Maderas. Ciencia y Tecnología
ISSN: 0717-3644
ananias@ubiobio.cl
Universidad del Bío Bío
Chile

Xavier, Lucía; Freire, M. Sonia; Vidal-Tato, Isabel; González-Álvarez, Julia

RECOVERY OF PHENOLIC COMPOUNDS FROM EUCALYPTUS WOOD WASTES USING ETHANOL-SALT-BASED AQUEOUS TWO-PHASE SYSTEMS

Maderas. Ciencia y Tecnología, vol. 19, núm. 1, 2017, pp. 3-14
Universidad del Bío Bío
Concepción, Chile

ISSN impresa 0717-3644
ISSN online 0718-221X
Maderas. Ciencia y tecnología 19(1): 3 - 14, 2017
DOI: 10.4067/S0718-221X2017005000001

RECOVERY OF PHENOLIC COMPOUNDS FROM EUCALYPTUS WOOD WASTES USING ETHANOL-SALT-BASED AQUEOUS TWO-PHASE SYSTEMS

Lucía Xavier[1], M. Sonia Freire[2], Isabel Vidal-Tato[2], Julia González-Álvarez[2,♠]

ABSTRACT

In this work the partition behavior of phenols using ethanol-salt-based aqueous two phase systems (ATPS) was evaluated. The aim was the recovery of phenolic compounds with antioxidant capacity from eucalyptus wood industrial wastes. Experiments were planned to study the influence of several parameters on phenols partition, including type of inorganic salt (ammonium sulphate, a mixture of monopotassium phosphate and potassium diphosphate and potassium diphosphate), tie-lie lenght (TLL), volume ratio (V_r), settlement time and temperature. Phenols could be recovered preferently from the top or bottom phases depending on the salt used. It was demostrated that tie-lie lenght, volume ratio and temperature had influence on phenols partition. The highest total phenols yield 5,36 mg gallic acid equivalent (100 mg oven dried wood)$^{-1}$ and FRAP antioxidant activity, 20 mmol AAE (100 g oven dried wood)$^{-1}$, was obtained using ATPS formed by 40.6% (w/w) ethanol and 12% (w/w) of ammonium sulphate at 65ºC. Analysis of the extract by RP-HPLC-ESI-TOF confirmed the presence of the phenolic compounds with potential antioxidant activity, namely, ellagic acid, myricetin 3-O-rhamnoside and quercetin 3-glucoside.

Keywords: Alcohol/salt system, aqueous two-phase systems, natural antioxidants, phenolic compounds, wood extraction.

INTRODUCTION

Aqueous two-phase system (ATPS) extraction is a liquid-liquid extraction technique that has been used for the extraction and partial purification of a wide variety of biomolecules (Rosa *et al*. 2010, Espitia-Saloma *et al*. 2014, Reis *et al*. 2014, Wu *et al*. 2014). ATPS form when an aqueous solution exceeds a critical concentration of two water soluble, but mutually incompatible, components (Albertsson 1986). This technique is now recognized as an alternative for the fractionation of biomolecules due to economic advantages and technological simplicity (Rosa *et al*. 2010, Espitia-Saloma *et al*. 2014). Additionally, ATPS could be considered as an integrated process, in which the insoluble components can be removed while at the same time the target product is purified (Rosa *et al*. 2010). This leads to lower energy costs as the steps of operation are reduced, getting more concentrated and purified biomolecules (Rosa *et al*. 2010).

There are many types of ATPS: polymer-salt, polymer-polymer, alcohol-salt, ionic liquid-salt, etc. (Rosa *et al*. 2010, Simental-Martínez *et al.* 2014). The most commonly used systems are based on polymer-polymer or polymer-salt. These systems have some drawbacks such as the high cost of polymers, the low segregation of the phases and the complications associated with recycling components (Ooi *et al.* 2009). In recent years alternative ATPS including alcohol-salt, ionic liquid-salt and micel-

[1]Instituto de Ingeniería Química. Facultad de Ingeniería. Universidad de la República Oriental del Uruguay, Montevideo, Uruguay.
[2]Departamento de Ingeniería Química, Escuela Técnica Superior de Ingeniería, Universidade de Santiago de Compostela, Santiago de Compostela, Spain.
♠Corresponding author: julia.gonzalez@usc.es
Received: 17.02.2016 Accepted: 01.10.2016

lar for recovery of biomolecules have been studied (Espitia-Saloma *et al*. 2014). Particulary, ATPS formed by alcohol and salts are of low cost and allow an easy recovery of the alcohol by evaporation or distillation (Reis *et al*. 2014). Moreover, alcohol-salt ATPS are of low viscosity, and usually led to high extraction efficiencies and purification levels in a single-step procedure (Reis *et al*. 2014). The low viscosity is industrially relevant as the cost of pumping fluids is reduced (Simental-Martínez *et al*. 2014). Alcohol-salt systems have received less attention because many biomolecules (such as proteins and enzymes) are incompatible with organic solvent phase, which can inactivate or denature the biomolecules (Ooi *et al.* 2009).

