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High-performance liquid chromatographic analysis of the pigments of blood-red prickly pear (*Opuntia ficus indica*)

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ABSTRACT

A method for the extraction, separation and evaluation of betalaines, the pigments of bood-red *Opuntia ficus indica* grown in Sicily, was studied. Tests with different solvent systems showed that extraction of the pigments with water–ethanol was the most complete. For the preparative separation of the betalaines, reversed-phase low-pressure column chromatography on an octadecylsilica bonded phase (RP-18) was the most suitable. Using phosphate buffer (pH 5)–methanol (85:15) the major yellow and red-violet pigments were sharply separated and recovered in good purity. Elution with the same mixture in the ratio 70:30 separated three minor yellow and red-violet components. The amounts of the yellow pigment were evaluated spectrophotometrically at 475 nm and of the red-violet pigment at 538 nm. For the evaluation of the purity of the recovered pigments, a reversed-phase high-performance liquid chromatographic method was applied using a LiChrosorb Select B column eluted with 0.1 *M* phosphate buffer (pH 5)–methanol (85:15), with spectrophotometric detection at 538 nm for the red pigments and 475 nm for the yellow pigment. The yellow pigment obtained was pure indicaxanthin, whereas in the red-violet pigments betanin and isobetanin were identified with another not yet characterized betalainic glucoside.

INTRODUCTION

Since the official EEC regulations have restricted the use of synthetic red colorants as additives in food, and the harmful ones have been banned, there has been a growth of interest in the application of natural pigments.

Betanin, also called "beetroot red", is accepted, among the natural pigments, by EEC and Italian regulations in force with the Sigle E162. It is used mainly to colour foods not requiring thermal treatment, such as yoghurt, confectionery, ice-cream, syrups and sausages. Betanin is the major compound of a group of glucosides called betacyanins whose aglycones are the enantiomers betanidin and isobetanidin, as shown in Fig. 1. The structures were elucidated by Wyler's group [1,2].

Betacyanins are the pigments of plants of the order Centrospermae, where they represent a taxonomic chemical constituent, like anthocyanins in some other plants. Betacyanins are frequently associated with yellow betaxanthins of similar biogenesis; the term betalaines includes the two classes of pigments. Various workers [3–5] have listed 44 betacyanins, which represent 37 plants in seven families; other new betacyanins were identified by surveying further 34 species of Cactaceae [6]. In *Opuntia ficus indica,* Piatelli and co-workers [7,8] identified and characterized two main pigments: a red-violet betanin, usually accompanied by minor amounts of its C-15 diastereoisomer isobetanin [7], and a yellow indicaxanthin [8] (Fig. 1).

Our studies of new sources of betanin colorant started with *Phytolacca decandra* berries, grown in Italy [9]. Extraction of the pigment was very simple, but it was necessary to remove a toxic saponin. The product obtained was tested for its pigmenting power and for its stability in model soft and alcoholic drinks [10].

Within a research programme of the Italian Ministry of Agriculture and Forestry for the development of tropical fruit cultivation and utilization in southern Italy, the pigments of blood-red prickly pear (*Opuntia ficus indica*) were of interest. We have

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Fig. 1. Prickly pear major pigments: 1 = betanin; 2 = isobetanin; 3 = indicaxantin.

therefore studied the modes of extraction and the evaluation of these pigments by using solid-phase techniques and high-performance liquid chromatographic (HPLC) analysis.

EXPERIMENTAL

Prickly pear Sanguigno fruits (*Opuntia ficus indica* L.) grown in Sicily were used. They were post-harvest ripened until the maximum colour values of the surface were reached: $L^* = 43.12 + 1.03$, $a^* = 22.16 + 1.55$, $b^* = 9.07 + 1.22$ (CIE 1976 L^*, a^*, b^* system). The fruits were peeled before homogenization.

Extraction of pigments

A 50-g amount of homogenized pulp was shaken with 100 ml of the following extracting solutions: (1) water; (2) dilute citric acid (pH 4.5); (3) ethanol–water (80:20); and (4) ethanol–dilute citric acid (pH 4.5) (80:20). After 5 min of magnetic stirring, the suspensions were centrifuged for 15 min at 1200 *g*. The extractions were repeated three times until there was total decolorization of the residue. The aqueous extracts were diluted to 500 ml in volumetric flasks. Before dilution of the aqueous–alcoholic extracts, the ethanol was removed by vacuum evaporation at 50°C in a rotary evaporator.

