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Colloidal gas aphrons based separation process for the purification and fractionation of natural phenolic extracts

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ABSTRACT

Following previous studies, the aim of this work is to further investigate the application of colloidal gas aphrons (CGA) to the recovery of polyphenols from a grape marc ethanolic extract with particular focus on exploring the use of a non-ionic food grade surfactant (Tween 20) as an alternative to the more toxic cationic surfactant CTAB. Different batch separation trials in a flotation column were carried out to evaluate the influence of surfactant type and concentration and processing parameters (such as pH, drainage time, CGA/extract volumetric and molar ratio) on the recovery of total and specific phenolic compounds. The possibility of achieving selective separation and concentration of different classes of phenolic compounds and non-phenolic compounds was also assessed, together with the influence of the process on the antioxidant capacity of the recovered compounds. The process led to good recovery, limited loss of antioxidant capacity, but low selectivity under the tested conditions. Results showed the possibility of using Tween 20 with a separation mechanism mainly driven by hydrophobic interactions. Volumetric ratio rather than the molar ratio was the key operating parameter in the recovery of polyphenols by CGA.

Keywords: Antioxidants; Colloidal gas aphrons; Grape marc; Phenolics; Surfactants

1. Introduction

Natural phenolic compounds have been investigated for their antioxidant and antimicrobial activity to quite some extent and this subject still raises a large interest due to the complex nature of the substances. Polyphenols are the biggest group of phytochemicals, with more than 8000 phenolic structures currently known, and among them over 4000 flavonoids identified (Löf et al., 2011). The polyphenols name includes different phenolic classes with even largelydifferent molecular

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; AOC, antioxidant capacity expressed as absorbance percent inhibition in the ABTS assay; CAE, caffeic acid equivalents; C_{APy}/C_{LPy}, concentration of compound y in the collapsed aphron phase/liquid phase at the end of separation trials; CGA, colloidal gas aphrons; CTAB, cetyltrimethylammonium-bromide; GAE_{TPI}/GAE_{FI}, gallic acid equivalents based on total phenol index/Folin Index; ME, malvidin-glucoside equivalents; $M_{y/feed}/M_{y/liq}$, total amount of compound y added in the feed/measured in the liquid phase in separation trials; QE, quercetin equivalents; RE_y, recovery of compound y in the aphron phase in separation trials; SF, separation factor; $V_{AP}/V_{LP}/V_{CGA}/V_{liquid\ drained}/V_{sample}$, volume of: aphron phase (measured after complete collapse)/liquid phase/CGA/liquid drained from complete collapse of CGA in the CGA characterisation/standard gallic acid solution or extract fed into the column in separation trials; ε/ε', gas hold-up from CGA characterisation/effective gas hold-up from separation trials.

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structures, such as non-flavonoid phenolic acids, non-flavonoid stilbenes, anthocyanins, flavonols, flavanols, tannins, flavones and flavanones. The majority of studies available in literature deal with the nutritional, biochemical, or chemical structure aspects of natural phenolic compounds while a few studies address the physico-chemical and colloidal properties (Tsao, 2010) which are, however, of high importance in the understanding of the behaviour and functionality of the substances. One of the main factors preventing efficient and widespread applications of natural phenolic compounds is their limited solubility in aqueous or lipid media (depending on the phenolic class and molecular hydrophobicity), with things even more complicated when dealing with natural phenolic extracts which contain mixtures of different phenolic compounds (Amendola et al., 2010; Menese et al., 2013; Spigno et al., 2013). Production of standard phenolic compounds from natural sources, including agro-food by-products which represent a unique low-cost resource, requires also important purification and fractionation steps with quite expensive technologies (strategies including sequential extraction, liquid–liquid partitioning and other complex and expensive processes based on membrane or chromatographic technologies have been proposed). As it concerns the polluting character of natural phenolic compounds, besides the intrinsic antimicrobial properties, limited solubility can also prevent their efficient bioremediation. Limited stability due to easy thermal and oxidative degradation of natural phenolic compounds during storage and processing is another heavy limit to their application. Finally, binding of some natural phenolic compounds to macromolecules, primarily proteins/enzymes and polysaccharides, but also minerals (such as iron and copper), may have important anti nutritional effects or limit their bioavailability after ingestion, compromising their so commonly claimed health benefits, or compromising food quality (see precipitation of proteins/polyphenols complexes in wine and fruit juices).

Besides the intensive use of emulsifiers in the food industry (Hasenhuettl and Hartel, 2010) some works have been reported about the interactions between surfactants and phenolic compounds. For example, a few works have shown the potential of specific surfactants in micellar form to: solubilise or precipitate specific phenolic compounds (Löf et al., 2011); alter their partitioning in oil-in-water emulsions (Richards et al., 2002; SØrensen et al., 2008); enable phenolic compounds analytical determination exploiting different affinities (Wang et al., 2007); protect phenolic compounds from oxidation (Lin et al., 2007); solubilise aromatic compounds (Yoshida and Moroi, 2000; Wei et al., 2012); improve phenolic compounds efficiency in topical formulations (Scognamiglio et al., 2013; Yutani et al., 2012). Other works have investigated the use of surfactants for the recovery of natural phenolic compounds from wastewaters (Gortzi et al., 2008; Katsoyannos et al., 2012).

