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Differential partitioning of betacyanins and betaxanthins employing aqueous two phase extraction

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ABSTRACT

Yellow betaxanthins and red purple betacyanins are genetically and structurally closely related pigments, hence making their separation from each other difficult and tedious. Fractionation of betalains into betacyanin and betaxanthin could be achieved by employing aqueous two-phase extraction (ATPE). Prior to fractionation, purification of betalains (devoid of sugars) was achieved employing ATPE comprising PEG 6000/ammonium sulphate. At a high tie line length (TLL) of 50.65%, phase volume ratio 1.96 and pH around 5, maximum differential partitioning was observed, wherein 92.62% of the betalains partitioned to the PEG-rich (top) phase while 72.38% of sugars partitioned to the salt-rich (bottom) phase. Fractionation of betalains into betacyanin and betaxanthin was achieved by multistage ATPE. At the end of the third stage ATPE, the bottom phase had only betaxanthin without any residual betacyanin. The fractions of betacyanin and betaxanthin were analyzed by spectrophotometric method and confirmed by HPLC.

1. Introduction

At present, there is a growing interest in colorants from natural sources. Recent problems with the safety of synthetic dyes have stimulated research on natural pigments. This is partly because of the need to expand the pool of colorants and partly because of the implication that being natural – they are safe. Betalains, the major pigments present in beet root (Delgado et al., 2000) have been known for their food (Azeredo, 2008) and medicinal applications (Kapadia et al., 2003). Betalains have two structural groups, the red-purple betacyanins (Fig. 1a) and the yellow betaxanthins (Fig. 1b). Conjugation of the substituted aromatic nucleus to the 1,7-diazaheptamethinium chromophore shifts the absorption maximum from 480 nm in betaxanthins to 540 nm in betacyanins. The structural difference enables a simultaneous spectrophotometric measurement of the pigment content without the need of chromatographic or electrophoretic separation.

Betacyanins and betaxanthins are reported to have different medicinal, food and pharmaceutical applications. Betacyanins have shown antiproliferative (Sreekanth et al., 2007), antioxidant (Cai et al., 2003) and radio protective activities (Vogt et al., 1999). Betaxanthins (indicaxanthin) have shown protective effects on human β-thalassemic red blood cells submitted *in vitro* to oxidative haemolysis by cumene hydroperoxide (Tesoriere et al., 2006). Betaxanthins and its derivatives were used for the preparation of a drug for the treatment of prediabetes and diabetes (Lugo radillo and Agustin, 2013). Several methods such as thin layer chromatography (Nilsson, 1970; Bilyk, 1981), gel filtration (Butera et al., 2002), counter current diffusion extraction (Wiley and Lee, 1978), Ion-Pair High-Speed Countercurrent Chromatography (Jerz et al., 2008), HPLC-electrospray ionization mass spectrometry (Stintzing et al., 2002), electrophoresis (Fernandez-Lopez and Almela, 2001), ion-exchange (Wilkins, 1987; Bokern et al., 1991) and column chromatography (Biswas et al., 2013) have been reported for separation of betacyanins and betaxanthins. In all these methods, though betacyanin and betaxanthin could be separated, they not be obtained in desirable quantities due to low yield. Therefore, these methods are not feasible for fractionation of betalains into betacyanins and betaxanthins at large scale. Hence, there exists a need for developing simple and effective methods for the separation and purification of these pigments.

The purpose of the present study is to explore a method to obtain betacyanin and betaxanthin in pure form employing aqueous two phase extraction (ATPE). This method has the potential to separate two closely related pigments (Patil et al., 2008), which conventionally requires chromatographic techniques, which are more analytical than preparative methods. ATPE has been used for the purification of proteins, enzymes (Rostami Jafarabad et al., 1992; Saravanan et al., 2007) and also for the separation of sugars from betalains (Chethana and Raghavarao, 2003; Chethana et al., 2007). In the present work, a method was developed to obtain

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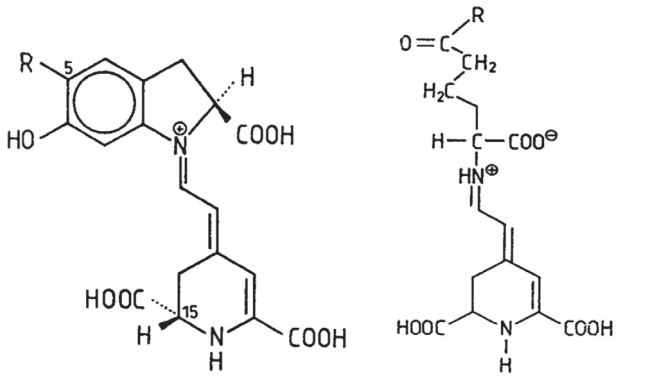

Fig. 1. (a) Structure of betacyanin; and (b) structure of betaxanthin.

the fractions of betacyanins and betaxanthins from betalains employing ATPE and their subsequent recovery devoid of phase forming components.