The spectra of the extracts were scanned in the visible range with a Philips PU 8800 spectrophotometer and the concentration of the pigments was estimated using A_{538} (1%) = 1120 for betanin [1,2] and A_{475} (1%) = 650 calculated from log A = 4.63 for indicaxanthin [8].

Thin-layer chromatography (TLC)

Preparative silica gel 60 plates (E. Merck, code 13895), 20 × 20 cm, 1 mm layer thickness, and cellulose plates (E. Merck, code 15275), 20 × 20 cm, 0.5 mm layer thickness, were used with two mobile

phases: acetic acid–methanol (40:60) and acetic acid–propanol–water (20:60:20), NH_2 F_{254} analytical HPTLC plates (F. Merck, code 15647) were used with the following mobile phases: 0.1 *M* phosphate buffer (pH 4 and 5) and 0.1 *M* phosphate buffer (pH 5)–acetonitrile (50:50, 60:40, 70:30, 80:20 and 90:10).

Low-pressure column chromatography (LPLC)

Preliminary tests were made with glass columns (10 cm × 2 cm I.D.), eluting by applying nitrogen pressure at the top in darkness. With LiChroprep NH_2 (E. Merck, code 13974), the mobile phases tested were 0.1 *M* phosphate buffer (pH 5)–acetonitrile (60:40, 70:30 and 80:20). With ICN Silica RP-8 and RP-18 (32-63) 60A (ICN Biochemicals, code 05010), the mobile phases tested were 0.1 *M* phosphate buffer (pH 5)–methanol (75:25, 80:20, 85:15 and 90:10).

Reversed-phase preparative column chromatography of the pigments

The above RP-18 phase was packed in a 50 cm × 5 cm I.D. column, then eluted by applying nitrogen pressure in dim light. Elution was performed in two steps, first with 0.1 *M* phosphate buffer (pH 5)–methanol (85:15) and second with 0.1 *M* phosphate buffer (pH 5)–methanol (70:30). The column was than flushed with isopropanol and conditioned again with the first eluent for a new separation.

HPLC of pigments

A Jasco HPLC system consisting of a Jasco Model 880 Pu pump, a Rheodyne Model 7125 injector and a Jasco Model 870 UV – VIS spectrophotometric detector was used. A Hibar RT 250-4, LiChrosorb RP-Select B (25 cm × 5 μm I.D.). (E. Merck, art. 19608) column was used, eluting with 0.1 *M* phosphate buffer (pH 5)–methanol (85:15) at a flow-rate of 0.5 ml/min.

For each sample the detection was applied at 475 and 538 nm (0.08 a.u.f.s.) using two runs to identify the yellow (Y) and red-violet (V) constituents at their particular absorption maxima.

Preparation of purified standards from a commercial red beet betanin concentrate

A commercial red beet betanin concentrate (IFF code 6200), purified by the already described preparative techniques, was used as a standard for betanin and isobetanin. The fractions obtained were subjected to HPLC using the above method.

Enzymatic hydrolysis of glucosides in the pigments

To distinguish aglycones from glucosides, the violet pigments were treated with β-glucosidase according to Piattelli and Minale [7] and Schwartz and Von Elbe [11]. Volumes of 10 ml of violet fraction V and of red beet extract were incubated at 37°C with 5 ml 0.001% β-glucosidase (Boehringer Mannheim, code 105422). Samples were removed immediately and at 30-min intervals and injected into the HPLC system to follow the disappearance of the glucoside peaks.

RESULTS AND DISCUSSION

Extraction of pigments

The visible absorption spectrum of the aqueous–alcoholic extract of the prickly pear was the same as that of the pressed juice. The presence of both yellow pigments absorbing at 475 nm and red-violet pigments absorbing at 538 nm was observed. The average amount obtained for the yellow pigments was about 40 mg per 100 g of fresh pulp and for the red-violet pigments, about 14 mg per 100 g, i.e., with a ratio of about 3:1. The yields of the pigments extracted using the solvents tried were not significantly different. Ethanol–water (80:20) extraction was chosen, because this solvent can recover the pigments completely from the pulp without co-extracting polysaccharides and other alcohol-insoluble solids. This simplifies the further purification of the coloured extracts.