ATPS composed of an organic solvent and an inorganic salt have been used to extract anthocyanins (Wu *et al*. 2014), rutin (Reis *et al*. 2014), lipase (Ooi *et al.* 2009), fucoxanthin (Gómez-Loredo *et al.* 2014), ascorbic acid (Reis *et al*. 2012), among others. However, at present, there is no literature regarding the partition behaviour of phenols applying alcohol-salt ATPS directly over a raw material without purification.

Simple phenols, polyphenolics and phenolic acid derivatives are present in many plant sources and can act as natural antioxidants for various uses (Moure *et al*. 2001). The search for cheap, renewable and abundant sources of antioxidant compounds is attracting worldwide interest. In this context, extensive research is being done focused on the exploitation of wastes that are abundant and low-cost, such as agricultural, forest or industrial residues. The forest industry generates large amounts of wastes of different compositions, which generally are burnt or wasted, although several extractable compounds could be recovered and used as high added value products. Several authors have reported the presence of low molecular weight phenolics compounds with demonstrated antioxidant capacity (gallic, vainillic and ellagic acids and syringilic, sinapic and vainillic aldehydes) in different parts of eucalyptus species (Conde *et al*. 1996, Eyles *et al.* 2003). In this research, ATPS formed by ethanol and different inorganic salts were investigated in order to study the partition behaviour of phenolic compounds from eucalyptus wood industrial wastes. The objetive was to investigate the effect of the salt used, tie line lenght (TLL), volume ratio (V_r) and temperature on the extraction efficiency and partition of phenols.

MATERIALS AND METHODS

Raw Material

Eucalyptus (*Eucalyptus globulus*) wood veneer trimmings were supplied by the company Aserpal S.A. (Grupo Losán S.A., Galicia, NW Spain) specialized in the elaboration of fine wood surfaces. Eucalyptus wood was pretreated in water at 75ºC for 16 h and, afterwards, veneers were obtained from slicing a wood block lengthways. The waste material used in this work were the trimmings which are obtained by squaring the veneers. They were air-dried till humidity reached equilibrium and prepared in pieces of 0,60 mm x 10 mm x 20 mm.

Chemicals

Monopotassium phosphate and sodium acetate 3-hydrate were purchased from Maiilnckrodt (Dublin, Irland), potassium diphosphate from Riedel-de Haën (Seelze, Germany). Sodium carbonate, acetonitrile, ethanol, ammonium sulphate, gallic acid, Folin-Ciocalteu's phenol reagent and ascorbic acid from Merck (Darmstadt, Germany). Iron(III) chloride hexahydrate from J.T.Baker (USA). Hydrochloric acid from Carlo Erba (Sabadell, Spain). TPTZ (2,4,6-tri(2- pyridyl)-S-triazine) from Fluka (Steinheim, Germany). Acetic acid from Dorwil (Buenos Aires, Argentina). HPLC standards: (+)-catechin hydrate, (-)-epicatechin, procyanidin B2, quercetin-3-O-rhamnoside, ellagic acid, isorhamnetin, kaempferol and tannic acid were purchased from Fluka (Steinheim, Germany); gallocatechin was from Sigma (Steinheim, Germany) and (-)-gallic acid was from Riedel-de Haën (Seelze, Germany).

Extraction and separation procedure

The three ATPS were selected using the phase diagram of the ternary systems found in literature: 1) water + ethanol + a mixture of monopotassium phosphate and potassium diphosphate (Reis *et al.* 2012), 2) water + ethanol + potassium diphosphate (Reis *et al.* 2012) and 3) water + ethanol + ammonium sulphate (Wang *et al.* 2010).

A predetermined quantity of salt was dissolved in water and the pH was measured (pH_l). Then, the corresponding quantity of ethanol according to the composition selected (Table 1) was added into the salt aqueous solution to form the ATPS. Finally, the biomass was added to the solution at a solid-liquid ratio of 1/10. The amount of liquid used in the extraction process was 50 g. The extractions were performed in a water bath with orbital shaking (UNITRONIC-OR, Selecta, Spain) at a shaking rate of 90 rpm.

After 90 min, the wood pieces were removed and the phases were separated under gravity in a separatory funnel. In order to establish the settlement time, phase separation was performed using different settlement times (1, 6 and 15 h). Additionally, the influence of the type of inorganic salt, tie lie length (TLL), volume ratio of both phases (V_r=volume top phase/volume bottom phase) and extraction temperature (25, 45 and 65ºC) were studied. All experiments were carried out in triplicate.

The partition coefficient of phenols, K, was determined as the ratio of the total phenol concentration in the top phase to that in the bottom phase, according to the following equation:

$$K = \frac{\left[GAE\ L^{-1}\right]_{TP}}{\left[GAE\ L^{-1}\right]_{BP}} \qquad (1)$$

where $[GAE\ L^{-1}]_{TP}$ is the total phenols concentration in the top phase and $[GAE\ L^{-1}]_{BP}$ is the total phenols concentration in the bottom phase.

