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Differential partitioning for purification of anthocyanins from *Brassica oleracea* L.

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ABSTRACT

Aqueous two phase extraction was employed as an alternate method for the downstream processing of anthocyanins. Polyethylene glycol (PEG) 4000/magnesium sulphate (14.8%/10.3% w/w) was found to be the most suitable system. Tie line length of 32.61% and volume ratio of 0.73 have resulted in maximum partitioning of anthocyanins to the PEG-rich phase (yield – 98.19%) and sugars to the salt-rich phase (yield – 73.16%). Multistage aqueous two phase extraction resulted in an increase in removal of sugars (to about 96.5%) with 91.9% yield of anthocyanins. The phase forming polymer (PEG 4000) was successfully separated from anthocyanins employing organic–aqueous extraction resulting in 2.3 fold increase in the concentration of anthocyanins. High performance liquid chromatography (HPLC)–Mass spectroscopy (MS)/MS was carried out to confirm the structural stability of anthocyanins.

1. Introduction

Awareness of the consumer as well as carcinogenic nature of most of the synthetic colours used in the food systems has resulted in an increase in the use of natural colours as an alternative [1–3]. Natural colours such as betalains from beet root, carotenoids from carrot and anthocyanins from grape are some of the examples, which have been evaluated in various food systems and observed to be relatively stable [4–6]. Anthocyanins are a group of phenolic compounds that belong to the flavonoid family and are responsible for red, purple and blue hues of plant fruits, flowers and leaves [7]. Anthocyanins are of interest also in cosmetic and pharmaceutical industries as they can be used as substitutes for synthetic colourants and antioxidants. Red cabbage (*Brassica oleracea* L.) is one among the many potential plant sources of anthocyanins. Red cabbage anthocyanins are highly acylated with ferulic, coumaric and sinapic acids, which accounts for the superior stability and colour characteristics of the pigments compared to those from other sources [8]. Stability of colourants is strongly influenced by light, temperature [9], sugars, oxygen, pH [2] and UV-light [10]. Free sugars and their degradation products in the extract of anthocyanins lead to the Maillard reaction and form brown compounds [11]. Hence, removal of sugars from the extract of anthocyanins is very much desirable for the stability of these pigments in order to facilitate their food applications. Several chromatographic techniques are used for the purification after extraction: Paper chromatography [12–14], thin layer chromatography [15,16], gas [17] or column chromatography with many adsorbents such as oxidized alum and silica gel [18] and Sephadex LH-20 [12,19]. In case of methods such as column chromatography, electrophoresis and conventional centrifugation scale up becomes uneconomical unless the product is of high value. Ultrafiltration (UF) also has been used for clarification of food colourants prior to concentration by reverse osmosis [20]. However UF could not be used for effective separation of solutes especially when they are of similar molecular weights. All these chromatographic techniques have drawbacks with respect to high cost of materials, low degree of separation and loss of yield.

Aqueous two phase extraction (ATPE) offers a potential alternative to existing methods, especially in the early processing stages, with regard to ease of scale up and scope for continuous operation for the separation and purification of desired biomolecules from a complex mixture [21–24]. The major advantages of ATPE are high capacity, biocompatible environment, low interfacial tension of phase systems, high yields, low process time and energy [23,25]. ATPE has potential to achieve purification and concentration of the product in a single step. ATPE has been used for the purification of broad range of proteins, enzymes [23] and closely related biomolecules [26]. Low molecular weight biomolecules such as B-phyco erythrin [27], geniposide from gardenia fruit [28], butyric acid [29] and citrinin [30] from fermentation broth, polyphenols [31], anthocyanins from grape juice, purple sweet potato and *Morus atropurpurea* Roxb. [32–34] were also purified using ATPE.

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In case of aqueous two-phase extraction (ATPE) employing ethanol/salt system, the removal of alcohol from anthocyanin solution is carried out at 40–42 °C (using rotary evaporator). The stability of anthocyanins is low at temperatures above 25 °C. Hence, polymer/salt system was employed for purification of red cabbage anthocyanins in the present study. Objectives of the present work are (1) to optimize the effect of process parameters such as phase forming salt, molecular weight of phase forming polymer, tie line length and phase volume ratio on the differential partitioning of anthocyanins and sugars into opposite phases during ATPE and also (2) to carryout multistage ATPE for the maximum possible removal of sugars in order to increase the purity of anthocyanins.

2. Materials and methods

2.1. Chemicals

Polyethylene glycol (PEG) (MW 1000 Daltons) and sodium sulphate were procured from Merck limited, Mumbai, India while PEG (MW 4000, 6000 Daltons) and dextrose were from Sisco Research laboratories, Mumbai, India. PEG (MW 8000, 20000 Daltons) were procured from Sigma Aldrich, St. Louis, USA while potassium phosphate, ammonium sulphate and magnesium sulphate were from Ranbaxy chemicals, Mumbai, India. All the chemicals used were of analytical grade.