Preparative chromatography of pigments

TLC. Preliminary tests were carried out by TLC on different adsorption layers using several solvent systems. The R_F values are given in Table I. The visible spectra of the red bands eluted both on silica gel and on cellulose indicated that this band was a mixture of violet and yellow compounds. The amino-bonded silica phase showed the best separation of the violet and yellow pigments in a shorter time than the other two phases. TLC was not considered for preparative purposes, because the coloured bands quickly faded after evaporation of the solvent even when protected from light and oxygen.

TABLE I

TLC RESULTS

Stationary phase	Eluent	R_F			Development time (min)
		Violet	Red	Yellow	
Silica gel	Acetic acid–methanol (40:60)	0.88	0.42	0.23	40
Cellulose	Acetic acid–propanol–water (20:60:20)	0.22	0.80	1.00	60
NH_2-silica gel	0.1 *M* phosphate buffer (pH 5)–acetonitrile (70:30)	0.80	–	0.06	20

Preparative liquid chromatography. As the weak anion-exchange capacity of amino-bonded silica gave the best results for the TLC separation of the yellow from violet pigments, this phase was tried in a column for preparative purposes. RP-8 and RP-18 phases, already utilized in the preparative HPLC of beetroot betanins [11], were also tested. All the solvent systems were at pH 5 because betanin has its maximum absorption and its highest stability at this pH. The results of these trials are given in Table II. With RP-18 the total elution of pigments was obtained by using two eluents with different concentrations of the organic modifier. The first eluent eluted the two major components, the yellow-orange fraction Y and the violet fraction V, successively. The less polar pigments that remained at the top of the column were eluted with the second eluent.

According to the absorption spectra reported in Fig. 2, fractions Y and V appeared as single coloured substances, whereas fractions FR, FV and FO appeared as mixtures of minor yellow and red violet pigments in different proportions (Fig. 3).

As the minor components were considered to be unimportant for utilization purposes, our research was developed to recover and to study the major fractions Y and V, choosing chromatography on an RP-18 column and single elution with 0.1 *M* phosphate buffer (pH 5)–methanol (80:15) for preparative purposes. The best means of removing the residual substances from the column was by flushing with isopropanol.

HPLC of Y and V fractions

Yellow fraction Y. HPLC separation on an end-capped RP-8 phase (LiChrosorb RP Select B) with detection at 475 nm displayed a single peak for this fraction. According to Piattelli *et al.* [8], this compound should be identified as indicaxanthin from the retention time and the absorption spectrum.

Red-violet fraction V. HPLC separation under the same conditions as above, with detection at 538

TABLE II

PREPARATIVE LIQUID CHROMATOGRAPHIC RESULTS

Stationary phase	Eluent	Order of elution
NH_2-silica	0.1 *M* phosphate buffer (pH 5)–acetonitrile (80:20)	Poorly separated
RP-8 silica	As above	Two band: yellow and red violet (mixture of violet and yellow).
RP-18 silica	1st eluent: 0.1 *M* phosphate buffer (pH 5)–methanol (85:15)	Two bands: Y = yellow-orange; V = violet
	2nd eluent: 0.1 *M* phosphate buffer (pH 5)–methanol (70:30)	Three minor bands: FR = rose; FV = red-violet; FO = orange

