not only in flowers and fruits, but also in stems, leaves, roots and storage organs, providing them with different colours, mostly red and blue, but also orange, purple and violet [2]. Furthermore, some studies have revealed their antioxidant activity [3–6], suggesting their beneficial effects on human health (cardiovascular-protective effects, reduction of the risk of cancer and heart disease, antiviral and anti-inflammatory effects, etc.) [7–9].

Anthocyanins are usually present in raw materials as a mixture of compounds. Thus, a separation step is needed prior to their identification. The technique most employed in their separation and identification is reversed-phase high-performance liquid chromatography (HPLC) with diode array detection (DAD), which has proved to be effective in the analysis of these pigments. The diode array detection allows the recording of the ultraviolet–visible (UV–vis) spectra from all the compounds separated in the chromatographic column, which can give useful information about the nature of the aglycone, glycosidation pattern and possibility of acylation with cinnamic acids [10]. However, there are a huge variety of different anthocyanins in nature and some of them present similar retention times and spectral characteristics,

---

[*] Corresponding author. Tel.: +34 923 29 45 37; fax: +34 923 29 45 15.
E-mail address: jcrivas@usal.es (J.C. Rivas-Gonzalo).

which makes their identification by only HPLC-DAD difficult. Therefore, other techniques are needed to carry out a more accurate identity assignment. Among these techniques, mass spectrometry and MS^n analysis, coupled to the HPLC-DAD technique, are being widely used in the identification of anthocyanins in different raw materials, such as fruits, flowers, leaves, tubers... [11–18]. The mass spectrum corresponding to each peak indicates the m/z ratio of the molecular ion and the MS^n analysis shows its fragmentation pattern from which information about the aglycone and the substituents can be obtained.

In the Andean region, there are several native crops that produce coloured tubers that have traditionally been used in human and animal diets. These crops grow easily and with high yields in poor soils in the severe climatic conditions of this region. The anthocyanin composition of the coloured tubers of one of these native crops (*Solanum stenotomum*) has already been determined by means of HPLC–DAD–MS [18]. The second most widely cultivated native tuber in the Andes is *Oxalis tuberosa*. Outside the Andean region, *O. tuberosa* is cultivated commercially in Mexico and New Zealand [19,20]. *O. tuberosa* belongs to the Oxiladaceae family. It is an annual herbaceous plant, with trifoliate or clover-like leaves. Its most common name is oca, but depending on the region it receives different names: quiba, ibia, apilla, miquichi.... The oca is cultivated as an important and good source of energy, with nutritional value similar to that of the potatoes. In the Andean region there are 27 cultivars of the oca and they differ one from another, above all, in the shape and colour of the tubers [21]. The tubers are cylindrical with buds and wrinkles on their surface and can be white, yellow, orange, pink and dark violet or almost black, as occurs in the variety isla oca, the variety studied in this work. The anthocyanin composition of these tubers has not been described yet. Only the anthocyanin composition of the purple leaves of a plant belonging to the same genus, *Oxalis triangularis*, has been reported [16]. The major anthocyanin found in this plant was malvidin-3-rutinoside-5-glucoside and the others were acylated derivatives containing one and two molecules of malonic acid.

The aim of this work was to determine the anthocyanin profile of isla oca tubers by means of HPLC–DAD–MS. The fact that these tubers have traditionally been used in human and animal diets and that they are easily grown in the Andean region, suggests that the tubers of *O. tuberosa* can be a potential source of anthocyanins to be employed as food colorants. The industrial use of these tubers should favour the economical development of the Andean region.

2. Experimental

2.1. Tuber pre-treatment

Tubers of isla oca were obtained from the region of Candelaria (Department of Cochabamba, Bolivia). In the country of origin, tubers underwent a pre-treatment in order to avoid the spoilage of the sample during the travel to Spain. Different pre-treatments were assayed and the one which produced the lowest anthocyanin degradation was finally employed. Thus, first of all, the tubers were immersed in boiling water for 15 minutes in order to inhibit enzymatic reactions. The coloured zones from the peel and the flesh of the tubers were removed and ground. Finally, this powder was freeze-dried and the concentrate of the tubers was sent to Spain for analysis.

2.2. Sample preparation

Forty milligrams of the concentrate were dissolved in ultra-pure water acidified with HCl to pH 0.5 and this solution was purified by solid phase extraction (SPE) in C18 cartridges (Sep pak®, Waters). The cartridges were activated by passing an MeOH solution acidified with 12 M HCl (0.01%), and equilibrated with water. The concentrate solution was carefully put into the cartridge, and then washed with water to remove sugars and substances which could interfere with the analysis. The elution of the anthocyanins was performed by the addition of acidified MeOH. The eluate was concentrated under vacuum and re-dissolved in water to a final volume of 5 mL.

