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Aqueous Two-phase Extraction of Anthocyanin from Fruits of *Garcinia indica*

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Abstract: *Synthetic colorants and flavouring compounds are posing serious health concerns and hence natural pigments are in the spotlight due to their health benefits and consumer acceptance. Anthocyanin pigments are considered as an important additive in food and medicine due to their antioxidant and anti-microbial properties along with attractive colour. Aqueous two-phase Extraction (ATPE) was used to purify anthocyanin from Garcinia indica from accompanying sugars and proteins. Various PEG-salt and water miscible alcohol-salts ATPS were screened for the extraction of anthocyanin and 1-propanol - magnesium sulfate system was found to be superior. The binodal curve of the selected system was developed at 303.15 K and the effect of 1-propanol and magnesium sulphate concentrations, was studied on the partitioning coefficient and yield of anthocyanin. The maximum yield (98.51 %) of anthocyanins with a partitioning coefficient of 70.36 and reduction of 85.92 % sugars was achieved in the system which contains the composition of 30 % (w/w) of 1-propanol and 32 % (w/w) $MgSO_4$.*

Keywords: *Anthocyanin, aqueous two-phase extraction, phase equilibrium*

1. Introduction

The use of natural colorants with antioxidant and biological properties is gaining popularity because of proven long term toxicological effects of some synthetic additives. Natural pigments can be called as healthy pigments now days as they are known to prevent cardiovascular, inflammatory and neurological diseases [1]. Anthocyanins are harmless pigments of the vascular plants, gaining importance due to their use as natural water-soluble colorants and easily incorporated in aqueous media [2]. Anthocyanins extraction from natural sources like grape, berries (blue berry and mulberry), red cabbage, kokum fruits, purple sweet potatoes, jamun fruits, etc., are reported in the literature. Nayak et al., in 2010 reported the highest content of anthocyanin, about 2.4 g/100 g in kokum fruit rinds as compared to other fruits and vegetables [3, 4]. *Garcinia indica*, known as kokum belongs to the family Guttiferae and mainly found in tropical Asia, Africa and Polynesian countries. Western Ghats of south India and north-eastern states of India are popular for kokum trees were they grow abundantly [5]. The presence of two different anthocyanins in *Garcinia indica* namely, cyanidin-3-glucoside and cyanidin-3- sambubioside in the ratio of 4:1 are reported [4], among more than 500 different varieties of anthocyanins.

The non-conventional liquid-liquid extraction, aqueous two-phase extraction (ATPE) is well known as versatile and powerful single step partial purification technique. It has been successfully applied for the separation and purification of proteins [6], nucleic acids [7], enzymes [8], flavor compounds [9], antioxidants and antibiotics [10].

Now a day's aqueous two-phase extraction (ATPE) has been utilized to separate anthocyanins from natural sources because of its mild conditions and high capacity. ATPE based on polyethylene glycol (PEG)/magnesium sulphate system was found better system for differential partitioning of anthocyanins from red cabbage and Jamun fruits [11]. Multistage ATPE was also studied to increase removal efficiency of sugars and other contaminants [12]. Recently, hydrophilic alcohols/salt systems are in spotlight applied to purify biomolecules [13]. ATPE systems formed from short-chain alcohols (ethanol, methanol, 1-propanol and 2-propanol) with inorganic salts (phosphate, sulfate and citrates) found stable and more economic [14, 15] along with giving very low surface tension [16]. Ethanol–ammonium sulphate system was identified as an economical ATPE system to extract and concentrate the anthocyanins from various sources like Mulberry [17], purple sweet potatoes [18] and Blueberry fruits [19]. Multistage and scale up extractions with Ethanol-NaH_2PO_4 also worked well for simultaneous extraction and purification of grape juice anthocyanins by removing the majority of sugars [20].

The ATPS consist of PEG, Ethanol and Propanol with different salts was investigated for partitioning of anthocyanins from *garcina indica*. The effect of 1-propanol and magnesium sulfate concentrations was studied on the partitioning coefficient, recovery of anthocyanins and removal of impurities like sugars.