The extraction efficiency (%EE), defined as the percentage ratio of the total phenol content in the top or bottom phase to the sum in both phases, was determined as follows:

$$\%EE = \frac{\left[GAE\ L^{-1}\right]_{jP} \times V_{jP}}{\left[GAE\ L^{-1}\right]_{BP} \times V_{BP} + \left[GAE\ L^{-1}\right]_{TP} \times V_{TP}} x\,100 \qquad (2)$$

where V_{TP} and V_{BP} are the volume of the ethanol rich-phase and inorganic-salt-rich phase, respectively and j denotes the top or the bottom phase depending whether the phenols migrate to the former or to the latter.

Table 1. Systems selected for the evaluation of the partition behavior of phenols.

Ethanol-$(NH_4)_2SO_4$						
System	Ethanol %(w/w)	$(NH_4)_2SO_4$ %(w/w)	H_2O %(w/w)	TLL %(w/w)	V_r	pH_1
1	25,0	19,5	55,5	45	1,0	5,2
2	26,0	20,0	54,0	52	1,0	5,3
3	27,0	20,5	52,5	57	1,0	5,2
4	15,0	28,0	57,0	57	0,1	5,2
5	40,6	12,0	47,4	57	2,8	5,2
Ethanol- KH_2PO_4/K_2HPO_4						
	Ethanol %(w/w)	KH_2PO_4/K_2HPO_4 %(w/w)	H_2O %(w/w)	TLL %(w/w)	V_r	pH_1
6	22,0	28,0	50,0	21	1,1	7,4
7	24,0	30,0	46,0	28	1,0	7,3
8	27,0	31,0	42,0	32	1,3	7,4
9	27,0	20,0	53,0	27	2,3	7,4
Ethanol- K_2HPO_4						
	Ethanol %(w/w)	K_2HPO_4 %(w/w)	H_2O %(w/w)	TLL %(w/w)	V_r	pH_1
10	18,0	21,5	60,5	38	1,1	9,6
11	20,0	22,5	57,5	46	1,0	9,7
12	24,0	23,5	52,5	57	1,1	9,8
13	35,0	9,3	55,7	46	6,4	9,7
14	10,0	31,3	58,7	46	0,3	9,7

TLL: tie line length; V_r: volume ratio of both phases

Total phenols content

Total phenols content was determined by the Folin-Ciocalteu method (Singleton *et al.* 1965): to 0,5 mL of an aqueous solution of the extract 2,5 mL of Folin-Ciocalteu reagent previously diluted with water (1:10, v/v) and 2 mL of a 75 g L^{-1} sodium carbonate aqueous solution were added. The mixture was kept for 5 min at 50ºC and, after cooling, the absorbance was measured at 760 nm in a Jasco V-530 UV-visible spectrophotometer. The phenols content was calculated as gallic acid equivalents (GAE) from the calibration curve of gallic acid standard solutions (2-40 mg mL^{-1}). The results were expressed as total phenols concentration (mg GAE L^{-1}) and as total phenols yield (TPY, mg GAE (100 mg of oven-dried (o.d.) wood)$^{-1}$), which takes into account both the amount (g) and the phenols concentration of each phase. All analysis were carried out in triplicate.

Ferric reducing antioxidant power (FRAP)

FRAP analysis was made according to Szollosi and Szollosi Varga (Szollozi *et al.* 2002). Briefly, 3,0 mL of freshly prepared FRAP reagent (2,5 mL of a 10 mmol L^{-1} TPTZ solution in 40 mmol L^{-1} HCl 2,5 mL of 20 mmol L^{-1} $FeCl_3$ and 25 mL of 300 mmol L^{-1} acetate buffer, pH 3,6) was added to 0,1 mL of diluted extract and incubated 5 min at 25 ºC. The absorbance was recorded at 593 nm against a blank containing 0,1 mL of distilled water. The results were expressed as (mmol of L-ascorbic acid equivalent

(AAE) (100 mg of oven-dried (o.d.) wood) $^{-1}$). The calculations were made using a standard curve of L-ascorbic acid standard solutions (0,1-0,8 mmol L^{-1}).

RP-HPLC-ESI-TOF mass spectrometry analysis

The selected samples were analyzed for its phenolic composition by RP-HPLC-ESI-TOF using an HPLC Agilent Technologies 1100 (Agilent Technologies, Germany) and the Bruker Microtof ESI-TOF analyzer (Bruker Daltonics, Germany). Phenolic compounds were separated using a Zorbax Eclipse XDB-C18 4,6 x 150 mm, 5 µm column (Agilent Technologies, Germany). A binary gradient of 2% aqueous acetic acid for mobile phase A and 0,5% acetic acid in water-acetonitrile (1:1, v:v) for mobile phase B at a flow rate of 1 ml min^{-1} was applied. The linear gradient was from 10 to 55% B for the time range from 0 to 50 min, from 55 to 100% B from 50 to 60 min and from 100 to 10% B from 60 to 65 min. The mass spectrometry analysis was performed in negative ion mode under the following conditions: analyzer TOF (time-of-flight), ionization source ESI (electrospray), capillary voltage at + 4,5 kV, nebulizer gas pressure at 32 psi, dry gas flow at 12 L min^{-1}, injection volume 10 µl. The sample and the standards were dissolved in water to a concentration in the range 100-200 ppm.

