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Research Article

Optimization of Separation of Bioactive Compounds in Daily Foods by Aqueous Two-Phase Flotation Coupled With HPLC-DAD

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Abstract

In this study, we have used Aqueous Two-Phase Flotation (ATPF) in order to recovery and concentrate bioactive phenolic compounds found in foods. Firstly, the effects of the operational conditions on the ATPF performance have been investigated using a model solution of Gallic Acid (GA). The optimal conditions were: [ammonium sulfate] = 450 g/L, pH = 3.8, air flow-rate = 37.5 mL/min, time flotation = 120 min, initial organic phase volume = 10.0 mL and polyethylene glycol 400 as solvent. Next, experiments were carried out with aqueous extracts of daily foods and the ATPF phases were analyzed by HPLC-DAD. In terms of total phenolic compounds content, the highest percentage of separation was obtained for clove extract with 38.80 % due to transfer of gallic acid and eugenol to PEG rich-phase. The results showed that ATPF is a promising technique for be applied for separation and purification of phenolic compounds mainly in natural extracts.

Keywords: Aqueous Two-Phase Flotation; Concentration; Foods; Gallic Acid; Polyethylene Glycol; Recovery

Introduction

Phenolic compounds are secondary metabolites existing in fruits and vegetables generated from the pentose phosphate, shikimate and phenylpropanoid pathways [1,2]. Recent studies have shown the benefits of these compounds for health mainly due to their antioxidant activities leading to inhibitory effects against cardiovascular diseases [3], cancer [4] and Alzheimer [5,6]. Nowadays, new phenolic components showing outstanding properties are being continuously documented, most being isolated from industry as well as agricultural wastes [7]. Anthocyanins, flavonoids, stilbenes and phenolic acids are found in high concentrations in the blueberry [8], pomegranate seed [9] and pineapple residues [10] also in orange peel [11] and rice bran [12], among others. Such progress has been made possible by improved purification protocols, which are generally formed by liquid-liquid and/or adsorption techniques. Despite this, the use of these traditional techniques presents some inconveniences like the excessive expenditure of solvents and the multiples steps [13]. Aqueous Two-Phase Flotation (ATPF) is a special type of liquid-liquid extraction coming from the combination of solvent sublation (a non-foaming flotation technique) and aqueous two-phase extraction [14,15]. The ATPF is based on the transfer of the surface-active components from a salt-rich phase to a phase rich in a hydrophilic organic phase promoted, mainly, due to the mechanism of adsorption to bubbles [16].

Due to the higher separation efficiency, higher concentration coefficient and lower solvent quantity necessary the ATPF has been extensively used to recover and to purify biomolecules such as enzymes [17,18,19,20], penicillin G [16], baicalin [21], lyncomycin [22], puerarin [23], [24], ortho-phenylphenol [25], sodium chlorophyllin [26], liquiritin and glycyrrhizic acid [27]. In addition, the ATPF can be used as a preconcentration step for High Performance Liquid Chromatography (HPLC), as reported [15,28,29]. In this sense, the ATPF system composed of polyethylene glycol/ammonium sulfate was used to recovery and concentrate bioactive compounds from daily foods. Firstly, we exploited a model solution

containing Gallic Acid (GA), a typical phenolic compound. GA (3,4,5-trihydroxybenzoic acid) has a phenyl group (Figure 1) that gives active- surface enough to absorb onto the gas-liquid interface. Also, it shows good solubility in polar solvents. Next, using the optimal conditions, the ATPF performance was evaluated with different aqueous food samples and bioactive compounds were identified by HPLC in solvent-rich phase after process.

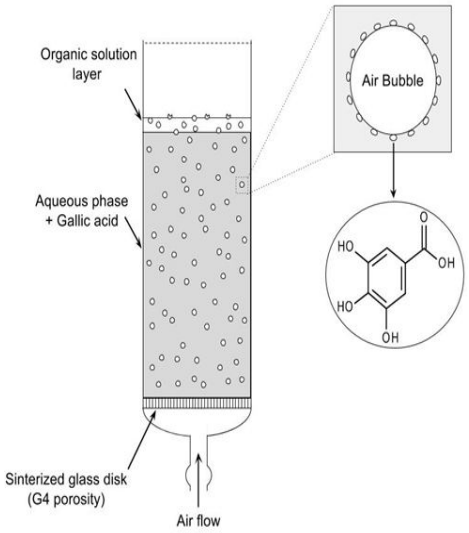

Figure 1: Illustration of the apparatus used in the ATPF. Marked is a typical bubble with the sublate (GA) adsorbed.