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2. Materials and Methods

2.1Materials

Analytical grade polyethylene glycols of molecular weight PEG 6000, Standard anthocyanin- Kuromanin chloride (cyanidin-3-O-glucoside) and standard protein BSA were purchased from Sigma Chem. Co. USA. Hydrochloric acid, sulphuric acid, phenol, sodium hydroxide, glucose and different salts like, ammonium sulfate, magnesium sulfate, mangenous sulfate, sodium citrate and sodium sulfate salts (> 99%) and HPLC-grade solvents like, 1-propanol, 2-propanol, and ethanol were procured from Merck company, India. Double distilled water was used throughout the experiment. Fresh kokum (*Garcinia indica*) fruits were procured from the local market near Mangalore, India.

2.2 Methods

The crude extract is prepared by mixing washed and cut pieces (rinds) of 1 kg fresh kokum fruits with 0.1% HCl water. After sufficient incubation the mixture is grinded in Morton piston. The extract was stored at 4^0 C for further use after filtered twice using muslin cloth and centrifuged at 12000 rpm for 30 min to remove fine particles.

The weight quantities of polymer, immiscible alcohols, salts and water were calculated from phase diagrams reported in the literature [12, 14] and added accordingly to the crude extract of anthocyanins (1g), making the total weight of the system 100% on w/w basis at 303.15 K (total 10g system prepared). After sufficient mixing the contents are kept for incubation for an hour to equilibrate and phase separation. The top and bottom phases were collected separately after clear separation. Noted the volumes, weight and anthocyanin and sugar concentrations were estimated.

Cloud point titration method was used to determine the binodal curve at 303.15 K for 1-Propanol and magnesium sulfate system.

To study the effect of phase component concentration on the partition coefficient of anthocyanin and sugars (K_a and K_s), required amount of $MgSO_4$ and 1-Propanol were added with 1 g of crude extract sample and made total weight of 10g with de-ionized water. After equilibration, the anthocyanin and sugar concentrations in both the phases were determined. The pH differential method was used to determine total monomeric anthocyanin content of the samples [21]. The pH of the anthocyanin samples was adjusted using a digital pH meter (digital pH meter Eutech Instruments, Singapore). Concentration of anthocyanins content as cyanidin-3-glucoside equivalents was calculated using the following equation [22] (Eq. (1) :

Anthocyanin concentration (mg/l) =

$$\frac{A \times MW \times Df \times 10^3}{\varepsilon \times L} \quad (1)$$

Where A is the total absorbance

$$\left(A_{\lambda_{max}} - A_{700}\right)_{\text{pH-1.0}} - \left(A_{\lambda_{max}} - A_{700}\right)_{\text{pH-4.5}}$$

Molecular weight of anthocyanin (MW) considered as per cyanidin-3-glucoside is 449.2 g/mol and the extinction coefficient (ε) are 26900 L/cm mol. Df is the dilution factor and L is the path length (1 cm).

Similarly, the total sugars present in the samples were determined through Dubois method [23], Glucose was used as a standard for the determination of sugars. The absorbance of the sample was recorded at 490 nm using UV spectrophotometer. The partition coefficient (K_a and K_s) of the anthocyanins/sugars and top phase anthocyanin yield (Y_{TA}) and salt-rich bottom phase sugars yield (Y_{BS}) were calculated using the eq. (2), (3) and (4), respectively [11,12,17].

$$K_a \text{ and } K_s = \frac{C_T}{C_B} \quad (2)$$

$$Y_{TA} = \frac{C_T V_T}{C_T V_T + C_B V_B} * 100 \quad (3)$$

$$Y_{BS} = \frac{C_B V_B}{C_B V_B + C_T V_T} * 100 \quad (4)$$

Where, C_T and C_B represent the concentrations of anthocyanins or sugars in the top phase and bottom phase. V_T and V_B represent the volume of top phase and bottom phase.

3. Results and Discussion

Initially, the crude extract rich with anthocyanins was prepared by employing three different extraction methods namely, aqueous extraction, 0.1 % HCl treatment and incubating with 50 % propanol. In all the techniques the fruit samples are mixed with solvents in 1:2 ratios [12]. The anthocyanins content was higher in propanol extract (151 mg/L) than the acidic water extract (143mg/L) and aqueous extract (130 mg/L). The 0.1 % HCl water extract was further used for the partitioning studies.