Statistical analysis

Data were reported as mean ± SD (standard deviation) of triplicate determinations. The existence of significant differences among the results for total phenols concentration and total phenols yield depending on the extraction conditions was analysed. For it, one-way analysis of variance (ANOVA) was used followed by the Tukey´s HSD or Dunnett T3 test, depending on whether equal variances could be assumed or not. All statistical tests were performed at a 5% significance level using PASW Statistics 18 software.

RESULTS AND DISCUSSION

Preliminary studies. Influence of settlement time on phenols recovery

The time required for the phases to separate could vary for different systems. It depends on the difference in density between phases and their viscosities, and the time needed for the small droplets, formed during shaking, to coalesce into larger drops (Albertsson 1986). In order to study the time at which the partition of phenols stabilizes, a system was selected for each type of ethanol-salt ATPS (Systems 5, 7 and 11, Table 1) and extraction was performed at 25ºC, using three different settlement times (1, 6 and 15 h) for phase separation.

Table 2 shows the total phenols yield of the separated phases. Except for the top phase of system 7, there was no significant difference among the total phenols yield at different settlement times. Then, for the next experiments a settlement time of 1 hour was considered enough to reach the thermodynamic equilibrium between phases. Comparing the results obtained for the three ATPS studied the highest TPY was obtained for the top phase of the ethanol/$(NH_4)_2SO_4$ system (2,81 mg GAE (100 mg of o.d. wood)$^{-1}$).

Table 2. Influence of settlement time on partition behavior of phenols in ethanol-salt ATPS at an extraction temperature of 25ºC.

System	Settlement time (h)	TPY_{TP} (mg GAE (100 mg o.d. wood) $^{-1}$)	TPY_{BP} (mg GAE (100 mg o.d. wood)$^{-1}$)
5	1	2,62 ^{a}±0,10	0,41^{a}±0,01
	6	2,81 ^{a}±0,10	0,43^{a}±0,03
	15	2,67^{a}±0,06	0,38^{a}±0,00
7	1	0,20^{a}±0,01	0,60^{a} ±0,13
	6	0,15^{b}±0,00	0,58^{a} ±0,00
	15	0,14^{b}±0,01	0,44^{a}±0,02
11	1	0,09^{a}±0,00	0,23^{a} ±0,02
	6	0,09^{a} ±0,01	0,23^{a}±0,04
	15	0,09^{a} ±0,01	0,21^{a}±0,04

Values are presented as mean±SD (n=3). In each column, values with different letters are significantly different ($p<0,05$).TPY: Total phenols yield; TP: top phase; BP: bottom phase.

Effect of the inorganic salt in phenols partition and recovery

To analyze the partition behavior of phenolics compounds in ethanol-based ATPS, three inorganic salts were studied: $(NH_4)_2SO_4$, a KH_2PO_4/K_2HPO_4 buffer solution and K_2HPO_4. The inorganic salts in aqueous solution confer different pH values to the ATPS, which are around 5,2; 7,4 and 9,7 for $(NH_4)_2SO_4$, KH_2PO_4/K_2HPO_4 and K_2HPO_4, respectively (Table 1). As noted in Figure 1, the ATPS studied present different behavior in phenols partitioning. The partition coefficient (K) varied between 0,14 and 2,97 when different salts were employed. At acid conditions (ethanol/$(NH_4)_2SO_4$) phenols migrated to the top phase (K>1) whereas at neutral and alkaline conditions (ethanol/KH_2PO_4/K_2HPO_4 or ethanol/K_2HPO_4) phenols partitioned to the bottom phase (K<1).

This phenomenon could be associated to the phase polarities coupled to the charged or not charged nature of some phenolics compounds (Shen *et al.* 2006). In the systems studied, there is a predominant hydrophobic phase (the ethanol rich one, the top phase) and a more hydrophilic phase (the salt rich one, the bottom phase). Many phenolic compounds present amphoterism (eg: gallic acid, ellagic acid, etc). Molecular form of phenolic compounds is more hydrophobic than the dissociation form, and when pH is below the pKa the molecular form is the predominant one. The interaction of the compounds with the upper or bottom phase will depend on the form of the molecules (Shen *et al.* 2006). It is expected that a higher affinity of the phenolic compounds by the hydrophobic upper phase would be achieved working at low pH.

Among the ATPS based on phosphate salts, phenols partition with KH_2PO_4/K_2HPO_4 ATPS was slightly better than with KH_2PO_4 ATPS (Figure 1). This difference must be related with the salting in and salting out behavior of molecules in aqueous media (K_2HPO_4 tends to fit into the salting in regime) (Reis *et al.* 2012). With respect to phenols recovery, the highest yield was achieved for the bottom phase of the ethanol/ KH_2PO_4/K_2HPO_4 ATPS 0,60 mg GAE per 100 mg o.d. wood (Table 2).