Surfactants and colloidal gas aphrons (CGA), which are surfactant-stabilized microbubbles (10–100 μm) generated by intense stirring of a surfactant solution at high speeds (>8000 rpm) (Jauregi and Dermiki, 2010), have been used for many separation processes such as protein and enzyme recovery (Fuda and Jauregi, 2006; Zidehsaraei et al., 2009; Cheng and Stuckey, 2012), carotenoids recovery (Dermiki et al., 2008; Alves et al., 2006); recovery of toxic wastes from soil, removal of dyes from wastewaters, stripping of dissolved gases and removal of dispersed oil droplets from water, bubble-entrained floc flotation. Depending on the type of surfactant used for the generation of CGA, e.g. cationic, anionic or non-ionic, the outer surface of the microbubble may be positively charged, negatively charged or neutral, to which oppositely charged or non-charged molecules will adsorb, resulting in their effective separation from the bulk liquid, and consequently the selectivity of adsorption can be controlled. CGA possess unique properties: (i) high interfacial area due to their small size; (ii) high stability compared to conventional foams; (iii) sufficient stability to allow them to be pumped from the generation point to the point of use without loss of their original structure; (iv) CGA can be easily separated from the bulk liquid without mechanical aid, as opposed to conventional liquid–liquid extraction methods and the aqueous two phase separations that need centrifugation for phase separation. If it is possible to use biodegradable and non-toxic surfactants, this could result in an environmentally friendly processes while the final product could also be safe for human consumption.

Previous research carried out by the authors (Spigno and Jauregi, 2005; Spigno et al., 2010;) demonstrated that the phenolic acid gallic acid in its anionic form (at neutral–basic pH conditions) can be recovered from aqueous solutions using CGA generated from the cationic surfactant cetyltrimethylammonium-bromide (CTAB). Gallic acid recovery was mainly affected by pH, ionic strength, surfactant/gallic acid molar ratio, mixing conditions and contact time. In further work it was demonstrated that total phenolic compounds could be recovered in high yield from a grape marc (the wine-making waste consisting in seeds and skins, which is typically removed after fermentation for red wines, and after pressing for white wines) ethanolic extract (Spigno et al., 2010). Here we take this work further onto investigating the application of CGA to the recovery of polyphenols from a grape marc ethanolic extract with particular focus on exploring the use of a non-ionic food grade surfactant Tween 20 as an alternative to a more toxic cationic surfactant. This could offer the additional advantage of extracting the polyphenols in a surfactant rich solution which could confer the product improved solubility properties. In particular this paper aims at getting a better insight into the following aspects of the application of CGA to the recovery of natural phenolic compounds from a crude ethanolic extract:

- Influence of surfactant type and concentration and processing parameters (such as pH, drainage time, CGA/extract volumetric and molar ratio) on the recovery of total and specific phenolic compounds.
- Selective separation and concentration of different classes of phenolic compounds and non-phenolic compounds.
- Influence of the process on the functional properties (antioxidant activity) of the recovered compounds.

2. Materials and methods

2.1. *Materials*

Gallic acid, caffeic acid, quercetin, CTAB (cetyltrimethylammonium bromide) and Tween 20 were supplied by Fluka (Milan, Italy); ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and malvidin-glucoside by Sigma (Milan, Italy); potassium persulphate by Carlo Erba (Milan, Italy); all other chemicals were of analytical grade.

Grape marc samples from two different red grape varieties (Barbera and off-skins fermented Pinot noir) were kindly provided by wineries in the Northern Italy. The samples were oven

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Table 1 – Experimental conditions of the separation trials carried out with colloidal gas aphrons (CGA).

Surfactant	Investigated variables	Fixed parameters	Evaluated outputs
CTAB	• CTAB concentration (0.5–1–2 mM) • Drainage time (3–25 min) • pH (2–6) • Volumetric ratio CGA/sample (1–23)	• Extract concentration (200 mg/L GAE_{TPI})	RE_{GAE}, RE_{ME}, SF_{GAE}, SF_{ME}, antioxidant activity of recovered aphron phase; liquid drainage.
Tween 20	• Drainage time • Extract concentration (200–5000 mg/L GAE_{TPI}) • Volumetric ratio CGA/sample (4–16) • In situ CGA generation	• Tween concentration (10 mM) • Not modified pH	RE and SF of: GAE_{TPI}, GAE_{FI}, WAE, CAE, QE, glucose, fructose, potassium. Antioxidant activity of recovered aphron phase; liquid drainage.

CAE: cinnamic acids equivalents; FI: Folin Index; GAE: gallic acid equivalents; ME: malvidin-glucoside equivalents; QE: quercetin equivalents; RE: percent recovery; SF: separation factor; TPI: total phenolics index.

dried at 60 °C until residual moisture content <5% and then milled (particle size <2 mm). Phenolic extracts were obtained by solvent extraction with aqueous ethanol according to the procedure reported in Amendola et al. (2010) slightly modified. Briefly, 125 g of powder was extracted with 1 L of 60% aqueous ethanol keeping the mixture stirred at 3500 rpm (mixer Silverson, L5M) for 2 h at 60 °C (by means of an electric heating plate). After extraction, the mixture was centrifuged at 5000 rpm for 10 min (Centrifuge ALC 4237R) and the supernatant (extract) recovered.

For the trials with Barbera marc, the extract was freeze-dried and the powder used to reconstitute a concentrated extract in ethanol 60%. The concentrated extract was then diluted with water to have a concentration of 200 mg/L of total phenolics (as total phenolics index). This step was carried out in order to reduce the ethanol content to about 10% to limit its antifoam effect (Alves et al., 2006).

For the trials with Pinot noir marc, the extract was concentrated under vacuum at 40 °C (Rotavapor Büchi R-114) until removing half the initial volume and then diluted with water until the desired concentration in total phenolics. This way, it was simulated an industrial application of the process where ethanol needs to be recovered.

2.2. CGA generation and characterisation

The CGA were generated from a surfactant solution stirred at 8000 rpm with the Silverson mixer for 5 min. CGA had been characterised in terms of gas hold-up and stability for CTAB and Tween 20 surfactant solutions in the generation vessel (Jauregi and Dermiki, 2010). However in this work the gas hold-up was measured taking into account the effect of pumping. To do so, after generation, 100 mL of the CGA were pumped into a volumetric cylinder and let to drain completely. The gas hold-up (ε) was then calculated from the measured drained volume ($V_{\text{liquid drained}}$) according to Eq. (1):

$$\varepsilon = \frac{V_{CGA} - V_{\text{liquid drained}}}{V_{CGA}} \quad (1)$$

Surfactant concentration in the generated CGA was estimated combining the drained volume with the molarity of the surfactant solution used to generate CGA.