2.3. HPLC–DAD analysis

HPLC–DAD analysis was performed in a Hewlett-Packward 1100 series liquid chromatograph (Agilent technologies, Waldbronn, Germany) and detection was carried out using a photodiode array detector. An AQUA C-18 reversed-phase, 5 μm, 125 Å, 150x4.6 mm column (Phenomenex®, Torrance, California, USA) thermostatted at 35 °C was used.

The HPLC conditions employed in this work had been previously optimised in our laboratory and used in the study of the *Solanum stenotomum* tubers [18]. The solvents used were: (A) an aqueous solution (0.1%) of trifluoracetic acid (TFA) and (B) 100% HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B for 5 min, from 10% to 15% B over 15 min, isocratic 15% B for 5 min, from 15% to 18% B over 5 min, and from 18% to 35% B over 20 min at a flow rate of 0.5 ml/min. Detection was carried out at 520 nm as the preferred wavelength. The spectra from 220 to 600 nm were recorded for all the peaks.

2.4. LC–MS analysis

LC–MS analyses were performed using a Finnigan LCQ MS detector (Thermoquest, San Jose, USA) equipped with an API source, using an ESI probe. The HPLC system was connected to the probe of the mass spectrometer via the UV cell outlet. Both the sheath and the auxiliary gas were a mixture of nitrogen and helium. The sheath gas flow was 1.2 L/min and the auxiliary gas flow 6 L/min. The capillary voltage was 4 V and the capillary temperature 195 °C. Spectra were recorded
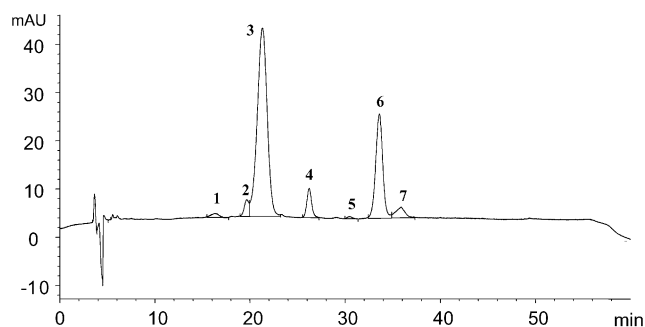
Fig. 1. Chromatogram recorded at 520 nm corresponding to the isla oca tuber concentrate.

in positive ion mode between m/z 120 and 1500. The mass spectrometer was programmed to do a series of three consecutive scans: a full mass, an MS^2 scan of the most abundant ion in the full mass, and an MS^3 of the most abundant ion in the MS^2. The normalised energy of collision was 45%.

3. Results and discussion

The anthocyanin composition of isla oca concentrate was determined by means of HPLC–DAD–MS techniques. The chromatogram of this sample, recorded at 520 nm is shown in Fig. 1. As can be seen, there are seven peaks in the chromatogram, but the MS, MS^2 and MS^3 analyses revealed that one of them, peak 2, corresponded to two different compounds (2a and 2b, respectively). The chromatographic and spectral features of all the compounds found in the isla oca concentrate are compiled in Table 1.

The major peak in the chromatogram is peak 3. Its UV–vis spectrum showed two absorption maxima, one at 275 nm and the other at 524 nm. The absence of a shoulder in the region of 440 nm was indicative of the substitution of the hydroxyl group in position 5 of the anthocyanin [22,10]. No significant absorbance within the range of 310–330 nm was observed, suggesting the absence of acylation of this compound with cinnamic acids [23,10]. This UV–vis spectrum, when compared to the spectra available in our in-house library, was identical to that of malvidin 3,5-*O*-diglucoside and the retention time of compound 3 coincided with that of this standard. The MS, MS^2 and MS^3 spectra and the frag-