Comparing all the ATPS studied, the highest total phenols yield was obtained for the top phase of the ethanol/$(NH_4)_2SO_4$ ATPS 2,81 mg GAE per 100 mg o.d. wood (Table 2). The influence of the inorganic salt on phenols extraction and phenols partition was previously studied for the same material using ATPS based on PEG 2000 and various salts (Xavier *et al.* 2014, 2015). With respect to phenols recovery, PEG2000/salt ATPS led to lower values than those found in this work for ethanol/salt ATPS (Xavier *et al.* 2014, 2015). Consequently, from a practical point of view, ethanol/salt ATPS would be more appropriate for phenols extraction from eucalyptus wood as higher yields were obtained and inherent complications related to the use of polymers (viscosity phase, difficulty in recycling the components, etc.) would be avoided.

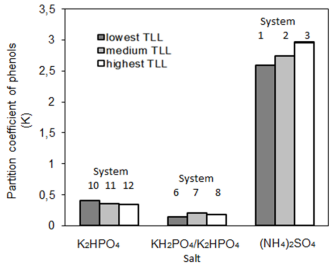

Figure 1. Partition coefficient of phenols, K, in several ethanol/salt ATPS in function of the tie line length (TLL) (Extraction temperature: 25ºC; Settlement time: 1 h).

Effect of the tie lie length on the partition behaviour of phenols

Many researchers have suggested that TLL, which relates the mass ratio between phases in equilibrium, could have influence on product recovery (Simental-Martínez *et al.* 2014). In order to examine the behaviour of phenol recovery, three different tie lie lengths (TLL) were selected for each ATPS at a V_r value around 1,0 to avoid any potential concentration effect (Table 1). As shown in Figure 1, for the ethanol /$(NH_4)_2SO_4$ ATPS, the highest K was attained for the highest TLL (57% w/w, system 3). This behaviour may be related with the hydrophobicity of both phases (Simental-Martínez *et al.* 2014). An increase in TLL resulted in an increase of ethanol and salt concentrations on the top and bottom phases, respectively. Thus, an increase in TLL resulted in a major difference among both phases, which led to increase the hydrophobicity of the ethanol phase favoring partitioning (Lucena de Souza *et al.* 2010, Simental-Martínez *et al.* 2014).

Regarding ethanol/K_2HPO_4 ATPS, phenols showed preference towards the bottom phase. A similar tendency was obtained when the TLL increased: the bottom phase becomes more hydrophilic favoring the fractionation towards such phase. In consequence, the lowest value of K was attained with the highest (57% w/w, system 12) TLL. A similar behaviour was found in the recovery of fucoxanthin using ethanol/potassium phosphate ATPS (Gómez-Loredo *et al.* 2014).

On the contrary, ethanol-KH_2PO_4/K_2HPO_4 ATPS did not show the same tendency. The lowest value of K was obtained for the lowest TLL (21% w/w, system 6) and the partition seems to be independent of the TLL.

Effect of volume ratio on the partition behaviour of phenols

To study the influence of V_r on product partitioning behavior, V_r values smaller and greater than 1 were evaluated at a fixed TLL (Table 1, Figure 2). Figure 2 depicts the extraction efficiencies data of phenols partition in ethanol based ATPS formed by different inorganic salts.

For the ethanol/$(NH_4)_2SO_4$ ATPS, an improvement in efficiency was achieved when V_r was increased. In these ATPS phenols prefer the upper phase. Therefore, by increasing V_r, an improvement in phenols recovery was achieved due to the major volume of the top phase. On the other hand, for the ethanol/K_2HPO_4 and ethanol/KH_2PO_4/K_2HPO_4 ATPS it was found that better extraction efficiencies were obtained decreasing V_r. When phenols migrate to the bottom phase, lower V_r values imply higher volumes of the bottom phase. This behaviour could be associated to the additional volume available which allows overcoming saturation problems, promoting the migration of the product towards the top or bottom phase depending on the case (Gómez-Loredo *et al.* 2014). Based on these results, systems 5, 7 and 14 were selected to study the influence of temperature.

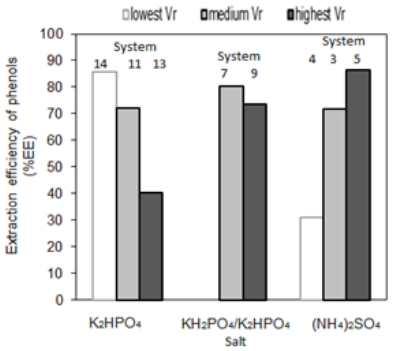

Figure 2. Extraction efficiency of phenols, %EE, in several ethanol/salt ATPS in function of the volume ratio V_r (Extraction temperature: 25ºC; Settlement time: 1 h).

