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# Fractionation of a Red Grape Marc Extract by Colloidal Gas Aphrons

Farid Dahmoune[a], Khodir Madani[a], Paula Jauregi[b], Dante M. De Faveri[c], Giorgia Spigno*[c]

[a]Laboratory of biomathematics, biochemistry, biophysics and scientometrics, Abderrahmane mira university of Bejaia, Bejaia 06000, Algeria
[b] Department of Food and Nutritional Sciences, University of Reading, Whiteknights, PO Box 226, Reading RG6 6AP, UK
[c]Istituto di Enologia e Ingegneria Agro-Alimentare, Università Cattolica Sacro Cuore, Via Emilia Parmense, 84, 29122 Piacenza (Italy)
giorgia.spigno@unicatt.it

The objective of the current study was to investigate the application of colloidal gas aphrons (CGA) as a potential low-cost technology for the fractionation of phenolic extracts obtained from off-skins fermented red grape skins with high sugar content. The trials were aimed to investigate the possibility of using CGA generated from the non-ionic surfactant Tween20 to separate phenolic compounds from non-phenolic compounds (minerals and sugars) and to fractionate different fractions of phenolic compounds (total phenols, cinnamic acids and anthocyanins). Separation tests were carried out in a 0.7 L flotation column investigating the effect of both the volumetric ratio CGA/extract sample and the molar ratio CGA/extract sample on both the recovery yields and the separation factor (the ratio between the concentration of a compound in the recovered aphron phase and in the discharged liquid phase). Results revealed the possibility of reaching high recovery yield even though with a poor selective fractionation of the different compounds present in the extract, with glucose and cinnamic acids showing the highest affinity for the aphron phase.

## 1. Introduction

Extraction of natural phenolic compounds from agro-food by-products has attracted both academic and industrial interest. However, plant crude extracts usually contain large amounts of non-phenolic substances, such as carbohydrates and minerals, and the concentration of the phenolic compounds may be low. To concentrate and obtain polyphenol enriched fractions, strategies including sequential extraction, liquid-liquid partitioning and other complex and expensive processes based on membrane or chromatographic technologies have been proposed. The use of surfactant-based methods for the treatment of aqueous streams and solid matrices to remove organic and inorganic contaminants, and for the recovery of valuable products, are promising new areas of great environmental and technological importance (Gortzi et al., 2008). In particular, colloidal gas aphrons (CGA) are surfactant-stabilized microbubbles (10 - 100 µm) generated by intense stirring of a surfactant solution at high speeds (> 8.000 rpm) (Yan et al., 2005). They were firstly postulated by Sebba (1987) to consist of a microbubble encapsulated in a thin aqueous film (“soapy shell”). CGA have been used for many separation processes of bioproducts such as protein, enzyme, carotenoids and dyes recovery (Dermiki et al., 2010). The most striking feature of CGA is their stability, which lets them generated externally to their point of use, and then to be transported by pumping. Generally, the stability of aqueous foam is determined by two different phenomena: the rate at which liquid drains from foam, and the rate at which the body of the foam breaks down. In the case of CGA, there is no perceptible breakdown of the microbubble until the great majority of the liquid has drained.

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Previous studies showed the potential of CGA to separate total phenolic compounds from a grape marc extract (Spigno et al., 2010) with the separation mechanism apparently predominantly driven by hydrophobic interactions (Spigno et al., 2011). This may lead to the development of low-cost purification processes for the production of bioactive ingredients with modified solubility properties. To get a better insight into the process mechanisms and potential application, the objective of the current study was to investigate the use of CGA for the fractionation of a natural grape waste phenolic extracts. In particular, the trials were carried out with CGA generated from the non-ionic surfactant Tween20 to separate phenolic compounds from non-phenolic compounds (potassium and glucose) and to fractionate different classes of phenolic compounds, considering also the influence on the antioxidant activity of the phenolic compounds.

## 2. Materials and Methods

### 2.1 Extract preparation

Waste grape marc were collected from a red grape cultivar (Pinot noir) immediately after destemming and pressing operations in the 2011 vintage from a winery in Northern Italy. Since grapes were off-skins fermented, the resulting marc was still rich in grape sugars. Marc were dried at 60°C in a hot air oven for 24 h and then grounded (final particle size < 2mm). The marc powder (125 g) was then 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 5.000 rpm for 10 min (Centrifuge ALC 4237R) and the surnatant (extract) recovered. The extract was concentrated under vacuum at 40°C (Rotavapor Büchi R-114) until half of the initial volume and then reconstituted with water up to the initial volume. This passage is necessary to partly recover ethanol (important from an industrial and economic point of view) and reduce ethanol content for the CGA trials, since high alcohol concentration is detrimental for CGA stability. The reconstituted extract was subdivided into 100 mL aliquots and then stored in freezer to avoid fermentation until use, while a 50 mL aliquot was used for chemical characterization according to the following analyses:

- ✓ Total phenols 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 phenols content by Folin-Ciocalteau analysis (Folin Index), expressing the results as $GAE_{FI}$ based on a calibration curve with standard gallic acid (García et al., 2011);
- ✓ Total anthocyans content expressed as WAE (Wine Anthocyans Equivalents) by absorbance reading at 538 nm of the sample diluted with chloridric ethanol and multiplying the absorbance by the conversion factor 26.6 (mg/l) for a standard solution containing all the 5 anthocyanidins typical of red wines: Delfinidin, Cyanidin, Petunidin, Peonidin and Malvidin (Amendola et al., 2010);
- ✓ Total cinnamic acids content by absorbance reading at 320 nm and expressed as caffeic acid equivalents (CAE) based on a calibration curve with standard caffeic acid (Spigno et al., 2007);
- ✓ Glucose content evaluated by a Megazyme enzymatic kit;
- ✓ Potassium content by atomic absorptions spectroscopy (Spigno et al., 2013);
- ✓ Antioxidant activity according to the radical ABTS test and expressing the results as percentage inhibition of radical oxidation (AOP%) (García et al., 2011).

### 2.2 CGA generation and characterization

The CGA were generated from a Tween20 10mM solution stirred at 8000 rpm with the Silverson mixer for 5 min. For CGA characterization, after generation, 100 mL of the foam were pumped into a volumetric cylinder and let completely drained to measure the drained volume. The gas hold up and surfactant concentration were then calculated from the measured drained volume and the foam volume as:

$$Gas\ hold\ up = \frac{V_{CGA} - V_{liquid\ drained}}{V_{CGA}} \quad (1)$$

$$Gas\ surfactant\ concentraion = \frac{V_{liquid\ drained} \cdot Molarity_{initial\ solution}}{V_{CGA}} \quad (2)$$

where *Molarity* is the molar concentration of the surfactant solution used to generate the CGA.

**2.3 Separation with CGA**

Trials were carried out in a flotation column (a glass column, internal diameter of 0.25 m, total height 0.4m) according to the experimental set-up schematised in Figure 1.

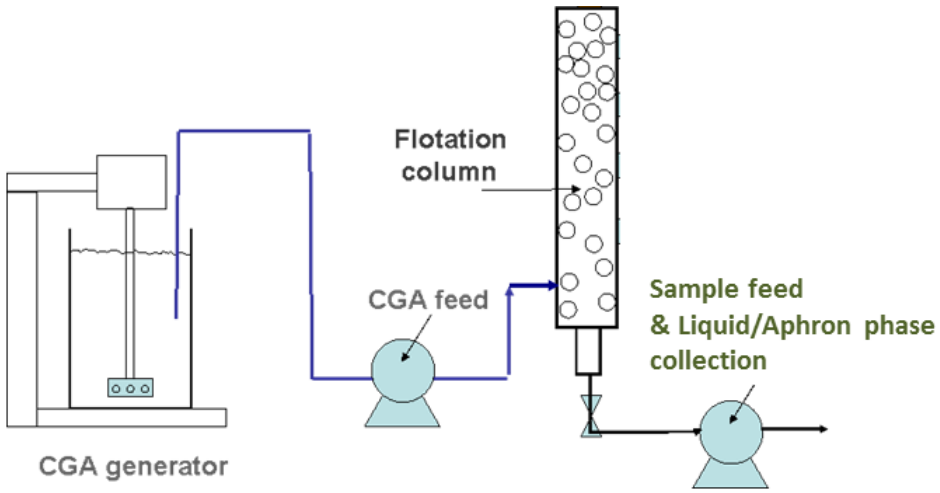


*Figure 1: Schematic diagram of the experimental set-up for the separation trials with colloidal gas aphrons (CGA).*

The extract solution was pumped into the column (from the bottom) by a peristaltic pump and then the CGA were pumped into the column (again from the bottom) at a speed rate such to take 5 min to fill the column (corresponding to the mixing time). Once filled the column, a 5 min contact time was waited for before separating the bottom drained aqueous phase and the top aphron phase. Both the phases were then pumped out the column taking note of the recovered volumes ($V_{LP}$ and $V_{AP}$ respectively). The liquid and aphron phase were characterised for the same analyses carried out on the initial extract. Based on the recovered $V_{AP}$ and its concentration ($C_{AP}$), and on the recovered $V_{LP}$ and its concentration ($C_{LP}$), the percentage recovery efficiency for each of the analysed compounds, was calculated as:

$$RE = \frac{C_{AP} \cdot V_{AP}}{(C_{AP} \cdot V_{AP}) + (C_{LP} \cdot V_{LP})} 100 \quad (3)$$

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:

$$SF = \frac{C_{Aphron\ phase}}{C_{Liquid\ phase}} \quad (4)$$

The following separation trials were carried out (in duplicate) to investigate the effect of both volumetric and molar ratio:

a. 16 Volumetric ratio $mL_{CGA}$/ $mL_{extract}$, using undiluted or diluted extract to have different total phenols concentration: 300 mg/L $GAE_{TPI}$ (conditions selected based on previous experimental work by Spigno et al., 2010); 1000 mg/L $GAE_{TPI}$ and 5700 mg/L $GAE_{TPI}$.
b. 8 and 4 Volumetric ratio $mL_{CGA}$/ $mL_{extract}$ using diluted extract (1000 mg/L $GAE_{TPI}$).