Stability was measured by measuring the drainage rate and as half-life, which is the time taken for half of the original liquid to drain.

2.3. Separation with CGA

Trials were carried out in a flotation glass column (i.d. 0.25 m, total height 0.4 m). First, the extract sample and then the CGA were pumped into the column by a peristaltic pump (Watson Marlow 505 U) from the bottom. The volumetric flow was regulated so that the mixing time would be 3.5–4 min. Once the column was filled, the mixture was left standing for a selected contact (or drainage time) before pumping out the separated bottom liquid phase and upper aphron phase. The latter was let to drain completely (collapsed aphron phase). The volumes of the separated liquid phase and collapsed aphron phase (V_{LP} and V_{AP}, respectively) were measured. Percent recovery of a specific compound y in the aphron phase (RE_y) was calculated based on the difference between the total amount of added y in the feed ($M_{y/feed}$) and the amount of y measured in the separated liquid phase ($M_{y/liq}$).

For some experiments the amount of y in the aphron phase was also calculated and the mass balance deviation was within 10%.

The separation factor (SF) was calculated to give an idea of the affinity of a compound to the aphron phase compared to the affinity to the liquid phase, based on the concentrations of the compound y in the aphron and in the liquid phase (C_{APy} and C_{LPy}) according to Eq. (2):

$$SF = \frac{C_{APy}}{C_{LPy}} \quad (2)$$

The different carried out separation trials are summarised in Table 1.

The volumetric ratio was calculated as the ratio between the volume of CGA and the volume of the sample fed into the column.

The molar ratio was calculated as the moles of surfactant to the moles of the compound y fed into the column; total phenols were determined as gallic acid equivalents and anthocyanins as malvidin glucoside equivalents therefore the molecular weights of these compounds were used to calculate the molar ratios corresponding to each volume ratio.

In the trials with "in-situ" CGA generation, the surfactant solution was mixed with the extract, stirred at 8000 rpm for 5 min and then pumped into the column and the separation was performed as above. The influence of mixing the feed (extract) with the CGA on the gas hold-up was assessed calculating an effective gas hold-up (ε') from Eq. (1) using as $V_{\text{liquid drained}}$ the difference between the total drained volume and the volume of the sample fed into the column ($V_{LP} + V_{AP} - V_{sample}$). All experiments were performed at least

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in duplicate and the results in this work are reported as means ± SD. Significance ($P < 0.01$) of the effect of investigated variable on evaluated outputs was established treating the data according to a general linear model (univariate ANOVA) and applying the post hoc Tukey's test to discriminate between means (IBM SPSS Statistics 19).

2.4. Analytical determinations

2.4.1. Phenolic compounds

The grape marc extracts and the recovered liquid and aphron phases were characterised for the total phenolics content and for the content of specific phenolic classes according to the following analyses.

- Total phenolics content by total phenol index (TPI) based on absorbance reading at 280 nm and expressing the results as gallic acid equivalents (GAE_{TPI}) by means of a calibration curve with standard gallic acid (Amendola et al., 2010).
- Total phenolics content by Folin–Ciocalteu analysis (Folin Index), expressing the results as GAE_{FI} based on a calibration curve with standard gallic acid (Amendola et al., 2010).
- Total anthocyanins content by absorbance reading at 538 nm of the sample diluted with chloridric ethanol and expressing the results as ME (Malvidin-glucoside Equivalents) based on a calibration curve with standard malvidin-glucoside (Amendola et al., 2010).
- Total cinnamic acids content by absorbance reading at 320 nm and expressing the results as caffeic acid equivalents (CAE) based on a calibration curve with standard caffeic acid (Spigno et al., 2007).
- Total flavonols content by absorbance reading at 370 nm and expressing the results as quercetin equivalents (QE) (Spigno et al., 2007).

For all the above spectrophotometric analyses, different calibration curves with aqueous ethanol, CTAB or Tween solutions were prepared and used depending on the analysed sample (initial extract, liquid and aphron phase).

2.4.2. Non-phenolic compounds

The grape marc extracts and the recovered liquid and aphron phases were characterised also for the content of compounds other than phenolics. After preliminary characterisation, the following compounds were selected based on their level in the extracts.

- Glucose and fructose content was evaluated by a Megazyme enzymatic kit (K-FRUGL).
- Potassium content was evaluated by Atomic Absorptions Spectroscopy (Perkin Elmer AAnalyst 300, Norwalk, CT, USA) based on calibration curve with standard K.

2.4.3. Antioxidant capacity

The antioxidant capacity of the grape marc extracts and the recovered liquid and aphron phases were assessed by the radical ABTS assay which is based on the ability of antioxidants to reduce the radical decreasing its absorbance at 734 nm (Amendola et al., 2010). Antioxidant capacity was expressed as absorbance percent inhibition (AOC).

3. Results and discussion

3.1. Separation with CTAB

In previous work by the authors it was demonstrated that CGA generated from the cationic surfactant CTAB could be applied successfully to the recovery of the standard phenolic acid gallic acid from water solutions (Spigno et al., 2010). In this work it was found that optimum recovery was obtained with CGA generated from a 1 mM CTAB aqueous solution at neutral to basic pH's, without buffer salts as these would increase ionic strength which will in turn reduce interactions between gallic acid and surfactant resulting in lower recoveries.

Here we applied the optimum conditions above to a Barbera marc extract diluted to 200 mg/L GAE_{TPI} as in the experiments with gallic acid. Similar recoveries were obtained for the extract as for the standard gallic acid (Fig. 1A). Slightly higher recovery was obtained for anthocyanins than for total phenolics based on the volumetric ratio (Fig. 1A), However, particularly at high volumetric ratios, higher separation factors were obtained for anthocyanins than for total phenolics which suggests that anthocyanins have a higher affinity for CGA than total phenols (Fig. 1B).