Effect of temperature on the partition behaviour of phenols

Table 3 shows that for all ATPS studied, the extraction of phenolics increased on both phases with increasing extraction temperature from 25 to 65ºC. The ethanol/$(NH_4)_2SO_4$ system resulted to be the most adequate for phenols recovery with a total phenols yield of 5,36 g GAE (100 g o.d. wood)$^{-1}$ at 65ºC. However, the partition behavior of phenols showed a different trend, as shown in Table 3. The selectivity decreased for all ATPS with increasing temperature. K diminished for the ethanol/$(NH_4)_2SO_4$ system and increased for the ethanol/phosphate systems. This trend was also observed in the partition of phenols using ATPS formed by PEG 2000 and different inorganic salts (Xavier *et al.* 2014, 2015). This behavior observed can be explained by the higher diffusion rate and solubility of the extracted substances when temperature is increased (Jokić *et al.* 2010).

As mentioned above, for all the ATPS studied, the highest total phenol yield was obtained at 65ºC. Additionally, the FRAP antioxidant capacity was determined for these extracts (Figure 3), and the highest value (20 mmol AAE (100 mg o.d. wood)$^{-1}$) corresponded to the ethanol/$(NH_4)_2SO_4$ system (System 5) with also the highest the total phenols yield (5,36 g GAE (100 g o.d. wood)$^{-1}$). Thus, this ATPS seems to be the most attractive alternative for phenols recovery from eucalyptus wood wastes in the sense of achieving the highest extraction yield and best extract properties.

Table 3. Total phenols yield and partition coefficient depending on extraction temperature (settlement time, 1 h).

Ethanol-$(NH_4)_2SO_4$ (System 5)				
Temperature (ºC)	TPY_{TP} (mg GAE (100 mg o.d.wood)$^{-1}$)	TPY_{BP} (mg GAE (100 mg o.d.wood)$^{-1}$)	K	V_r
25	2,62^{a}±0,10	0,41^{a}±0,01	2,31±0,11	2,8
45	3,83^{b}±0,08	0,57^{b}±0,04	1,79±0,13	2,7
65	5,36^{c}±0,05	0,63^{c}±0,04	1,39±0,08	2,6
Ethanol- KH_2PO_4/K_2HPO_4 (System 7)				
25	0,20^{a} ±0,01	0,60^{a}±0,05	0,20±0,01	1,1
45	0,30^{b}±0,01	0,73^{b}±0,01	0,32±0,01	1,1
65	0,25^{c}±0,01	0,66^{ab}±0,06	0,34±0,01	1,2
Ethanol- K_2HPO_4 (System 14)				
25	0,07^{a}±0,01	0,40^{a}±0,05	0,57±0,02	0,3
45	0,10^{b}±0,01	0,58^{b}±0,01	0,75±0,03	0,2
65	0,10^{ab}±0,02	0,95^{c}±0,04	0,90±0,06	0,1

Values are presented as mean±SD (n=3). In each column, values with different letters are significantly different ($p<0,05$).
TPY: total phenols yield; TP: top phase; BP: bottom phase; K: partition coefficient; V_r: volume ratio.

Consequently, the results obtained with the selected ethanol/$(NH_4)_2SO_4$ ATPS were compared with those obtained for the same material using conventional extraction with ethanol or ethanol-water under similar extraction conditions (total phenols yield: 2,46-4,74 g GAE (100 g o.d. wood)$^{-1}$; FRAP antioxidant activity: 22,35-40,91 mmol AAE (100 mg o.d.wood)$^{-1}$, respectively) (Fernández-Agulló *et al.* 2015). Total phenols yield was considerably higher using the selected ATPS whereas FRAP antioxidant capacity was slightly lower. However, there are many advantages of ATPS extraction process. First of all, a primary recovery and partial phenols purification is achieved in a single unit operation, thereby reducing equipment needs. Furthermore, phenols were obtained in a lower volume than with conventional extraction which facilitates extract separation from the top ethanol-rich phase. Finally, note that ethanol recovery is an essential stage, in particular if one intends to apply this system at a large scale.

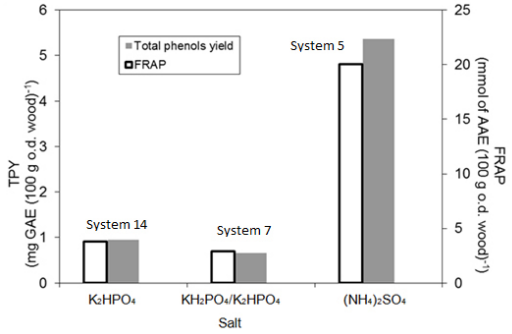

Figure 3. Total phenols yield (TPY) and FRAP antioxidant activity for the top phase extract of system 5 and the bottom phase extracts of systems 7 and 14 at 65ºC.