Materials and Methods

Chemicals and Apparatus

The phenolic compounds catequin, eugenol, gallic acid, quercetin, syringic acid, vanillic acid and vanillin were acquired by Sigma-Aldrich (MO/USA). Polyethylene Glycol (PEG) showing molar mass of 400, 1500, 4000, 6000 Da, ethanol, isopropanol, n-propanol, n-butanol and ammonium sulfate were purchased from Synth (São Paulo/Brazil). Beer, clove, garlic and potato were acquired from a local market. Ultra-purified water used in the ATPF experiments was obtained using a MiliQ system. The apparatus used to carry out the ATPF experiments is shown in (Figure 1). It consisted of a glass-column of 2.64 cm (i.d.) diameter and capacity of 180 mL. The column was equipped at the bottom with a sintered glass disc of G4 porosity coupled to a compressed air system that was used to supply the bubbles to the column. The air flow-rate was measured by using a rotameter RMA-1-SSV (Dwyer, USA) coupled to the compressor by a regulator.

Optimization of Separation Parameters

At beginning of this study, we have investigated the influence of the operational conditions on the ATPF performance. In this approach, the type of hydrophilic solvent, the pH of bottom phase, the concentration of ammonium sulfate, the sublate concentration, the air flow-rate, the flotation time as well as the volume of organic phase were evaluated in order to increase the recovery and the concentration of the sublate using the ATPF system. The sublate concentration as well as the percentage of PEG solution were fixed at 500 µg/mL and 80 % (w/w), respectively. In this case, the separation efficiency in percentage (E%), the distribution ratio (D) and the concentration coefficient (α), as shown in equation (1-3) were used to evaluate the ATPF performance.

$$E\% = \left(1 - \frac{C_w}{C_{wi}}\right) \times 100 \quad (1)$$

$$D = \frac{C_{PEG}}{C_w} \quad (2)$$

$$\alpha = \frac{C_{PEG}}{C_{wi}} \quad (3)$$

Where C_w is the Gallic concentration at salt-rich phase for a given time t, C_{wi} is the initial gallic acid concentration at salt-rich phase and C_{PEG} is the gallic acid concentration at PEG-phase for a given time t. Then, experiments were performed with food samples, which were prepared with deionized water in the following mass loading: 50 % (w/v) for beer, 1 % (w/v) for clove, 5 % (w/v) for garlic and 5 % (w/v) for potato. The operational conditions of the ATPF used were defined according to the previous experiments with the model solution. All experiments were carried out in duplicate at room temperature. Statistical analysis was carried out using the Tukey's test by means of the software Statistica 7.0 (Stat Soft, Microsoft/USA) at a significance level of 95 % ($p < 0.05$).

Determination of Phenolic Compounds Concentration

In ATPF assays using a model solution of GA, the quantification of sublate concentration in the aqueous and organic phases was carried out using UV spectrometry at $\lambda = 262$ nm using a calibration curve. The effects of salt and organic solvents concentration as well as the pH were considered on the record. In the assays involving extracts of foods, the Folin-Ciocalteau was used for quantification of total phenolic compounds, as shown in Song FL, Gan RY, Zhang Y, Xiao Q, Kuang L, et al. [30] with some modifications. Firstly, 0.5 mL of samples was reacted with 2.5 mL of 2 M Folin reagent during 5 min, and then the mixture was added 2.0 mL of 75 g/L sodium carbonate solution. The readings were performed on the ThermoSpectronic Genesys 10 UV/ Vis spectrophotometer at 760 nm after 20 min of incubation and GA was used as standard.