mentation pattern allowed the confirmation of the identity of peak 3. The mass spectrum of the peak presented an intense signal at m/z 655, corresponding to the molecular ion. This molecular ion was fragmented in the MS^2 analysis, yielding signals at m/z 493 and 331. The first fragment ion was the more abundant and was generated by the loss of one glucose moiety (162 amu). The other corresponds to the anthocyanidin malvidin and was originated by the loss of 324 amu (two glucoses). The MS^3 spectra showed a signal at m/z 331, indicating and confirming the presence of two glucose moieties. The fragmentation pattern was useful to assign the positions of substitution. When an anthocyanidin is substituted with two sugars in positions 3 and 5, respectively, the fragmentation of the molecular ion leads to the formation of three fragment ions, one corresponding to the aglycone, another corresponding to the 3-*O*-glycoside and the last corresponding to the 5-*O*-glycoside. If both sugars were the same, only two signals would appear in the MS^2 spectrum: one corresponding to the aglycone and the other corresponding to the two possible monoglycosides [24,25]. Taking into account the analytical data and this fact, peak 3 was identified as malvidin 3,5-*O*-diglucoside.

The second peak, in quantitative terms, was peak 6. Its UV–vis spectrum showed a shoulder in the region of 440 nm, which indicated that position 5 of the anthocyanidin was not substituted. Furthermore, this spectrum and the retention time of this peak were identical to those of malvidin 3-*O*-glucoside, suggesting the assignment of this identity to compound 6. The data obtained in the MS, MS^2 and MS^3 analyses helped us to confirm it. The molecular ion of this compound showed an m/z ratio of 493 and in the MS^2 analysis it was fragmented generating only one fragment ion (m/z 331). As seen previously, this ion corresponded to the anthocyanidin malvidin and was originated by the loss of one glucose moiety. In the MS^3 analysis, the ions originated from the fragmentation of the aglycone confirmed its identity. Thus, peak 6 was identified as malvidin 3-*O*-glucoside.

Peak 2, as previously mentioned, contained two different compounds, denominated respectively, 2a and 2b. Compound 2b showed a signal in the MS spectrum at m/z 625 and was fragmented in the MS^2 analysis in two ions, with signals in the MS^2 at m/z ratios 463 and 301. The latter corresponded to the anthocyanidin peonidin, and the former with the monohexoside of peonidin, which was fragmented, in the MS^3

Table 1
Chromatographic and spectral characteristics of the compounds found in isla oca tuber concentrate

Peak number	R_t (min)	Molecular ion: M^+ (m/z)	Fragment ions in MS^2 (m/z)[a]	Fragment ions in MS^3 (m/z)[a]	Absorbance maxima (nm)
1	16.4	641	479, 317	317	274, 523
2a	19.5	465	303	303	275, 523
2b	19.7	625	463, 301	301	278, 523
3	21.3	655	493, 331	331	275, 524
4	26.2	479	317	317, 302	277, 525
5	30.4	463	301	301	
6	33.6	493	331	331	278, 525
7	35.9	697	535, 493, 331	331	275, 524

[a] The fragment ions are shown in this table in order of their relative abundance: the first ion, in each case, is the most abundant.

analysis, originating again the ion corresponding to peonidin. Thus, this compound should contain peonidin and two hexoses linked to the aglycone in different positions. Taking into account the identity assigned to compounds 3 and 6, it can be assumed that both sugars are glucoses. Therefore, compound 2b was identified as follows: peonidin 3,5-*O*-diglucoside, which was consistent with its elution order [26].

Peak 5 showed a signal at m/z 463 in the MS spectrum, corresponding to the molecular ion, which was fragmented in the MS^2 analysis originating only one ion (m/z 301), corresponding to peonidin. The fragmentation pattern was identical to that of malvidin 3-*O*-glucoside (peak 6). Peak 5 was, therefore, identified as peonidin 3-*O*-glucoside.

Peak 1 presented a molecular ion with an m/z ratio of 641. As in peaks 2b and 3, this ion generated two fragment ions in the MS^2 analysis, corresponding to the monoglucoside and aglycone of petunidin (m/z 479 and 317, respectively). The aglycone was originated again from the fragment ion of m/z 479 in the MS^3 analysis. Peak 1 was identified as petunidin 3,5-*O*-diglucoside.

The third important peak, in quantitative terms, was peak 4. Its UV–vis spectrum showed a shoulder in the region of 440 nm and was identical to that of the monoglucoside of petunidin. The retention time and MS data agreed with this proposed identity. The molecular ion had an m/z ratio of 479 and the fragment ion generated in its fragmentation corresponded to the anthocyanidin petunidin. Thus, peak 4 was identified as: petunidin 3-*O*-glucoside.

The chromatographic and spectral data (signals at m/z 465 in the MS spectrum, and at m/z 303 in the MS^2 spectrum) of peak 2a allowed us to propose the following identity for it: delphinidin 3-*O*-glucoside. The presence of three hydroxyl groups in the B-ring of this anthocyanidin provokes the earlier elution of this compound in relation to the other monoglucosides present in this sample.

