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Separation of betalains from berries of *Phytolacca americana* by ion-pair high-speed counter-current chromatography

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Abstract

The first preparative fractionation of betalain pigments by means of ion-pair high-speed counter-current chromatography (IP-HSCCC) from berry extracts of *Phytolacca americana* (Phytolaccaceae) is presented. A novel HSCCC solvent system consisting of 1-butanol–acetonitrile–water (5:1:6, v/v/v) was applied using ion-pair forming trifluoroacetic acid at low concentration (0.7%, v/v). Affinity of polar betacyanins and betaxanthins to the organic stationary phase of the biphasic HSCCC solvent mixture was considerably improved. Partitioning coefficient values and influence of increasing trifluoroacetic acid additions to the biphasic solvent mixture were measured for all identified betacyanins and betaxanthins. Gentle separation by IP-HSCCC of the injected pigment extract (900 mg) yielded sufficient amounts of the principal pigments 15*S*-betanin/15*R*-isobetanin. The pure epimers separated by C_{18}-HPLC were immediately studied by one- and two-dimensional NMR. In the recovered fractions, minor concentrated betacyanins and betaxanthins were significantly enriched by IP-HSCCC and were detected for the first time in the extracts of *P. americana*. IP-HSCCC and C_{18}-HPLC were shown to be complementary techniques in the isolation procedure of recovering minor concentrated, highly polar and chemically instable betacyanins and betaxanthin from complex plant matrices. Altogether, identification of 17 betalains was achieved by HPLC-diode array detection-electrospray ionization MS/MS in the HSCCC fractions with their respective isomers, also resulting in the tentative elucidation of betacyanins with novel salicylic acid substitution pattern in the berry extracts of *P. americana*.

Keywords: Betanin; Betalains; Betacyanins; Betaxanthins; Ion-pair counter-current chromatography; IP-HSCCC; HPLC-DAD-ESI-MS/MS; NMR; *Phytolacca americana*; Phytolaccaceae

1. Introduction

High-speed counter-current chromatography (HSCCC) has been proved to be a very useful technique for preparative isolation of natural compounds from plant extracts [1,2]. In this study, a separation of betalains, from the extracts of dark violet pokeberry fruits – *Phytolacca americana* – (Phytolaccaceae) is presented. Because of high levels of saponins, the *Phytolacca* species were proposed as biological molluscicides for the control of bilharziosis transmitting snails in Ethiopia [3,4]. On the other hand, betalains, having strong antioxidant properties, are arising as interesting chemopreventive natural compounds [5–8]. Therefore, in connection to their good colorant properties, a growing interest in betalains has been noticed during the past decade [9,10] and some of these compounds have not been identified yet.

Betalains (red-violet betacyanins and yellow-orange betaxanthins) are water soluble plant pigments. Betacyanins can be considered as condensation products of betalamic acid with cyclodopa or its *O*-glycosylated (in most cases 5-*O*-glucosylated) derivatives. Frequent esterification of the *O*-glycosides with acids such as ferulic, *p*-coumaric or malonic acid was also confirmed [11,12]. Closely related betaxanthins are condensation products of betalamic acid with amines or amino acids. Betalains exist in two diastereomeric forms differing by the configuration of the C-15 carbon (Fig. 1) [11,12].

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Betanin, the simplest 5-*O*-glucosylated betacyanin, and its C-15 isoform are frequently derived from red beet root (*Beta vulgaris* L.). Another edible rich source of betacyanins are recently investigated species of *Hylocereus* cacti containing betanin, phyllocactin and hylocerenin as the most abundant pigments [10,13,14].

The basic structural study performed on *P. americana* fruit pigments [15] revealed prebetanin (betanin 6′-*O*-sulphate) as the predominant betacyanin identified together with betanin and betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside. Further research on cell cultures derived from stem explants of *P. americana* confirmed the presence of another pigment, lampranthin II (betanidin 5-*O*-(6′-*O*-*E*-feruloyl)-β-D-glucopyranoside) [15].