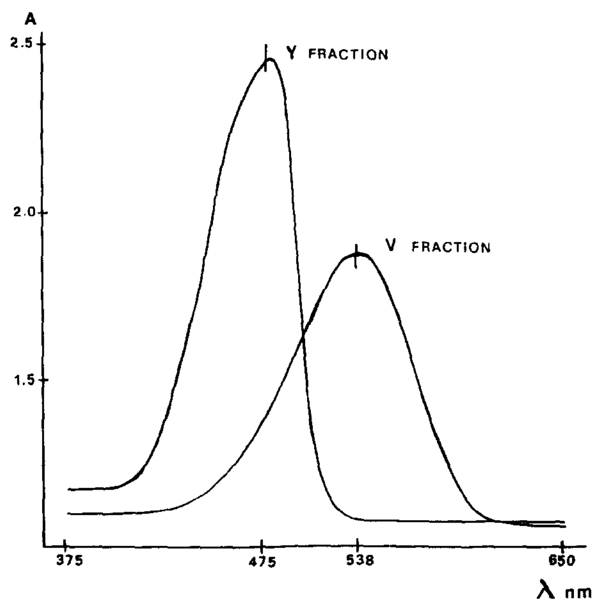

Fig. 2. Absorption spectra of yellow (Y) and violet (V) bands eluted from the RP-18 column with 0.1 *M* phosphate buffer (pH 5)–methanol (80:15).

nm, showed three major peaks eluting at 6.47 min (peak A), 11.05 min (peak B) and 28.30 min; other smaller peaks present are believed to be degradation products. The same HPLC procedure was also applied to a previously purified commercial betanin concentrate. Comparing the retention times of the peaks of the two chromatograms of the prickly pear pigments and that of red beet, we can identify peak A as betanin and peak B as isobetanin, while the peak at 28.30 min is not present in the beetroot extract.

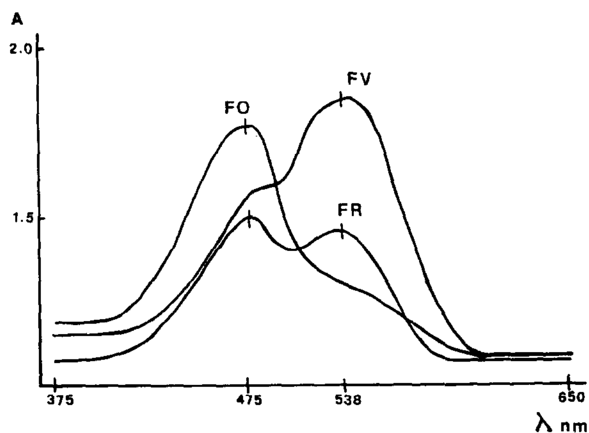

Fig. 3. Absorption spectra of the less polar bands FR = rose, FV = red violet and FO = orange, eluted from the RP-18 column with 0.1 *M* phosphate buffer (pH 5)–methanol (70:30).

Evidence in support of this identification was provided by treatment of both the betanin extracts, *Opuntia ficus indica* (V) and beetroot, with β-glucosidase. According to Schwartz and Von Elbe [11], the enzymatic hydrolysis of betanin and isobetanin gives rise to aglycone peaks that have higher retention values than to their parent compounds. As shown in Fig. 4, enzymatic hydrolysis of the beetroot extract yields betanidin and isobetanidin exclusively (peaks A1 and B1). A same trend is observed in Fig. 5 for the hydrolysed fraction V: peaks A and B, which diminished, are the betacyanin glucosides, whereas the peaks A1 and B1 are the corresponding less polar aglycones. Hence the compounds present in that red-violet fraction V were identified as A = betanin (6.47 min), A1 = betanidin (10.05 min), B = isobetanin (11.05 min) and B1 = isobetanidin (18.64 min). The different order of elution of betanins and betanidins compared with the reported

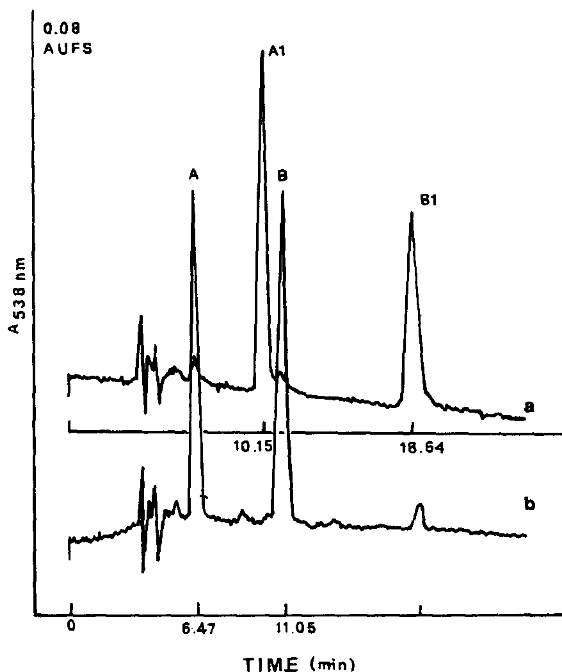

Fig. 4. HPLC of beetroot extract before (b) and after (a) treatment with β-glycosidase: A = betain; B = isobetanin; A1 = betanidin; B1 = isobetanidin.