RP-HPLC-ESI-TOF mass spectrometry results

The top phase extract obtained with the ethanol/ammonium sulphate ATPS (System 5) at 65ºC, with the highest total phenol yield and FRAP antioxidant activity, was analized by RP-HPLC-ESI-TOF mass spectrometry. The following phenolic compounds were identified in the extract based on their molecular weight and/or retention time of the standard compounds: myricetin 3-O-rhamnoside (peak 3), quercetin 3-glucoside (peak 3), quercetin 3-glucoronide (peak 4) and ellagic acid (peak 5) (Table 4, Figure 4). As shown in Figure 4. ellagic acid is the main compound present in the extract. Indeed, all these compounds typically occur in bark, leaves or needles of eucalyptus sp (Conde *et al.* 1996, Eyles *et al.* 2003) and have demonstrated antioxidant capacity (Romani *et al.* 1999, Moure *et al*. 2001).

Table 4. Phenolic compounds identified in the eucalyptus wood top phase extract obtained with the ethanol/ammonium sulphate ATPS (System 5) at 65ºC.

Compound	[M-H]-(m/z)	Retention time (min)
Ellagic Acid	301[a]	26,4[a]
Quercetin 3-glucoronide	477[b]	26,0[b]
Myricetin 3- O-rhamnoside	463[b]	19,1[b]
Quercetin 3-glucoside	463[b]	19,1[b]

[a], according to standards; b, based on molecular weight.

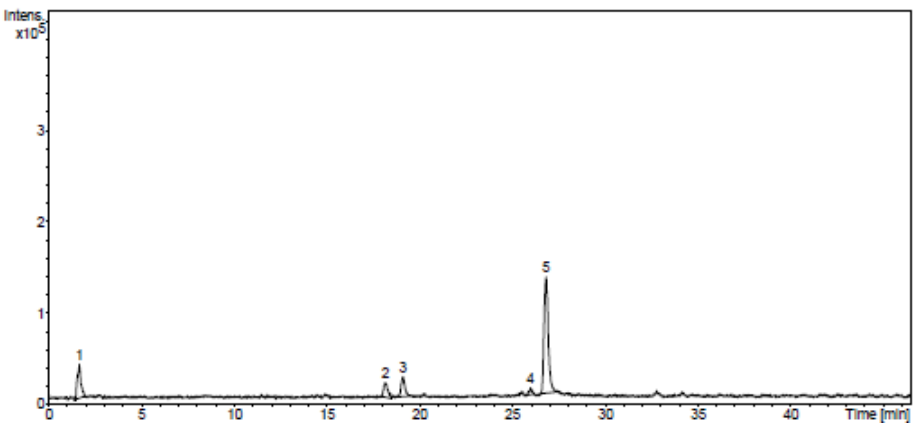

Figure 4. HPLC chromatogram of the eucalypt wood top phase extract obtained with the ethanol/ammonium sulphate ATPS (System 5) at 65ºC with identified peaks numbered: (3) myricetin 3-O-rhamnoside and quercetin 3-glucoside, (4) quercetin 3-glucoronide and (5) ellagic acid.

CONCLUSIONS

The potential of ethanol-salt aqueous two-phase systems for phenols recovery from eucalyptus wood wastes was demonstrated, offering an alternative to conventional extraction. Phenols recovery and partition depended on the inorganic salt selected (ammonium sulphate, a mixture of monopotassium phosphate and potassium diphosphate and potassium diphosphate) and, for each ATPS, on the tie-lie lenght, volume ratio and extraction temperature used. The ethanol-$(NH_4)_2SO_4$ ATPS at the highest tie line length 57% (w/w), volume ratio (2,8) and temperature (65ºC) showed the best results for phenol recovery and primary purification: 5,36 mg gallic acid equivalent (100 mg oven dried wood)$^{-1}$ and FRAP antioxidant activity, 20 mmol AAE (100 g oven dried wood)$^{-1}$. Analysis of the extracts by RP-HPLC-ESI-TOF confirmed the presence of the phenolic compounds with potential antioxidant activity.

ACKNOWLEDGMENTS

This work was partially financed by Comisión Sectorial de Investigación Científica (CSIC) from Uruguay through the project "Iniciación a la investigación: CSIC-UDELAR-INI 2013".

REFERENCES

Albertsson, P. 1986. Partition of cell particles and macromolecules. J. Wiley and Sons, New York.

Conde, E.; Cadahia, E.; Garcia Vallejo, M.C.; Diez, R. 1996. Composición polifenólica de madera, corteza y hojas de *Eucalyptus camaldulensis*, *E. globulus* y *E. rudis*. *Investigación Agraria Sistemas y Recursos Forestales* 5:333-350.

Espitia-Saloma, E.; Vázquez-Villegas, **P.; Aguilar, O.; Rito-Palomares, M. 2014.** Continuous aqueous two-phase systems devices for the recovery of biological products. *Food and Bioproducts Processing* 92:101-112.

Eyles, A.; Davies, N.W.; Mohammed, C. 2003. Wound wood formation in *Eucalyptus globulus* and *Eucalyptus nitens*: anatomy and chemistry. *Canadian Journal of Forest Research* 33:2331-2339.