The known lability of betalains, e.g. their low resistance to elevated temperatures (especially of purified pigments) [10,16–21], makes the isolation and purification of higher quantities of betalains for further investigations (e.g. medicinal, pharmaceutical or analytical research) problematic. In addition, new interesting betalain structures are usually present in the plant material at low concentration and their isolation requires tedious and time consuming procedures. Therefore, our research was directed to the application of HSCCC which had already proved to be a promising technique of isolation of natural compounds [1,2]. The main purpose of this contribution was to study the isolation possibilities of the most abundant betalains and the minor betalains on a preparative scale from berries of *P. americana* by HSCCC. The possibility of enrichment of less hydrophilic betalains by HSCCC (e.g. for structural elucidation by NMR techniques) which usually exist in much lower concentration level than the hydrophilic ones (especially betanin) in plant material was studied. In addition, the isolation of betaxanthins from betacyanins existing in *P. americana* as well as the separation between the polar betacyanins was investigated. This is the first report on the presence of betaxanthins in *P. americana* as well as on a successful isolation (fractionation) of the wide range of betalain structures from a natural source using HSCCC.

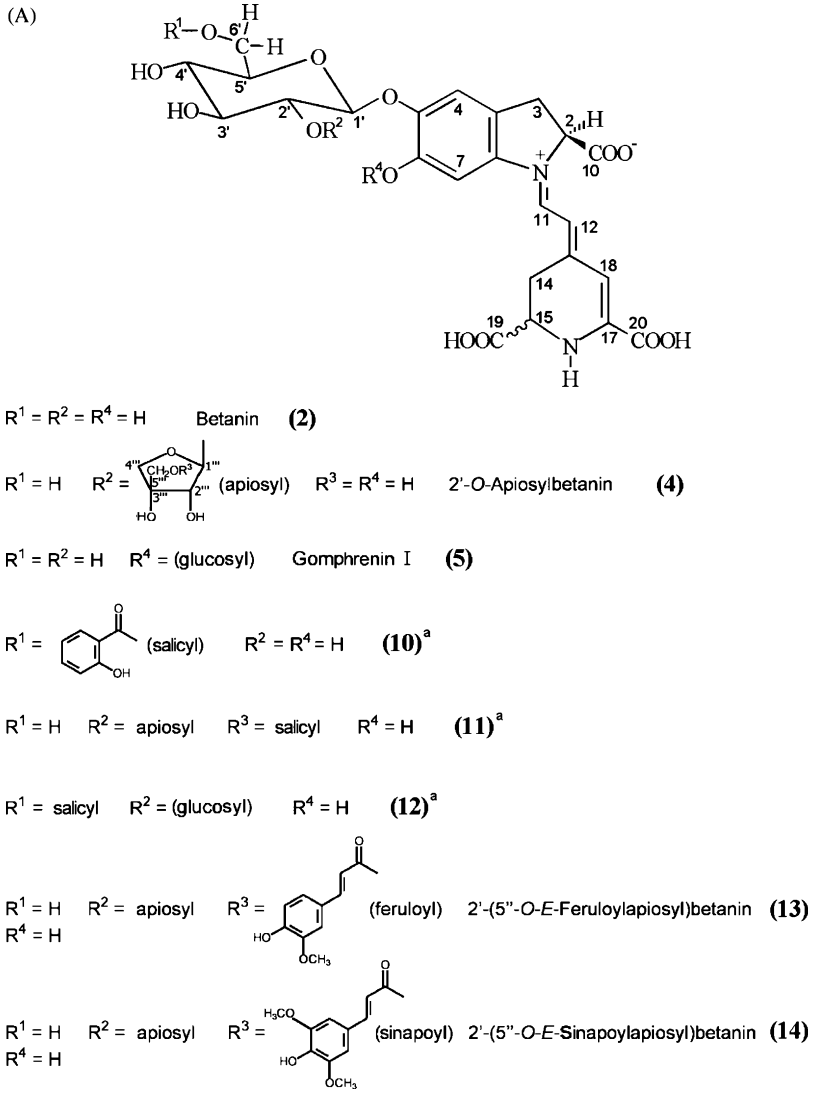

Fig. 1. Chemical structures of betacyanins (A), betaxanthins (B) and purification artefacts (betanidin, 17- and 2-decarboxybetanin and neobetanin) (B) present in the HSCCC fractions of *P. americana* berries. [a]Tentatively identified.