HPLC-DAD Analysis

The identification of phenolic compounds was carried out by HPLC using the chromatographic platform Accela (Thermo Scientific, USA), the system has a diode array detector as well as an automatic sample injector and automatic fraction collector. In these experiments a Hypersil GOLD RP-C18 column with 50 x 2.1 mm (Thermo Scientific, USA) was used. The elution was performed

at a flow rate of 0.6 mL/min using as mobile phase a mixture of water/acetic acid (99.0:1.0 v/v) (A) and acetronitrile (B) at 25 oC. Detection was performed at 280 and 255 nm and the samples were eluted by the following gradient: 100 % of A as initial condition, 90 % of A and 10 % of B in 1 min, 30 % of A and 70 % of B in 3 min, 100 % of B in 4 min and 100 % of A in 5 min.

Results And Discussion

Effect of the Type of Organic Solvent

For ATPE, the choice of the type of organic solvent plays a key role for the formation of a biphasic system. Also, it affects the partition behavior of the target molecule (Han et al. 2014) [15].

Therefore, a preliminary investigation was carried out in order to observe the effect of the type of organic solvent on the recovery and concentration of GA using ATPF. In this case, it was used as organic phase aqueous solutions of PEG (molar mass of 400, 1500, 4000 and 6000) and alcohols (ethanol, n-propanol, isopropanol and n-butanol). The experiments using PEG 1500, PEG 4000 and PEG 6000 showed higher viscosity due to the organic phase also they showed foam at the top of the column. These facts hampered the record of these experiments. For the experiments using the PEG 400 none of these effects were observed. As can be seen in (Table 1), the values for the separation efficiency as well as for the distribution ratio for the GA were higher for the PEG than for the alcohols solutions. The strongest polarity of PEG allows a more easily dissolution of the sublate showing polar structures than given better performance in ATPF [16,23,27]. Then, the best performance shown by the organic solvent was that shown by the PEG 400.

Type of hydrophilic solvent	Separation efficiency (E%)	Concentration coefficient (α)	Distribution ratio (D)
PEG 400	49.68±1.57^{a}	2.70±0.09^{a}	5.18±0.34^{a}
ethanol	17.41±1.77^{b}	1.78±0.17^{bc}	2.15±0.15^{c}
isopropanol	19.61±1.00^{b}	1.88±0.00^{b}	2.30±0.01^{b}
n-propanol	18.90±0.15^{b}	1.55±0.05^{c}	1.91±0.06^{d}
n-butanol	13.55±1.86^{c}	1.35±0.04^{d}	1.53±0.05^{e}

Table 1: ATPF performance for the different organic solvents assayed. Experiments were carried out at pH = 4.0, [ammonium sulfate] = 450 g/L, % organic solvent (w/w) = 80.0, [GA] = 500 µg/mL, air flow-rate = 25 mL/min, t_f= 30 min, volumetric ratio = 0.1. The lowercase a, b, c, d and e are used for performance comparison between organic solvents; values with the same letter in one column are not significantly different from each other ($p < 0.05$).

Effectof the pH of Aqueous Phase

The pH plays an important role in the ATPF system since it can reduce the stability of the sublate in the aqueous phase as well as increase the solubility in the organic phase [14,21,24,27].

In this study, the effect of the pH of the aqueous phase was assayed in the range from 2.0 to 8.0 by adding HCl and NaOH. It was observed that PEG/ammonium sulfate showed just a single phase for pH lower than 2.0. Also, occurred the decomposition of $NH4^{+}$ to NH3.H2O (with the system showing a strong ammonia odor) for pH higher than 7.0. According to (Figure 2a), the efficiency of separation, the concentration coefficient and the distribution ratio were kept constant for the pH range from 2.0 to 4.0, then reducing to higher values of the pH. The gallic acid has four hydroxyls able to become ionizable with pKa values of 4.0, 8.7, 11.4 and >13.0) [31]. Overall results for the pH considered were about fifty percent for the efficiency of separation, approximately 2.7 for the concentration coefficient and 4.8 for the distribution ratio.

In the experiments with pH ≤ 4.0, probably most Gallic acid molecules were in a non-acidified mode then likely to adsorb onto the bubble. Also, since the aqueous solution of ammonium sulfate and GA showed pH near 4.0 without need to add acid or base, we have kept the pH value for the next steps.