The higher recoveries obtained with the real extract, might have been due to the stabilising effect of the sample on the CGA, as evident from the lower volumes of drained liquid phase (Fig. 1C). Other compounds present in the real extract such as sugars most probably contributed to an increase in the viscosity of the liquid within the aphrons which resulted in reduced drainage rate.

Further separation trials at constant volumetric ratio (15.33) and variable drainage time were conducted (Fig. 2) in order to see if increased drainage led to an increase concentration of total phenolics and/or anthocyanins in the aphron phase as observed in our previous work for asthaxantin (Dermiki et al., 2008). Recovery decreased at increased drainage time following a linear trend (r^2 0.985 and 0.978 for ME and GAE, respectively) which implied that phenolic compounds drained together with the liquid phase (Fig. 2A). However, the separation factor increased with drainage time particularly for anthocyanins following a nonlinear trend. A very similar trend was followed by the ratio of drained liquid phase to the volume of collapsed aphron phase (Fig. 2B). This again supports that anthocyanins have more affinity for the CGA than polyphenols.

Overall a higher selectivity for anthocyanins than for total phenolics was found. Although at the pH conditions of our experiments (pH of the recovered liquid phase 3.5–4.36 in the range of volumetric ratios studied) anthocyanins are not expected to be charged since the flavilium form (cationic form) exists only at pH ≤2 these molecules are polarised with high electron density in the aromatic rings which may explain their affinity for the positively charged CGA generated from CTAB. However, it is expected that hydrophobic interactions will also play an important role in their separation.

To verify the pH effect, additional trials were carried out at pH 2 (with 1 M HCl) in both the CTAB solution and the extract. The pH reduction led to a small increase in recovery of total phenolics and a reduction in the recovery of anthocyanins, particularly at low volumetric ratios (Fig. 3A). The separation factor increased for total phenolics but not for anthocyanins (Fig. 3B). A reduction in recovery can be explained based on repulsive electrostatic interactions between the positively

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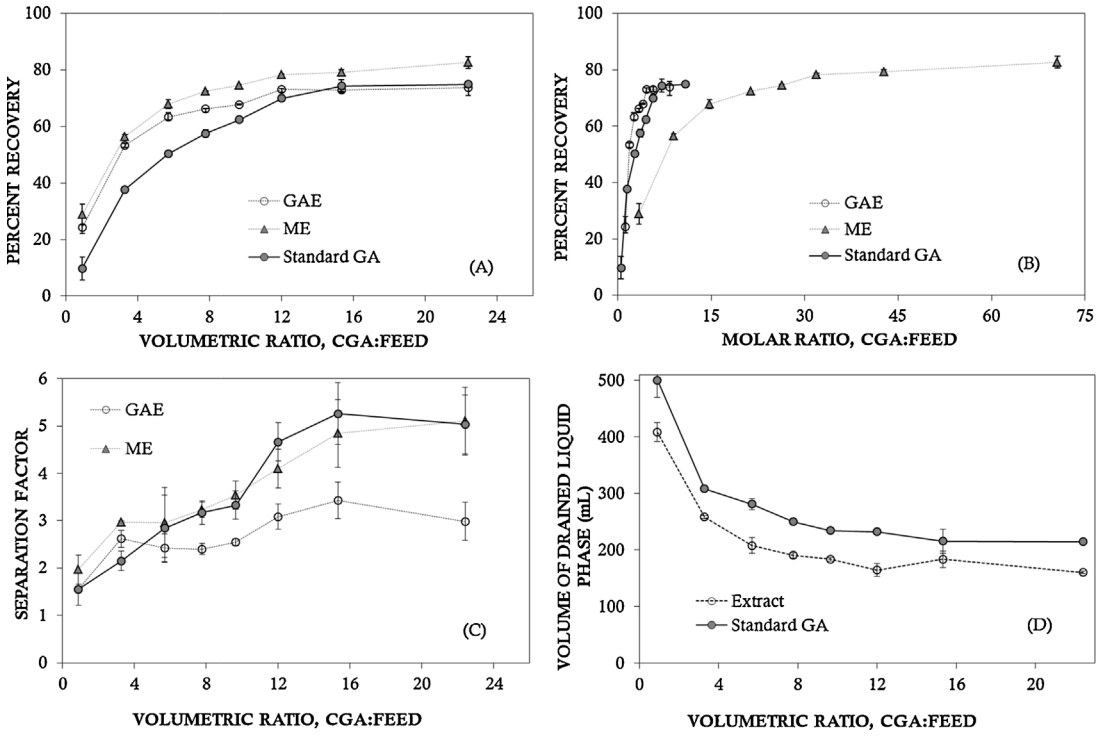

Fig. 1 – Results of separation trials carried out with colloidal gas aphrons (CGA) (generated from CTAB 1 mM) and sample consisting in standard gallic acid (GA) solutions or Barbera marc extract (GAE: gallic acid equivalents as total phenolics index; ME: malvidin-glucoside equivalents). (A and B) Recovery as a function of volumetric ratio or molar ratio, respectively. (C and D) Separation factor or volume of drained liquid phase as a function of volumetric ratio, respectively. Error bars indicate ± SD.

charged cationic surfactant and anthocyanins at this pH. However, the recovery and separation factor were still high which shows that separation of anthocyanins occurs also due to hydrophobic interactions; at this pH a proportion of anthocyanin molecules will not be charged and will be complexed with other phenolic compounds.

Trials carried out at pH 2 and 0.5 mM CTAB, confirmed the above results, a lower recovery of anthocyanins at this pH. However, the recovery of the total phenolics decreased also contrary to what was observed at pH 2 with 1 mM CTAB. This is likely to be not a pH effect but an effect of CGA stability. In fact, 0.5 mM is below the critical micellar concentration of CTAB (reported to be 0.8–1 mM at 25 °C), with a consequent decrease in CGA stability, a higher drainage rate and a lower effective gas hold-up.