2.2. Preparation of anthocyanins crude extract

Red cabbage was procured from local super market. Leaves were cut into small pieces and extraction was carried out by maintaining the ratio of cut leaves (250 g) to extraction media (500 mL) at 1:2 while providing thorough contact in a mixing unit (Singer FP-450, India). The extract of anthocyanins obtained was filtered (using a muslin cloth) to remove coarse particles. The filtrate obtained was centrifuged (Eltek-TC 4100D, Elektrocrafts, Mumbai, India) at 6000 rpm for about 15 min to remove the fine suspended particles. Ascorbic acid (0.1% w/v) was added to inhibit the activity of polyphenoloxidase which otherwise causes enzymatic browning. The crude extract of anthocyanins (~600 mL, concentration – 0.32 mg/mL) thus obtained was stored for 7 days at 4–6 °C and required quantities were taken for different aqueous two phase extraction experiments.

2.3. Aqueous two phase extraction

2.3.1. Standardization of process parameters

Predetermined weighed quantities of polymer and salt, selected from different phase diagrams reported in the literature [22,35] were added to the crude extract of anthocyanins, making the total weight of the system 100% on w/w basis. Total mass of the phase system was maintained constant at 20 g in all the experiments, however, its volume was found to vary between 17 and 19 mL due to the variation in phase density (with the phase composition). The compositions of phase systems employed in each experiment are given in the results and discussion section and corresponding tables. The contents were mixed thoroughly using a magnetic stirrer for about an hour to equilibrate and the mixture was allowed for phase separation. After clear separation, the top and bottom phases were collected, volumes were noted and subjected to analysis of anthocyanins as well as sugars.

2.3.2. Multistage aqueous two phase extraction

The overall process flow diagram for multistage extraction of anthocyanins is shown in Fig. 1. First stage extraction was carried out using the standardized process conditions. Second stage

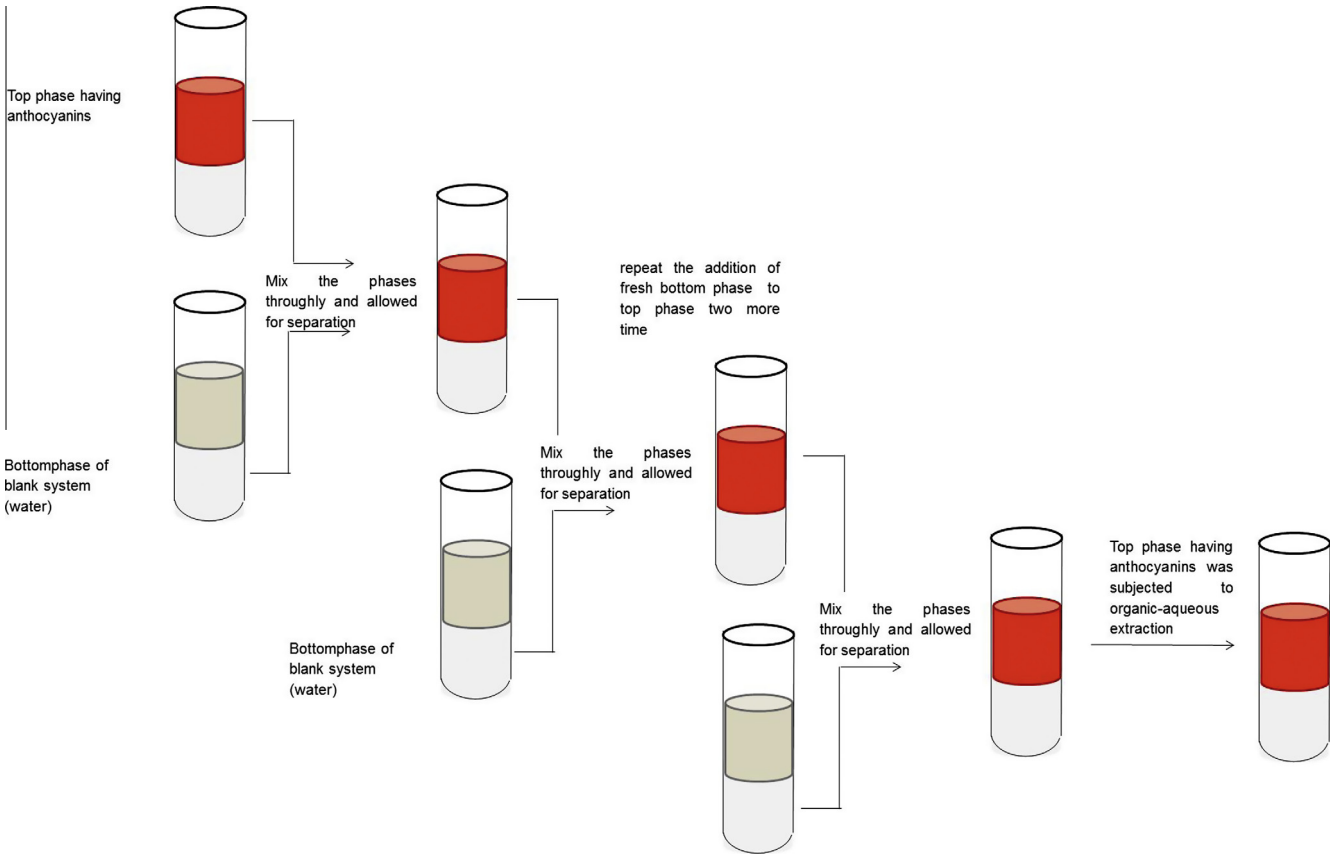

Fig. 1. Overall flow chart for multistage aqueous two-phase extraction.