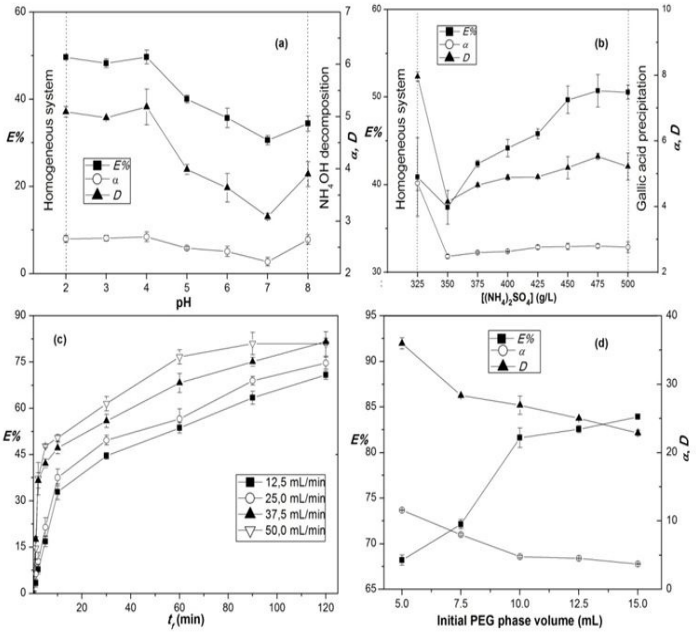

Figure 2a-2d: Effect of some operational conditions on the ATPF performance: **(a)** pH of the Bottom Phase, **(b)** Ammonium Sulfate Concentration of the Bottom Phase, **(c)** Initial Gallic Acid Concentration, **(d)** Time Flotation and Air Flow-Rate.

Effect of the Ammonium Sulfate Concentration

In order to keep higher values of sublate concentration in the organic phase during the ATPF process, the aqueous phase volume should higher than organic [18]. Thus, the salt concentration in the aqueous phase plays a key role to the existence of two immiscible phases in the PEG/ammonium sulfate system [16,21,23,27]. Therefore, the influence of the ammonium sulfate concentration in

the ATPF was investigated in the range from 350 g/L to 500 g/L, (Figure 2b) shows the results obtained. As can be seen in (Figure 2b), the highest values for separation efficiency, separation coefficient as well as distribution ratio were obtained for those experiments in which the ammonium sulfate concentration were highest. Similar to the influence of the pH, the addition of salt to the aqueous phase reduced the GA solubility as well as led to an increase on the sublate adsorption to the bubbles. For the experiments in which the ammonium concentration was 325 g/L it was observed a vigorous mixing at aqueous-organic interface. This, in general, led to a loss of volume for the organic phase and, consequently, to higher values of concentration coefficient and distribution ratio, 4.70 and 7.96, respectively. Also, operating the system with ammonium concentration higher than 500 g/L it was observed a marginal increase of the ATPF performance due to the GA precipitation. Therefore, based on these results and considering the lower quantity of ammonium sulfate used, we have chosen the value of 450 g/L for this salt concentration.

Effect of the Air Flow-Rate and Flotation Time

The air flow-rate is an important variable to ATPF once it affects the gas-liquid interface area by unity of aqueous phase volume by minute. Thus, it is closely linked to the flotation [19,32]. Therefore, both the effect of the air flow-rate and flotation time on the separation efficiency of the ATPF system was investigated as shown in (Figure 2c). We highlight that in this study the air flow-rate ranged from 12.5 mL/min to 50 mL/min and the efficiency values were estimated from 1 min until 120 min. As can be seen, the separation efficiency for the GA increased to both a higher flow-rate and a higher flotation time. It can be seen that to all curves the separation efficiency can be divided in two regions. Firstly, between 5-10 minutes in which there is an abrupt increment in this parameter, i.e. highest rate of separation. Secondly, after this fast rate there is a reduction on the mass transfer until 120 minutes. Even though it can allow a fast equilibrium in the system, higher gas flow-rate (≥50 mL/min) should be avoid once it induces to a turbulent mixing at aqueous-organic interface as well as to accumulation of bubbles at top of the column [23]. Therefore, an air flow-rate of 37.5 mL/min and the flotation time of 120 min were chosen for the next step.