Furthermore, in all the trials, it was found that the antioxidant activity of total phenolics recovered in the aphron phase is reduced compared to total phenolics drained in the liquid phase (Fig. 4). This can be due to oxidation during recovery or to a selective partition of phenolics between the two phases.

Table 2 – Results (in terms of recovery efficiency RE) for the separation trials carried out with colloidal gas aphrons generated from Tween 20 10 mM, at different volumetric ratios ($ml_{CGA}/ml_{extract}$) and different molar ratios (mol_{Tween}/mol_{GAETPI}). V_{AP} and V_{LP} is the recovered volume of aphron and liquid phase, respectively.

$ml_{CGA}/ml_{extract}$		16	16	16	8	4
$g_{GAETPI}/L_{extract}$		0.3	1.0	5.4	1.0	1.0
mol_{Tween}/mol_{GAE}		90.7	27.2	4.8	13.6	6.8
V_{LP}/V_{AP}		1.25	1.00	0.83	1.25	2.50
Effective gas hold-up		0.53	0.60	0.60	0.60	0.55
RE	GAE_{TPI}	77.29^{c}	75.59^{d}	78.47^{b}	61.75^{c}	48.25^{c}
	GAE_{FI}	69.42^{b}	72.86^{bcd}	76.33^{b}	55.40^{b}	41.21^{b}
	CAE	75.92^{c}	78.90^{e}	84.51^{c}	60.66^{bc}	54.56^{d}
	ME	NE	69.76^{b}	76.36^{b}	68.90^{d}	24.56^{a}
	QE	70.75^{b}	79.28^{e}	84.28^{c}	60.18^{bc}	54.03^{d}
	Glucose	44.52^{a}	73.03^{cd}	70.98^{a}	66.09^{cd}	46.99^{c}
	Fructose	46.20^{a}	71.30^{bc}	70.62^{a}	46.09^{a}	45.34^{bc}
	Potassium	70.88^{b}	52.38^{a}	72.21^{a}	68.40^{d}	43.92^{bc}
FI/TPI (Aphron phase)		1.88	1.76	2.02	1.12	0.76
FI/TPI (liquid phase)		2.82	1.52	1.78	1.46	1.01

GAE: gallic acid equivalents according to total phenol index (TPI) or Folin Index (FI). CAE: caffeic acid equivalents. ME: malvidin-glucoside equivalents. QE: quercetin equivalents. NE: not evaluated. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post hoc test.

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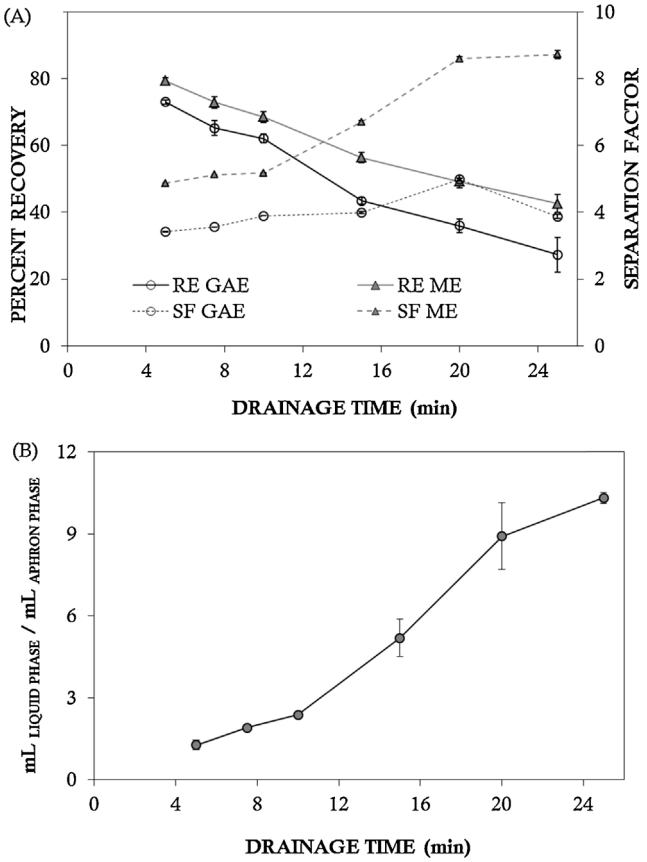

Fig. 2 – Results of separation trials carried out with colloidal gas aphrons (CGA) (generated from CTAB 1 mM) and Barbera marc extract (GAE: gallic acid equivalents as total phenolics index; ME: malvidin-glucoside equivalents) with a constant volumetric ratio of 15.33 $mL_{CGA}/mL_{extract}$. (A): recovery as a function of drainage time. (B): the volumetric ratio of drained liquid phase to drained aphron phase as a function of drainage time. Error bars indicate ± SD.