Finally, the last peak in the chromatogram was peak 7. The compound corresponding to this peak eluted after the monoglucoside of malvidin, the least polar of the monoglucosides of anthocyanins, which means that this compound should possess some kind of acylation in its structure. The UV–vis spectrum of the peak did not show an additional shoulder in the region of 309–330 nm, indicating that the acylating agent was not a cinnamic acid, but an aliphatic one. Furthermore, it was practically identical to that of peak 4, suggesting that peak 7 should be a derivative of malvidin 3,5-*O*-diglucoside (Fig. 2). This compound showed a signal at m/z 697 in the MS spectrum, corresponding to the molecular ion. The fragmentation of this ion yielded three ions at m/z ratios 535, 493 and 331, the first being the most abundant. They were originated, by the loss of 162, 204 and 366 amu, respectively. The presence of the ions at m/z 331 and 493 indicated that compound 7 was a malvidin derivative possessing at least one glucose moiety. The loss of 162 amu in the signal of the molecular ion to generate the ion of m/z 535 was indicative of the presence of another glucose moiety. In the
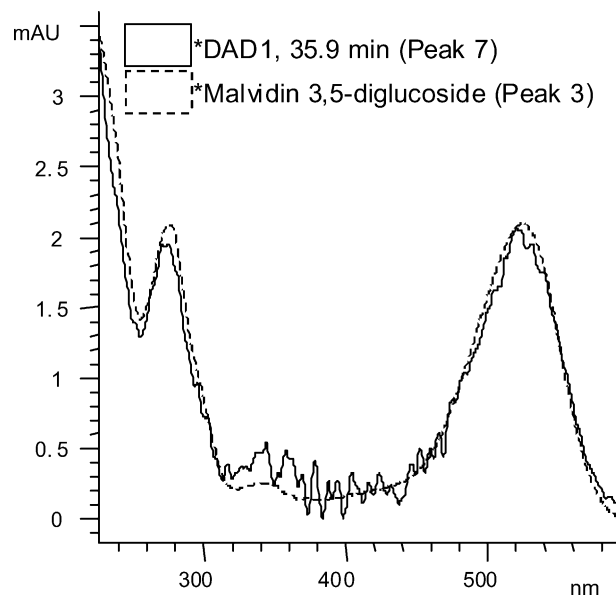

Fig. 2. UV–vis spectra corresponding to peak 7 (—) and peak 3 (···).

Table 2
Identity proposed for all the compounds found in the concentrate of isla oca tubers

Peak number	Proposed identity
1	Petunidin 3-*O*-glucoside-5-*O*-glucoside
2a	Delphinidin 3-*O*-glucoside
2b	Peonidin 3-*O*-glucoside-5-*O*-glucoside
3	Malvidin 3-*O*-glucoside-5-*O*-glucoside
4	Petunidin 3-*O*-glucoside
5	Peonidin 3-*O*-glucoside
6	Malvidin 3-*O*-glucoside
7	Malvidin 3-*O*-acetylglucoside-5-*O*-glucoside

MS^3 analysis, the ion of m/z 535 was fragmented, originating only one ion at m/z 331, by loss of 204 amu. This loss can be attributed to the simultaneous loss of one glucose moiety and one acetic acid moiety (162 + 42). The loss of the acetic acid moiety was not observed since the cleavage of the linkage between the sugar and the acid is not usually produced in the fragmentation conditions employed in this kind of analysis [25,27,28]. Thus, the identity proposed for this compound was: malvidin 3-*O*-acetylglucoside-5-*O*-glucoside.

The identity proposed for every compound found in the isla oca tuber concentrate is listed in Table 2.

4. Conclusions

This study has demonstrated, once again, the utility of HPLC–DAD–MS in the analysis of anthocyanins in plant materials, in this case in a concentrate made from tubers of *O. tuberosa*. This technique has allowed the identification of eight different anthocyanins: 3,5-*O*-diglucosides of petunidin, peonidin and malvidin, 3-*O*-glucosides of delphinidin, petunidin, peonidin and malvidin and the acetylated deriva-

tive of the 3,5-*O*-diglucoside of malvidin. All of them are commonly found in nature. To our knowledge, this is the first time that the anthocyanin composition of these tubers has been described. Since these tubers have been traditionally used in human and animal diets in the Andean region and, since they grow easily in the severe climatic conditions of this region, they could be used for the extraction of anthocyanins to be employed as food colorants.