Fernández-Agulló, A.; Freire, M.S.; González-Álvarez**, J. 2015.** Effect of the extraction technique on the recovery of bioactive compounds from eucalyptus (*Eucalyptus globulus*) wood industrial wastes. *Industrial Crops and Products* 64:105-113.

Gómez-Loredo, A.; Benavides, J.; Rito-Palomares, M. 2014. Partition behavior of fucoxanthin in ethanol-potassium phosphate two-phase systems. *Journal of Chemical Technology and Biotechnology* 89:1637-1645.

Jokic, S.; Velic, D.; Bilic, M.; Bucic-Kojic, A.; Plannic, M.; Tomas, S. 2010. Modelling of the process of solid-liquid extraction of total polyphenols from soybeans. *Czech Journal of Food Sciences* 28(3):206-212.

Lucena de Souza, R.; Barbosa, J.M.P.; Zanin, G.M.; Lobão, M.W.N.; Soares, C.M.F; Lima, A.S. 2010. Partitioning of porcine pancreatic lipase in a two-phase system of polyethylene glycol/potassium phosphate aqueous. *Applied Biochemistry Biotechnology* 161:288-300.

Moure, A.; Cruz, J.M.; Franco, D.; Dominguez, J.M.; Sineiro, J.; Domínguez, H.; Nuñez, M.J.; Parajo, J.C. 2001. Natural antioxidants from residual sources. *Food Chemistry* 72:145-171.

Ooi, C.W.; Tey, B.T.; Hii, S.L.; Kamal, S.M.M.; Lan, J.C.W.; Ariff, A. 2009. Purification of lipase derived from *Burkholderia pseudomallei* with alcohol/salt based aqueous two-phase systems. *Proceedings of the Biochemical Society* 44:1083-1087.

Reis, I.A.O.; Santos, S.B.; Santos, L.A.; Oliveira, N.; Freire, M.G.; Pereira, J.F.B.; Ventura, S.P.M.; Coutinho, J.A.P ; Soares, C.M.F ; Lima, A.S. 2012. Increased significance of food wastes: Selective recovery of added-value compounds. *Food Chemistry* 135:2453-2461.

Reis, I.A.O. ; Santos, S.B.; Pereira, F.D.S.; Sobral, C.R.S.; Freire, M.G.; Freitas, L.S.; Soares, C.M. F.; Lima, A. S. 2014. Extraction and recovery of rutin from acerola waste using alcohol-salt-based aqueous two-phase systems. *Separation and Purification Technology* 49:656-663.

Romani, A.; Pinelli, P.; Mulinacci, N.; Vincieri, F.F.; Tattini, M. 1999. Identification and quantitation of polyphenols in leaves of *Myrtus communis* L. *Chromatographia* 49:17-20.

Rosa, P.A.J.; Ferreira, I.F.; Azevedo, A.M.; Aires-Barros, M.R. 2010. Aqueous two-phase systems: A viable platform in the manufacturing of biopharmaceuticals. *Journal of Chromatography A* 1217:2296-2305.

Shen, S. ; Chang, Z.; Liu, H. 2006. Three-liquid-phase extraction systems for separation of phenol and p-nitrophenol from wastewater. *Separation and Purification Technology* 49: 217-222.

Simental-Martínez, J.; Montalvo-Hernándeza, B.; Rito-Palomares, M.; Benavides, J. 2014. Application of aqueous two-phase systems for the recovery of bioactive low-molecular weight compounds. *Separation Science and Technology* 49:1872-1882.

Singleton, V.L.; Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture* 16:144-158.

Szollozi, R.; Szollozi-Varga, I. 2002. Total antioxidant power in some species of *Labiatae* (Adaptation of FRAP method). *Acta Biologica Szegediensis* 46(3-4):125-127.

Wang, Y.; Yan, Y.S.; Hu, S.P.; Han, J.; Xu, X.H. 2010. Phase diagrams of ammonium sulfate + ethanol/1-propanol/2-propanol + water aqueous two-phase systems at 298.15 K and correlation. *Journal of Chemical & Engineering Data* 55:876-881.

Wu, Y.; Wang, Y.; Zhang, W.; Han, J.; Liu, Y.; Hu, Y.; Ni, L. 2014. Extraction and preliminary purification of anthocyanins from grape juice in aqueous two-phase system. *Separation and Purification Technology* 124:170-178.

Xavier, L.; Freire, M.S.; Vidal-Tato, I.; González-Álvarez, J. 2014. Aqueous two-phase systems for the extraction of phenolic compounds from eucalyptus (*Eucalyptus globulus*) wood industrial wastes. *Journal of Chemical Technology and Biotechnology* 89:1772-1778.

Xavier, L.; Freire, M.S; Vidal-Tato, I.; González-Álvarez, J. 2015. Application of aqueous two phase systems based on polyethylene glycol and sodium citrate for the recovery of phenolic compounds from eucalyptus wood. *Maderas-Ciencia y Tecnología* 17:345-354.