3.1Selection of suitable ATPS

The partitioning of target biomolecule in ATPE depends on size, charge, and hydrophobicity of target biomolecule and system properties like concentration of phase forming components (salt and polymer/alcohols), phase volume ratio and pH. Polymer–polymer ATP systems are not preferred much because of high cost and handling problems due to high viscosity, which makes the polymer-salt systems advantageous [24]. Short chain alcohol-salt systems are also preferred because of low cost, high water content, low surface tension, faster phase separation, easily recyclable and more suitable for hydrophilic compounds [17]. Hence, eight different ATPSs consist of different salts like magnesium sulfate, sodium sulfate, ammonium sulfate, sodium citrate, and manganese sulfate with PEG 6000, 1-Propanol and ethanol were considered for the selective partitioning of *garcina indica* anthocyanins by leaving the other impurities as shown in Figure 1.

The different compositions of the phase forming components of the systems were considered based on the bi-nodal curve of the respective systems. In all the selected systems the anthocyanins found to selectively partition towards top phase (PEG or alcohol rich phase) by leaving the impurities in the salt rich bottom phase. The PEG6000-$MgSO_4$ and PEG6000 - $(NH_4)_2SO_4$ systems provided a partition coefficient of 15.70 and 6.43 with a yield of 90 and 70 %, respectively. Both the system exhibits a native pH between 4 and 5. These systems are also worked well for anthocyanins from red cabbage and jamun fruits as reported earlier by Chandrasekhar and Raghavarao [11, 12]. The Ethanol- $(NH_4)_2SO_4$ system was considered for the partitioning since this system was used extensively for the recovery of anthocyanins from Mulberry (*Morus atropurpurea Roxb.*) [17], Purple sweet potatoes [18] and Blueberry (*Vaccinium uliginosum Linn*) [19]. Around a yield of 83% *garcina indica* anthocyanin was noticed with this system.

Further the phase forming ability of the ATPS consisting of 1-Propanol with different salts like, $MgSO_4$ / $(NH_4)_2SO_4$/ $MnSO_4$/ $Na_3C_6H_5O_7$/ Na_2SO_3 were examined and the anthocyanin partitioning was carried out in all the mentioned system. The 1-Propanol-$MgSO_4$ system showed a very high partitioning of anthocyanin in the top phase with a partition coefficient of 25.57 and yield of 93.58 % w/w (Figure 1). However, the $(NH_4)_2SO_4$ and $MnSO_4$ salts also provide a significant yield and partitioning coefficient but lesser than 1-Propanol- $MgSO_4$ system. The sodium salts namely, $Na_3C_6H_5O_7$ and Na_2SO_3 failed to provide a significant yield due to the degradation of anthocyanins. It was observed that the partitioning and yield of the *garcina indica* anthocyanin was majorly depends on the native pH of the ATPS.

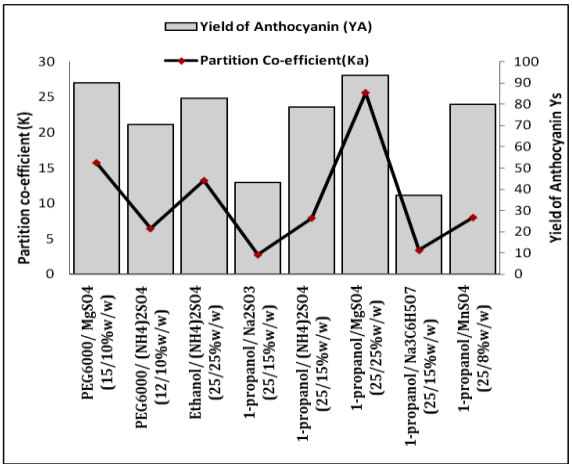

Figure 1: *Effect of different ATPE systems on partitioning of Anthocyanin and yield. K_a represents partition co-efficient of anthocyanin and Y_A represents yield of anthocyanin*