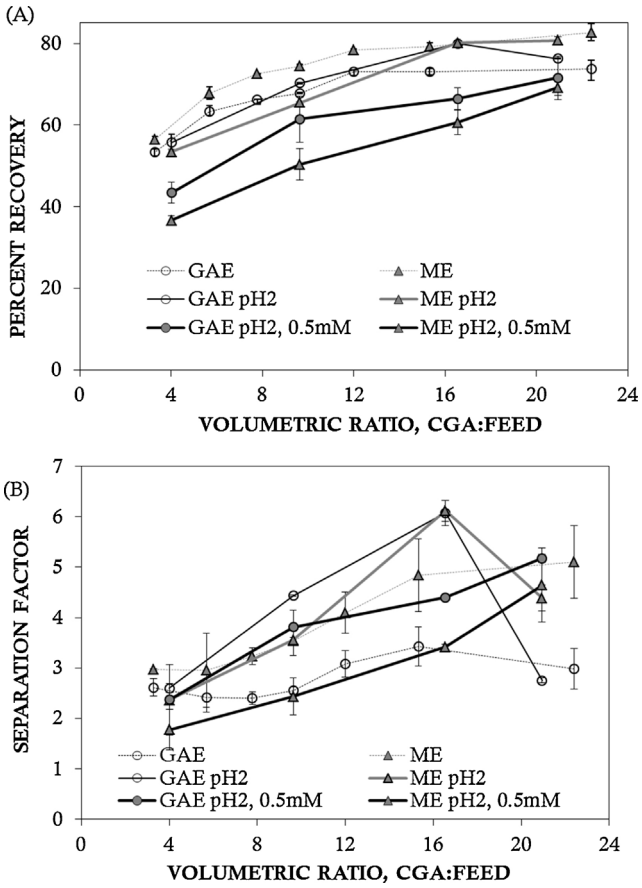

Fig. 3 – Results of separation trials carried out with colloidal gas aphrons (CGA) (generated from CTAB 1 mM and 0.5 mM, modifying or not the pH to 2) and Barbera marc extract (GAE: gallic acid equivalents as total phenolics index; ME: malvidin-glucoside equivalents). (A): recovery; (B): separation factor as a function of volumetric ratio. Error bars indicate ± SD.

3.2. *Separation with Tween 20*

CGA were generated from the non-ionic, food-grade surfactant Tween 20 at a fixed concentration of 10 mM. This concentration was used based on characteristics of CGA such as, stability (half-life) = 407 s and gas hold-up = 60% which, were similar to those of the CGA generated from CTAB, stability (half-life) = 493 s and gas hold-up = 63%.

Compared to the trials with CGA generated from CTAB 1 mM (without pH correction), lower anthocyanins recovery and separation factor were obtained with CGA(Tween 20) than CGA(CTAB), but only slightly lower recovery and higher separation factor of total phenolics (Fig. 5A and B). These results confirm that the separation of polyphenols is driven by both electrostatic and hydrophobic interactions in the case of CGA generated from CTAB whilst with CGA generated from Tween 20, only hydrophobic interactions drive the separation hence the lower recovery with Tween. CGA generated from Tween 20 had also lower stability and gas hold-up than CGA generated from CTAB (Fig. 5C). Particularly at low volumetric ratio (4) Tween 20 had very low effective gas hold-up (about 0.2) which coincides with low recovery of polyphenols. The reduction in gas hold-up leads to a reduction in interfacial area hence lower recovery; a similar observation was made by the authors on the recovery of astaxanthin (Dermiki et al., 2010).

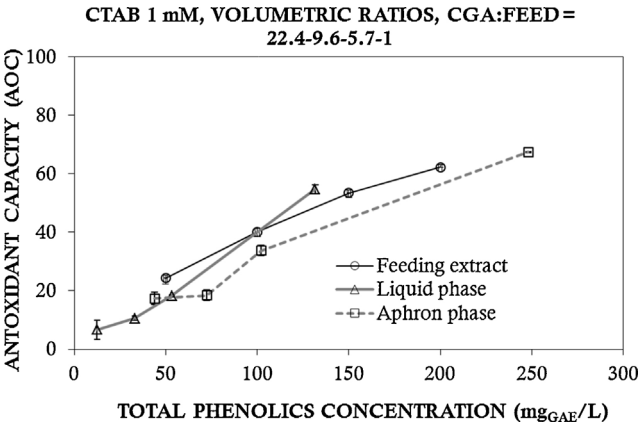

Fig. 4 – Comparison between the antioxidant capacity as a function of total phenolics concentration (GAE: gallic acid equivalents as total phenolics index) for the initial Barbera marc and the recovered liquid and aphron phase, for separation trials carried out with colloidal gas aphrons generated from CTAB (without pH modification) and different volumetric ratios. Error bars indicate ± SD.

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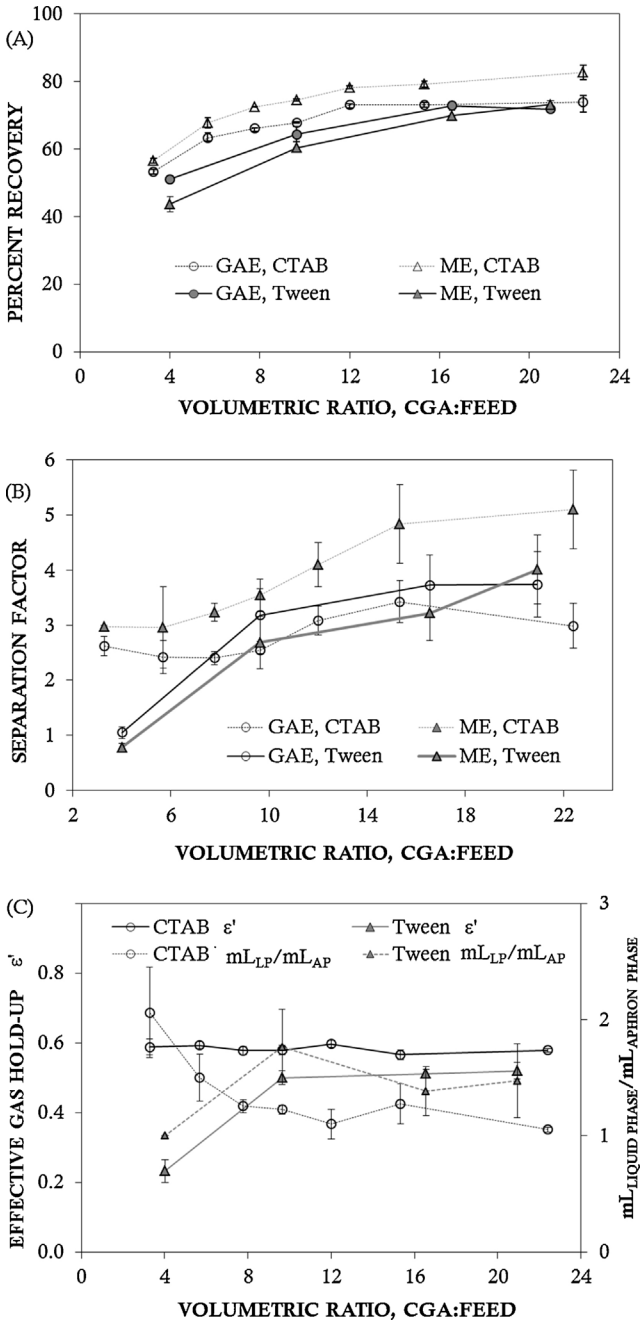