Effect of theOrganic Phase Volume

According to Sebba [33], studying a system for removing ions by complexation using a surfactant, the performance of solvent sublation is independent of the volumetric ratio used. Also, Caballero M, Cela R, Perez-Bustamante JA [34] reported that the effect of the organic phase on the sublation performance is insignificant until the saturation of the phase by the sublate. However, recent reports [16,17,35] point that the increase of the height of the top layer allows a higher solvation of the sublate and thus a higher separation efficiency. In order to clarify the effect of the variable in the ATPF system, we have changed the organic phase volume from 5.0 mL to 15.0 mL. As it can be seen in (Figure 2d), the lower the initial PEG phase volume the lower the separation efficiency. However, for the distribution ratio a contrary behavior can be seen. For the concentration coefficient, value of initial PEG volume higher than 10 mL does not change considerably this parameter. In order to ensure simultaneously a good separation and the GA concentration from the aqueous phase to the organic, the initial PEG phase volume of 10.0 mL was chosen. Overall, for this condition a separation efficiency of 86% and values of concentration coefficient and distribution ratio of 4.70 and 23.0 were obtained, respectively. In summary, the above results showed that the optimum conditions to operate the ATPF system was use the PEG 400 as the organic solvent, to keep the pH of organic phase at 4.0, the ammonium sulfate concentration in the aqueous phase with 450 g/L, GA concentration of 500 µg/mL, an air flow-rate of 37.5 mL/min, a flotation time of 120 min and an initial PEG phase volume of 10.0 mL. Through the HPLC-DAD analysis, the phenolic compounds were identified by overlapping with the peaks found in the standard chromatogram (Figure 3).

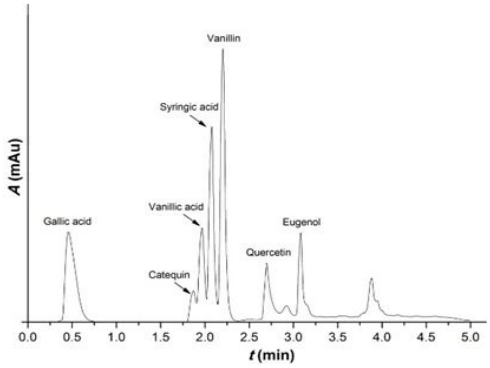

Figure 3: Effect of Initial PEG Phase Volume on the ATPF Performance. Experiments Were Carried Out with [Ammonium Sulfate] = 450 g/L, pH = 4.0, % PEG (w/w) = 80.0, [GA] = 500 µg/mL, Air Flow-Rate = 37.5 mL/min, t_f= 120 min.

Application of ATPF for Separating Bioactive Compounds from Foods

To demonstrate the applicability of the technique to real samples, in this section the performance of ATPF was evaluated in the separation and identification of bioactive compounds of different daily foods. Under optimal conditions, the experiments were conducted with aqueous samples of beer, clove, garlic and potato. In addition, as before, their results were expressed in terms of E %, α e D. According (Table 2), the responses assume different values in each extract, which can be attributed to the difference in the total phenolic content as well as in the structures of the present species. Another aspect to be observed is the positive correlation between the initial TPC concentration and the performance of the technique, since beer and clove samples of 438.97 and 1035.25

mg GAE/mL, respectively, reached the highest of E %, 36.72 and 38.80. Alternatively, experiments with garlic (287.73 mg GAE/mL) and potato samples (285.56 mg GAE/mL) obtained minor E % values, 21.44 and 22.37, respectively. In this section, we compare the ATPF system with the traditional ATPE in relation to separation and concentration of GA from a model solution. To ensure the two-phase formation, the volumetric ratio of the ATPE system was 1:4 (volume of organic phase /volume of aqueous phase), while the volumetric ratio for the ATPF 1:10. When the GA model solution was used, as can be seen in (Table 2), the values for separation efficiency and distribution ratio were lower to ATPF (E%= 81.62; D = 26.94) compared to those shown by the ATPE system (E%= 96.21; D = 54.81). However, the concentration coefficient for the ATPF system (α = 4.76) was more than 1.8-fold that shown by the ATPE (α = 2.53). On the other hand, the ATPF system showed better performance to both concentration coefficient and distribution ratio (D= 27.92; α= 5.72) than ATPE (D= 22.08; α = 2.63) when the guava leaves extract was used, with GA content equal to 37.36 µg/mL. Also, the process separation efficiency was slightly lower in the ATPF (E%= 80.28) compared to ATPE (E%= 88.73). Therefore, the results showed that ATPF can efficiently be used to recovery and concentration of phenolic compounds of natural extracts, not only GA as can be seen in (Figure 4b).