The ATPS formed with 1-propanol - $MgSO_4$/ $MgSO_4$ / $(NH_4)_2SO_4$/ $MnSO_4$/ $Na_3C_6H_5O_7$/ Na_2SO_3 has the pH of 3.9, 5.2, 5.9, 8.8 and 9.5, respectively. As the pH moves from acidity to basicity the yield was found to decrease, since the anthocyanin molecules are stable at acidic pH than basic pH. Hence, the maximum yield was obtained for 1-propanol/ $MgSO_4$ system which offers the pH of 3.9. The1-propanol/MgSO4 system was selected for further studies since it showed a good yield of *garcina indica* anthocyanin. In general, most of the anthocyanin from other sources has a better stability below the pH value of 4. However it was observed that the garcina indica anthocyanins able to with stand upto the pH value of 6 with 1-propanol as one of the phase forming component.

3.2 Effect of phase components on partitioning of anthocyanins and sugars

With the aim to partition the anthocyanins in any one of the phases of ATPS by leaving the other impurities like total sugars and proteins in the other phase, the experiments were conducted to improve the partitioning coefficient and yield of anthocyanins by varying the system conditions of 1-propanol/ $MgSO_4$ system. The effects of 1-propanol and $MgSO_4$ concentration on the separation of anthocyanins were analysed by selecting the appropriate concentration from the phase diagram developed (Figure 2).

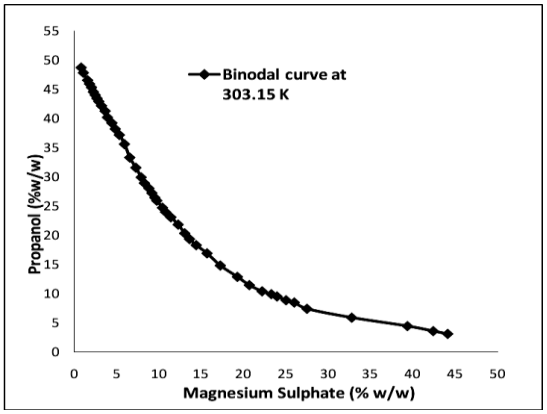

Figure 2: *Binodal curve for 1-propanol and MgSO4 system at 303.15^0 K*

The effects of various salt concentrations were studied at constant 1-propanol concentration. From the Figure 3, it was observed that the partitioning coefficient of anthocyanins increases with increasing salt concentration, which indicates that the anthocyanins present in the salt phase was expelled to the top phase due to the salting out phenomena of the system. The $MgSO_4$ salt molecules prefer to surround by the same molecules than the other molecules, hence the anthocyanins are shifted from salt rich bottom phase to alcohol rich top phase. The maximum partitioning was achieved between 30-32 % (w/w) $MgSO_4$ concentrations irrespective of 1-propanol concentration (Fig 3). Higher anthocyanins partitioning coefficient (Ka) between 66.27 to 70.36 is observed for the system contains 30 % (w/w) 1-

propanol and 30-32 % (w/w) $MgSO_4$ with the maximum yield of 98.60 % by removing 85.92 % sugars.

Even though the anthocyanin molecules are preferred to partition in top phase, the associated proteins and sugars are partitioned towards bottom phase due to the ionic effect of the salts present in bottom phase. Lower the partitioning coefficient of the sugar is better for the removal of sugar from the top phase where anthocyanin molecules are partitioned. It was observed that the partitioning coefficient of sugar decreased with increasing concentration of $MgSO_4$ (Fig. 3). Since the native pH of the system is in acidic range (3.9 pH), the proteins and the associated sugar molecules become more hydrophilic than the anthocyanin molecules and they prefer to stay in the salt rich phase.

Salt concentration more than 15 % w/w is required to get higher yield of sugars in bottom phase. Maximum sugar partitioning (Ks= 0.08) towards bottom phase (92.10 % yield) is observed with 25% w/w 1-propanol and 30-32 % w/w $MgSO_4$ system (Figure 4).