Fig. 5 – Results of separation trials carried out with colloidal gas aphrons (generated from CTAB 1 mM or Tween 20 10 mM) and Barbera marc extract (GAE: gallic acid equivalents as total phenolics index; ME: malvidin-glucoside equivalents). (A): recovery; (B): separation factor and (C) the volumetric ratio of drained liquid phase to drained aphron phase and the effective gas hold-up as a function of volumetric ratio. Error bars indicate ± SD.

Contrary to what was observed with CGA generated from CTAB no loss of antioxidant activity was observed in recovered fractions with CGA generated from Tween 20 (data not shown here).

Overall 16 was found to be an optimum volumetric ratio where recovery of both total phenolics and anthocyanins reached a plateau. Further experiments were carried out in order to investigate the applicability of the process to different phenolic extracts, total phenolics load and selectivity of different classes of phenolic compounds and non-phenolic compounds.

Compared to Barbera extract, the Pinot extract was characterised by a 1.6 times higher content of total phenolics, but a 5 times lower amount in anthocyanins (Barbera grape is, in fact, well-known for its high anthocyanins content). Overall composition of Pinot extract was: 5.43 ± 0.16 g/L GAE_{TPI}; 8.74 ± 1.32 g/L GAE_{FI}; 2.54 ± 0.03 g/L CE; 161.46 ± 2.28 mg/L ME; 8.10 ± 0.086 g/L glucose; 10.42 ± 0.16 g/L fructose and 767.5 ± 0.14 mg/L potassium.

Separation experiments were carried out at different volumetric ratios and different dilutions of the feed extract (0.3–1.0–5.4 g/L GAE_{TPI}). The results are reported in Tables 2 and 3.

Very similar recovery of total phenolics was obtained to that obtained with Barbera extract.

When the volumetric ratio was kept constant, it was observed, for almost all the compounds, a general increase in the recovery with decreasing molar ratio (Table 2). This might have been due to the higher CGA stability observed when a more concentrated extract was used; with the stabilising effect possibly due to other compounds in the extract as observed previously for the Barbera extract. Furthermore, contrary to what was observed with CGA generated from CTAB, the volumetric ratio has a major effect on the recovery instead of the molar ratio. For example in experiments at a constant volumetric ratio of 16 but varying molar ratio, the recovery is almost the same (Table 2). However, experiments at increased volumetric ratio (4–16) at constant feed concentration show an important increase in recovery (48.25–75.59) of GAE. In the trials with concentrated extract (molar ratio 4.8), the formation of aggregates was observed in the recovered aphron phase. Since these aggregated could not be completely solubilised during sample analysis, an underestimation of the recovery was probably done.

In summary maximum recovery is obtained at high volumetric ratio and concentrated extract, which results optimal also for the selectivity of the process. In fact, looking at the separation factor (Table 3), in these trials it was observed a higher affinity for the aphron phase by cinnamic acids and flavonols, which tend to be smaller molecules compared to anthocyanins and other phenolic compounds. However, it was still not possible to selectively separate anthocyanins (confirming their probable association with other molecules), or non-phenolic compounds such as sugars and minerals.

Analysis of antioxidant activity (Fig. 6) confirmed that only a slight reduction in the antiradical activity of the phenolic compounds could be achieved using Tween 20.

The occurrence of a certain selectivity and/or oxidation of the compounds during the separation is shown also by the ratio between the total phenolic index and the Folin Index. In fact, even though both methods are used to quantify total phenolics, the total phenolic index is based on the characteristic absorption of the aromatic ring at 280 nm (with different molar extinction coefficients depending on the molecular structure), while the Folin Index is based on the ability of the compound to reduce the Folin reagent which depends on the molecular structure and oxidative status of the antioxidant. It follows that a change in the Folin Index/total phenolic index ratio indicates a change in the composition and/or oxidation of the phenolic compounds.

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Table 3 – Results (in terms of separation factor, SF) for the separation trials carried out with colloidal gas aphrons generated from Tween 20 10 mM, at different volumetric ratios ($ml_{CGA}/ml_{extract}$) and different molar ratios (mol_{Tween}/mol_{GAETPI}). V_{AP} and V_{LP} is the recovered volume of aphron and liquid phase, respectively.

$ml_{CGA}/ml_{extract}$	16	16	16	8	4
$g_{GAETPI}/L_{extract}$	0.3	1.0	5.4	1.0	1.0
mol_{Tween}/mol_{GAE}	90.7	27.2	4.8	13.6	6.8
V_{LP}/V_{AP}	1.25	1.00	0.83	1.25	2.50
Effective gas hold-up	0.53	0.60	0.60	0.60	0.55
	SF	SF	SF	SF	SF
GAE_{TPI}	4.10^{c}	3.00^{c}	3.78^{b}	1.91^{bc}	2.27^{c}
GAE_{FI}	2.71^{b}	2.60^{bc}	3.35^{ab}	1.48^{ab}	1.71^{b}
CAE	3.82^{c}	3.64^{d}	5.70^{c}	1.84^{bc}	2.93^{d}
ME	NE	2.24^{b}	3.43^{b}	2.66^{d}	0.79^{a}
QE	2.88^{b}	3.74^{d}	6.60^{d}	1.79^{b}	2.88^{d}
Glucose	0.95^{a}	2.62^{bc}	3.03^{a}	2.32^{cd}	2.18^{bc}
Fructose	1.02^{a}	2.41^{bc}	3.03^{a}	1.01^{a}	2.03^{bc}
Potassium	2.89^{b}	1.06^{a}	3.21^{a}	2.56^{d}	1.91^{bc}

GAE: gallic acid equivalents according to total phenol index (TPI) or Folin Index (FI). CAE: caffeic acid equivalents. ME: malvidin-glucoside equivalents. QE: quercetin equivalents. NE: not evaluated. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post hoc test.