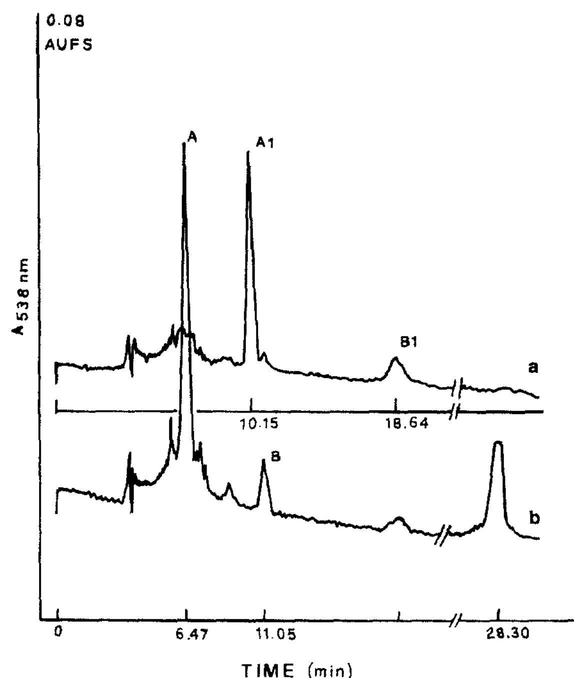

Fig. 5. HPLC of the V fraction before (b) and after (a) treatment with β-glucosidase: A = betain; B = isobetanin; A1 = betanidin; B1 = isobetanidin; peak at 28.30 min = a not previously quoted betanin glucoside.

[11] can be attributed to the difference in polarity between RP-8 used here and RP-18 used previously [11]. Hence the interactions of these compounds with the stationary phase may change.

For the red-violet compounds with a retention time of 28.30 min, we could not find any information in the literature. It was less polar than the other betanins, it absorbed between 538 and 540 nm at pH 5 and it was hydrolysable by β-glucosidase. The absorption spectrum and the RP-HPLC analysis exclude the identification of this peak as neobetanin, an orange compound claimed by Strack *et al.* [12] as a major constituent of *Opuntia ficus indica* pigments. That this substance could be a glucoside of a low-polarity betacyanin is the only possible suggestion at present.

CONCLUSIONS

The red-violet major pigment extracted from blood-red prickly pear presented some differences from the red beet betanin concentrate. It is constituted mainly of betanin and of another betacyanin glucoside, with traces of isobetanin and isobetanidin, while the red beet colorant concentrate contains mainly betanin and isobetanin, in about equal amounts.

The selected techniques of extraction with an aqueous–alcoholic solvent and preparative column chromatography on RP-18; allowed us to recover the two major yellow and red violet pigments from blood-red prickly pears. By using an aqueous-alcoholic extraction medium, the enzymatic activity can be hampered, and also polysaccharides and other alcohol-insoluble compounds are left behind in the residue pulp. Moreover, this method avoided the formation of artifacts. In fact, only traces of isobetanin and isobetanidin were found, indicating that the epimerization was minimized during the preparative process [5,7,11]. Hence reversed-phase low-pressure chromatography can be used for both practical and research purposes without causing alterations.

The developed isocratic RP-HPLC method was suitable for monitoring the composition of both the yellow and red-violet compounds. With this method, in the red-violet fraction a so far uncharacterized betacyanin glucoside was observed. According to our results (not shown), the optimum stability of the pigments was at pH 5. This pH proved to be efficient for the separation of the compounds with an end-capped fine particle size, slightly polar RP-8 phase, such as the LiChrosorb Select B column used here.

The purity of the peaks was also checked using diode-array spectrophotometric detection, confirming the high resolution of this column. The extension of this method to the determination of the detected compounds is being developed.

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