2. Materials and methods

2.1. Chemicals

Polyethylene glycol (PEG MW 1000, 1500, 4000, 6000, 8000) and dextrose were procured from Sisco Research Laboratories, Mumbai while ammonium sulphate ($(NH_4)_2SO_4$), magnesium sulphate ($MgSO_4$), sodium sulphate (Na_2SO_4) and potassium salts (KH_2PO_4, K_2HPO_4) were from Ranbaxy Chemicals. Phenol, sulphuric acid and ascorbic acid were procured from Himedia. C-18 column was used (SPD, Japan) for HPLC and the solvents used were of HPLC grade. All the other chemicals used were of analytical grade.

2.2. Preparation of crude extract of betalains

Fresh beetroots were procured from the local supermarket. The crude extract of betalains was prepared as reported in literature (Nilsson, 1970). Beetroots were washed, peeled and sliced into small pieces, to which water containing ascorbic acid (0.1% w/v) was added and allowed to stand for an hour before grinding and filtration. The filtrate was centrifuged at 8000 rpm for 10 min. The crude extract of betalains was stored at 4 °C and used it for the experiments.

2.3. Purification of betalains

Predetermined quantities of polymer and salts, as per the phase diagram reported in literature (Albertsson, 1986; Zaslavsky, 1995), were weighed and added to the crude extract to make the total weight of the system to 100% on w/w basis. The contents were mixed thoroughly using a magnetic stirrer for equilibration (2 h) and were allowed for about 8 h for phase separation. After the phase separation, the volumes of PEG-rich (top) and salt-rich (bottom) phases were noted and analyzed for betalains content. All the experiments were carried out at 25 ± 2 °C. After standardizing the process parameters, multistage ATPE was carried out in order to remove maximum possible sugars from PEG-rich top phase followed by organic-aqueous extraction employing chloroform for the removal of PEG, the phase forming polymer, from this phase.

2.4. Fractionation of betalains into betacyanins and betaxanthins

The overall process flow diagram for fractionation of betalains into betacyanin and betaxanthin is shown in Fig. 2. The fractionation of betalains into betacyanin and betaxanthin was achieved (Fig. 3) during multistage aqueous two phase extraction. The fresh top phase (system prepared using water) was added to the bottom phase containing betalains. The mixture was stirred for 5 min for equilibration and allowed for 30 min for phase separation. The phases were separated and the pigment concentrations were analyzed using spectrophotometric method.

2.5. Removal of phase forming components

The top phase (containing betacyanin) obtained after multistage ATPE was subjected to organic-aqueous extraction employing chloroform for the removal of phase forming polymer (PEG). The bottom phase (containing betaxanthin), obtained after the third stage of multistage ATPE, was subjected to Ultrafiltration (Amicon 8060, capacity −50 mL) in order to remove the phase forming salt (Ammonium sulphate). Polysulphone membrane disc (45 mm diameter) of 300 kDa molecular weight cut-off (M/s Permionics, Baroda) was used. Ultrafiltration was carried out by keeping the pressure, stirring speed and temperature constant throughout the experiment at 1 atm, 250 rpm, 25 ± 2 °C, respectively. The transmembrane flux (L/m^2 h) was calculated at regular intervals of 0.167 h using following equation

$$J = \frac{V}{A \times T} \tag{1}$$

where A the area (m^2) of membrane disc; V the volume (L) of permeate and T the time (hr) interval.

2.6. Analytical procedures

2.6.1. Estimation of betacyanin and betaxanthin

Betacyanins and betaxanthins were estimated by spectrophotometric method at 540 and 480 nm (Nilsson, 1970), which are the absorption maxima of betacyanin and betaxanthin, respectively. The concentration was calculated using extinction coefficient of 1120 and 750 for betacyanin and betaxanthin, respectively.

2.6.2. Estimation of total sugars

The Dubois method (Dubois et al., 1956) was used for the estimation of total sugars present in the betalains extract. The sugars have absorption maxima at 480 nm. Dextrose was used as a standard for the determination of sugars.

2.6.3. Tie line length

The tie line length (TLL) of the aqueous two phase system was calculated from its reported phase diagram (Albertsson, 1986) according to the following equation

$$\text{TLL}\ (\%) = \sqrt{(C_{pt} - C_{pb})^2 + (C_{st} - C_{sb})^2} \tag{2}$$

where C_{pt} and C_{pb} are concentrations of PEG (%, w/w) in the top and bottom phases, respectively and C_{st} and C_{sb} are concentrations of salt (%, w/w) in the top and bottom phases, respectively.

2.6.4. Phase volume ratio

The phase volume ratio (V_r) is defined as the ratio of volume of the PEG-rich (top) phase to that of the salt-rich (bottom) phase at equilibrium and was calculated using the following equation

$$V_r = \frac{V_T}{V_B} \tag{3}$$