Acknowledgements

References

[1] J.M. Jones, Food Safety, Eagan Press, St Paul, MN, 1992.
[2] J.B. Harborne, R.J. Grayer, in: J.B. Harborne (Ed.), The Flavonoids, Advances in Research since 1980, Chapman and Hall, London, 1988, p. 1.
[3] I. Tedesco, G.L. Russo, F. Nazzaro, M. Russo, R. Palumbo, J. Nutr. Biochem. 12 (2001) 505.
[4] M. Netzel, G. Strass, C. Kaul, I. Bitsch, H. Dietrich, R. Bitsch, Food Res. Int. 35 (2002) 213.
[5] P.J. Tsai, J. McIntosh, P. Pearce, B. Camden, B.R. Jordan, Food Res. Int. 35 (2002) 351.
[6] I. Serraino, L. Dugo, P. Dugo, L. Mondello, E. Mazzon, G. Dugo, A.P. Caputi, S. Cuzzocrea, Life Sci. 73 (2003) 1097.
[7] P. Bridle, C.F. Timberlake, Food Chem. 58 (1997) 103.
[8] F. Delgado-Vargas, A.R. Jiménez, O. Paredes-López, Crit. Rev. Food Sci. Nutr. 40 (2000) 173.
[9] F.C. Stintzing, R. Carle, Trends Food Sci Technol. 15 (2004) 19.
[10] V. Hong, R. Wrolstad, J. Agric. Food Chem. 38 (1990) 708.
[11] A. Ordaz-Galindo, P. Wesche-Ebelin, R.E. Wrolstad, L. Rodriguez-Saona, A. Argaiz-Jamet, Food Chem. 65 (1999) 201.
[12] R.L. Prior, S.A. Lazarus, G. Cao, H. Muccitelli, J.F. Hammerstone, J. Agric. Food Chem. 49 (2001) 1270.
[13] S. de Pascual-Teresa, C. Santos-Buelga, J.C. Rivas-Gonzalo, J. Sci. Food Agric. 82 (2002) 1003.
[14] W. Mullen, M.E.J. Lean, A. Crozier, J. Chromatogr. A 966 (2002) 63.
[15] F. Lopes-da-Silva, S. de Pascual-Teresa, J.C. Rivas-Gonzalo, C. Santos-Buelga, Eur. Food Res. Technol. 214 (2002) 248.
[16] E.A. Pazmiño-Durán, M.M. Giusti, R.E. Wrolstad, M.B.A. Glória, Food Chem. 75 (2001) 211.
[17] L.E. Rodriguez-Saona, M.M. Giusti, R.E. Wrolstad, J. Food Sci. 63 (1998) 458.
[18] C. Alcalde-Eon, G. Saavedra, S. de Pascual-Teresa, J.C. Rivas-Gonzalo, Food Chem. 86 (2004) 441.
[19] C.R. Sperling, S.R. King, in: J. Janick, J.E. Simons (Eds.), Advances in New Crops, Timber Press, Portland, p. 428.
[20] National Research Council, Lost Crops of the Incas: Little Known Plants of the Andes with Promise for Worldwide Cultivation, National Academic Press, Washington, DC, 1989.
[21] F. Terrazas, G. Valdivia, La Dinámica Espacial de la Conservación in situ: Manejo de la Diversidad Genética de Tubérculos Andinos en Sistemas de Mosaico, Candelaria, Cochabamba, Bolivia, 1999. Information from: http://www.condesan.org/infoandina/Foros/insitu99/terrazas.htm.
[22] P. Ribéreau-Gayon, Les Composés Phénoliques des Végétaux, Dunod, París, 1968.
[23] K.R. Markham, Techniques of Flavonoid Identification, Academic Press, New York, 1982.
[24] M.C. Oliveira, P. Esperança, A. Ferreira, Rapid Commun. Mass Spectrom. 15 (2001) 1525.
[25] M.M. Giusti, L.E. Rodríguez-Saona, D. Griffin, R.E. Wrolstad, J Agric. Food Chem. 47 (1999) 4657.
[26] A. Heier, W. Blaas, A. Droβ, R. Wittkowski, Am. J. Enol. Vitic. 53 (2002) 78.
[27] D. Favretto, R. Flamini, Am. J. Enol. Vitic. 51 (2000) 55.
[28] C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos-Buelga, J.C. Rivas-Gonzalo, Anal. Chim. Acta 513 (2004) 305.