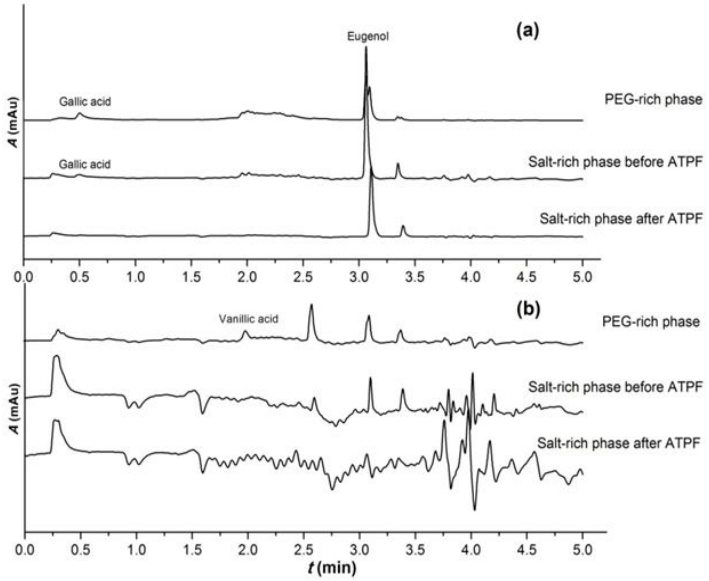

Figure 4: HPLC profiles of the phases involved in the ATPF using model solution (a-c) and guava leaves extract (d-f). Salt-rich phase before the ATPF system (a, d), salt-rich phase after the ATPF (b, e) and PEG-rich phase after ATPF (c, f).

Besides, GA and others phenolic compounds such as tannins and flavonoids are present in great quantity in the guava leaves extract [36]. Compared to other separation techniques the complexity of the beer and potato samples hindered the accurate detection of any patterns in the phases of the ATPF system (data not shown). However, it was possible to observe that gallic acid, eugenol and vanillic acid were strongly captured by the PEG-rich phase in the other samples, as shown in (Figure 4). While concentrations of gallic acid and eugenol reached 0.207 mg/mL and 3.351 mg/mL, respectively, in the top phase of the experiments using clove extract, vanillic acid was concentrate from garlic extract to 0.025 mg/mL. In summary, ATPF shows some advantages such as simple operation, lower cost, softer conditions, lower PEG quantity need as well as higher concentration coefficient.

Response variable	Beer	Clove	Garlic	Potato peel
Separation efficiency (E%)	36.72±0.13	38.80±0.11	22.37±0.13	21.44±0.38
Concentration coefficient (α)	2.19±0.01	2.31±0.01	1.33±0.01	1.28±0.02
Distribution ratio (D)	3.00±0.00	7.35±0.98	1.39±0.00	1.33±0.03

Table 2: Performance between ATPE (1:4) and ATPF (1:10) for the Model Solution and Guava Leaves Extract.

Conclusion

A PEG/ammonium sulfate system has been successful used to separate and concentrate phenolic compounds by means of the ATPF process. The effect of some process variables on the separation efficiency, concentration coefficient as well as distribution ratio showed that optimum condition was given when PEG 400 was used as the organic solvent, pH of the aqueous phase was 4.0, ammonium sulfate concentration was 450 g/L, GA concentration was 500 µg/mL, the air flow-rate was 37.5 mL/min with a flotation time of 120 minutes and the volume of organic phase was 10 mL. Overall, the ATPF system working with a model solution achieved a separation efficiency of 81.62 % with a concentration coefficient of 4.76. In relation to compounds identified by HPLC-DAD, gallic acid and eugenol were recovered in clove extract, while in garlic extract vanillic acid was captured. Finally, this technique has a quite good potential for separating as well to concentrate bioactive compounds of natural extracts.

Acknowledgements

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