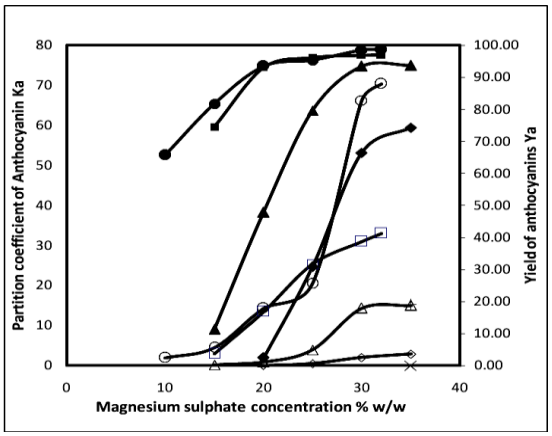

Figure 3: Effect of 1- propanol and magnesium sulfate concentration on partitioning co-efficient (K_a) and % yield of anthocyanins. K_a at different 1-Propanol concentration represented as (w/w) 30% (o), 25% (□), 20% (Δ), 15% (◊), 10% (x) and % yield as 30% (●), 25% (■), 20% (▲), 15% (♦), 10% (+)

However the partitioning coefficient was found to decrease at higher salt concentration above 32 % due to the salting out effect and the non-availability of the free volume of solvent to dissolve them. The trend observed in the present work is similar to the anthocyanin partitioning in Ethanol/ $(NH_4)_2SO_4$ ATPS reported by Hua et al [19].

Similarly, the experiments were conducted by varying the 1-propanol concentration at a constant salt concentration of 32 % (w/w) to study the effect of 1-propanol concentration on the partitioning of anthocyanins in 1-propanol/ $MgSO_4$ system. The anthocyanins partitioning coefficient was found to increase along with corresponding decrease in the partitioning coefficient of sugars, as the concentration of 1-propanol increases.

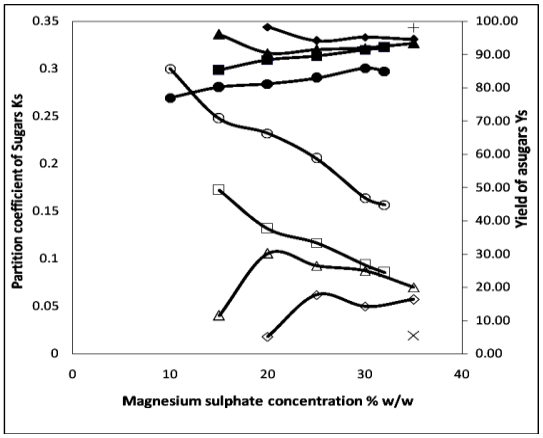

Figure 4: *Effect of 1- propanol and magnesium sulfate concentration on partitioning co-efficient (K_S) and % yield of sugars in bottom phase. K_s at different 1-Propanol concentration represented as (w/w) 30% (o), 25% (□), 20% (Δ), 15% (◊), 10% (x) and % yield as 30% (●), 25% (■), 20% (▲), 15% (♦), 10% (+)*

The impurities like sugars and proteins have higher interaction with the salt rich phase due to the higher hydrophilicity and hence the anthocyanins molecules are transferred to the alcohol phase. As the concentration of alcohol increases, the free volume available for the dissolution of anthocyanin in the system also increases. The combined effect of hydrophobic force and the free volume of the system favour the partitioning of anthocyanin in to the top phase and proteins associated with the sugars to the bottom phase. The maximum partitioning of anthocyanin (Ka of 70.36) with lower partitioning of sugar (Ks of 0.156) and was achieved at 30% (w/w) of 1-propanol (Figure 3 and 4).

4. Conclusions

An aqueous two-phase system comprising the hydrophilic alcohol and an inorganic salt i.e. 1-propanol and magnesium sulfate, is found to be suitable for the partial purification of anthocyanins from *Garcina indica* fruit rinds. The concentration of 30 % (w/w) 1-propanol and 30-32% (w/w) $MgSO_4$ system resulted in maximum yield of 98.60 % anthocyanin with partition co-efficient 70.36. Maximum sugar removal of 92.10 % towards the bottom phase was achieved with 25% (w/w) 1-propanol and 30-32 % (w/w) $MgSO_4$ system. This ATPS can be a suitable system for the extraction and preliminary purification of sensitive natural pigments. After appropriate optimization of the system and operating variables, it can be considered for the industrial scale purification of anthocyanins.

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