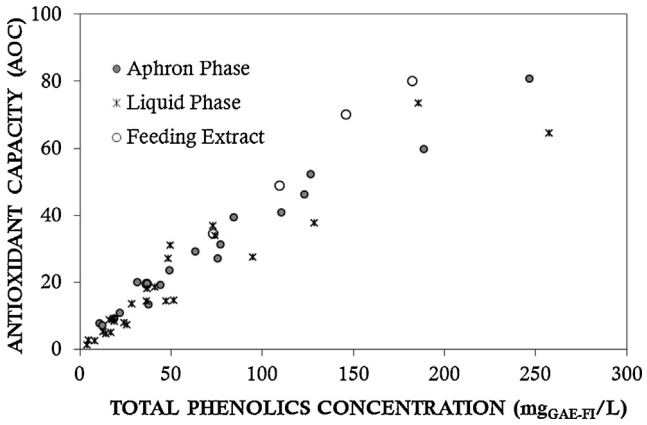

Fig. 6 – Comparison between the antioxidant capacity as a function of total phenolics concentration (GAE: gallic acid equivalents as Folin Index) for the initial Pinot noir marc extract and the recovered liquid and aphron phase, for the different separation trials carried out with colloidal gas aphrons generated from Tween 20.

3.2.1. *In situ CGA generation*

The last trials were carried out by generating the CGA directly from a mixture of surfactant solution and extract with a volumetric ratio of 8 $mL_{Tween}/mL_{extract}$ (corresponding to a theoretic volumetric ratio of 18.56 $mL_{CGA}/mL_{extract}$, based on the gas hold-up of Tween 20 10 mM). Two different extract concentrations (0.3 and 5.4 g/L GAE_{TPI}) were used, corresponding to molar ratio of 45.3 and 2.5 mol_{Tween}/mol_{GAE}, respectively. Results are reported in Table 4. Decidedly lower recovery and selectivity, compared to the conventional separation trials, were obtained. In particular, the trials with the undiluted extract showed a uniform partition of all the analysed compounds in the two phases (separation factors around 1 and the same ratio Folin Index/total phenolic index), which suggests that when CGA are generated in the presence of the extract, the solubilisation of polyphenols by the micelles competes strongly with the flotation of polyphenols by the CGA.

Table 4 – Results (in terms of recovery efficiency RE and separation factor SF) for the separation trials carried out with colloidal gas aphrons generated from Tween 20 10 mM mixed with Pinot noir marc extract, at different molar ratios (mol_{Tween}/mol_{GAETPI}). V_{AP} and V_{LP} is the recovered volume of aphron and liquid phase, respectively.

$g_{GAETPI}/L_{extract}$	0.3		5.4	
mol_{Tween}/mol_{GAE}	45.3		2.5	
V_{LP}/V_{AP}	1.69		0.77	
	RE	SF	RE	SF
GAE_{TPI}	41.96^{ab}	1.23^{ab}	55.07^{a}	0.94^{a}
GAE_{FI}	50.94^{c}	1.78^{c}	55.65^{a}	0.97^{a}
CAE	41.69^{ab}	1.21^{ab}	57.12^{a}	1.03^{a}
ME	52.44^{c}	1.87^{c}	55.91^{a}	0.98^{a}
QE	45.83^{b}	1.43^{b}	55.07^{a}	0.95^{a}
Glucose	37.98^{a}	1.04^{a}	58.24^{a}	1.09^{a}
Fructose	38.51^{a}	1.06^{a}	48.27^{b}	0.72^{b}
FI/TPI (Aphron phase)	2.12		1.50	
FI/TPI (liquid phase)	1.46		1.46	

GAE: gallic acid equivalents according to total phenol index (TPI) or Folin Index (FI). CAE: caffeic acid equivalents. ME: malvidin-glucoside equivalents. QE: quercetin equivalents. NE: not evaluated. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post hoc test.

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4. Conclusions

In this work we have shown that CGA generated from CTAB can effectively extract phenolic compounds. Slightly higher recoveries than when applied to standard solution of gallic acid are obtained which is thought to be due to an stabilising effect by the feed. Furthermore slightly higher recoveries and separation factors are obtained for anthocyanins showing a slightly higher affinity of CGA generated from CTAB for these compounds. This results together with those obtained at pH = 2 suggest that polyphenols partition to the CGA mainly due to hydrophobic interactions but electrostatic interactions enhance the partitioning as shown by a higher recovery and separation factor obtained for anthocyanins at pH >2. This could also explain why lower recovery and separation factor were obtained for phenolic compounds with CGA generated from Tween 20 than with CGA generated from CTAB as with the former partitioning into the CGA phase is driven by hydrophobic interactions only. Maximum recovery (78% GAE and 76% ME) and separation factor (4 GAE and 3 ME) were obtained at the highest volumetric ratio and higher extract concentration with CGA generated from Tween 20. Moreover, the extraction with CGA generated from Tween 20 did not lead to a substantial loss of antioxidant capacity contrary to the extraction with CGA generated from CTAB. Overall, here it has been shown that the extraction of phenolic compounds with CGA generated from Tween 20 could be a promising alternative to other separations such as organic extraction.

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