Based on the total phenols content of the extract (as TPI) and the gas surfactant concentration Eq. ( 2), the molar ratio was calculated as $mol_{Tween}/mol_{GAE}$.

**2.4 Statistics**

All the values are reported as means of replicates. The significance of the difference in the RE and SF for the different analyzed compounds, under the same working conditions (molar ratios or volumetric ratios),

was assessed by ANOVA and Tukey's post-hoc test for means discrimination at a confidence level of 99% (IBM SPSS Statistics 19).

## 3. Results and discussion

CGA generated from aqueous solution of Tween 20 10mM showed a gas hold up of 0.57 and a production yield of 2.32 $mL_{CGA}$ /$mL_{Tween,}$ in agreement with previous works (Spigno et al., 2010; Spigno et al., 2011). The grape marc extract showed the following composition: 5.43 ± 0.16 g/L $GAE_{TPI}$; 8.74 ± 1.32 g/L $GAE_{FI}$; 2.54 ± 0.03 g/L CAE; 298.04 ± 4.20 mg/L WAE; 8.10± 0.086 g/L glucose; 767.5±0.14 mg/L potassium.
For almost all the considered compounds, it was observed a general increase in the recovery efficiency with decreasing molar ratio, which might be also due to the increased measured ratio between the volume of recovered aphron phase and the volume of drained liquid phase ($V_{AP}/V_{LP}$ in Table 1).

*Table 1: Results (in terms of recovery efficiency RE and separation factor SF) for the separation trials carried out at constant volumetric ratio (16) and different molar ratios ($Mol_{Tween}/mol_{GAE}$). $V_{AP}$: recovered volume of aphron phase; $V_{LP}$: recovered volume of liquid phase. NE: not evaluated. GAE: gallic acid equivalents according to total phenol index (TPI) or Folin index (FI). CAE: cinnamic acids equivalents. WAE: wine anthocyans equivalents. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test.*

| **$Mol_{Tween}/mol_{GAE}$** | 90.7 | 27.2 | 4.8 | 90.7 | 27.2 | 4.8 |
|---|---|---|---|---|---|---|
| **$V_{AP}/V_{LP}$** | 0.8 | 1.0 | 1.2 | 0.8 | 1.0 | 1.2 |
| **Measured gas hold-up** | 0.53 | 0.60 | 0.60 | 0.53 | 0.60 | 0.60 |
| | **RE** | **RE** | **RE** | **SF** | **SF** | **SF** |
| **$GAE_{TPI}$** | $77.29^{d}$ | $75.59^{d}$ | $78.47^{b}$ | $4.10^{d}$ | $3.00^{c}$ | $3.78^{b}$ |
| **$GAE_{FI}$** | $69.42^{b}$ | $72.86^{c}$ | $76.33^{ab}$ | $2.71^{b}$ | $2.60^{bc}$ | $3.35^{ab}$ |
| **CAE** | $75.92^{cd}$ | $78.90^{e}$ | $84.51^{c}$ | $3.82^{cd}$ | $3.64^{d}$ | $5.70^{c}$ |
| **WAE** | NE | $69.76^{b}$ | $77.61^{a}$ | NE | $2.24^{b}$ | $3.92^{a}$ |
| **Glucose** | $44.52^{a}$ | $73.03^{cd}$ | $70.98^{a}$ | $0.95^{a}$ | $2.62^{bc}$ | $3.03^{a}$ |
| **Potassium** | $70.88^{bc}$ | $52.38^{a}$ | $72.21^{a}$ | $2.89^{bc}$ | $1.06^{a}$ | $3.21^{a}$ |

Since the CGA were pumped into the flotation column from the bottom through the extract sample already loaded, the higher extract concentration might have reduced the destabilising effect of this step, probably due to the presence of like-surfactant-behaving components in the natural extract. Furthermore, in the trials with undiluted extract and extract at intermediate concentration, recovery might have been underestimated since the aphron phase showed the formation of agglomerates that could not be solubilised for the quantification (Figure 2).

*Figure 2: Picture of agglomerates formed in the aphron phase recovered from the separation trials carried out with undiluted extract.*

Considering the calculated separation factor (Table 1), smaller molecules (potassium and cinnamic acids) showed a higher affinity for the aphron phase, even though not high selective separation could be obtained. In the trials at intermediate molar ratio, the separation factors of all the analysed components

were very close each other; while in the trials with the highest molar ratio the sugars showed the lowest affinity values.

Additional trials were carried out at constant inlet extract concentration but different volumetric ratios with consequently also different molar ratios (Table 2). The effect of the volumetric ratio appears higher than that of the molar ratio, since for similar molar ratios (6.8 and 4.8) but different volumetric ratios (4 and 16), the recovery efficiency dramatically decreased, while the separation factors tended to slightly decrease.

Analysis of antioxidant activity showed that the CGA separation process slightly lowered the antiradical activity, particularly of the phenolic compounds recovered in the aphron phase (Figure 3). This might be due to a combined effect of real oxidation during the separation process and of surfactant interaction with the radical analysis.

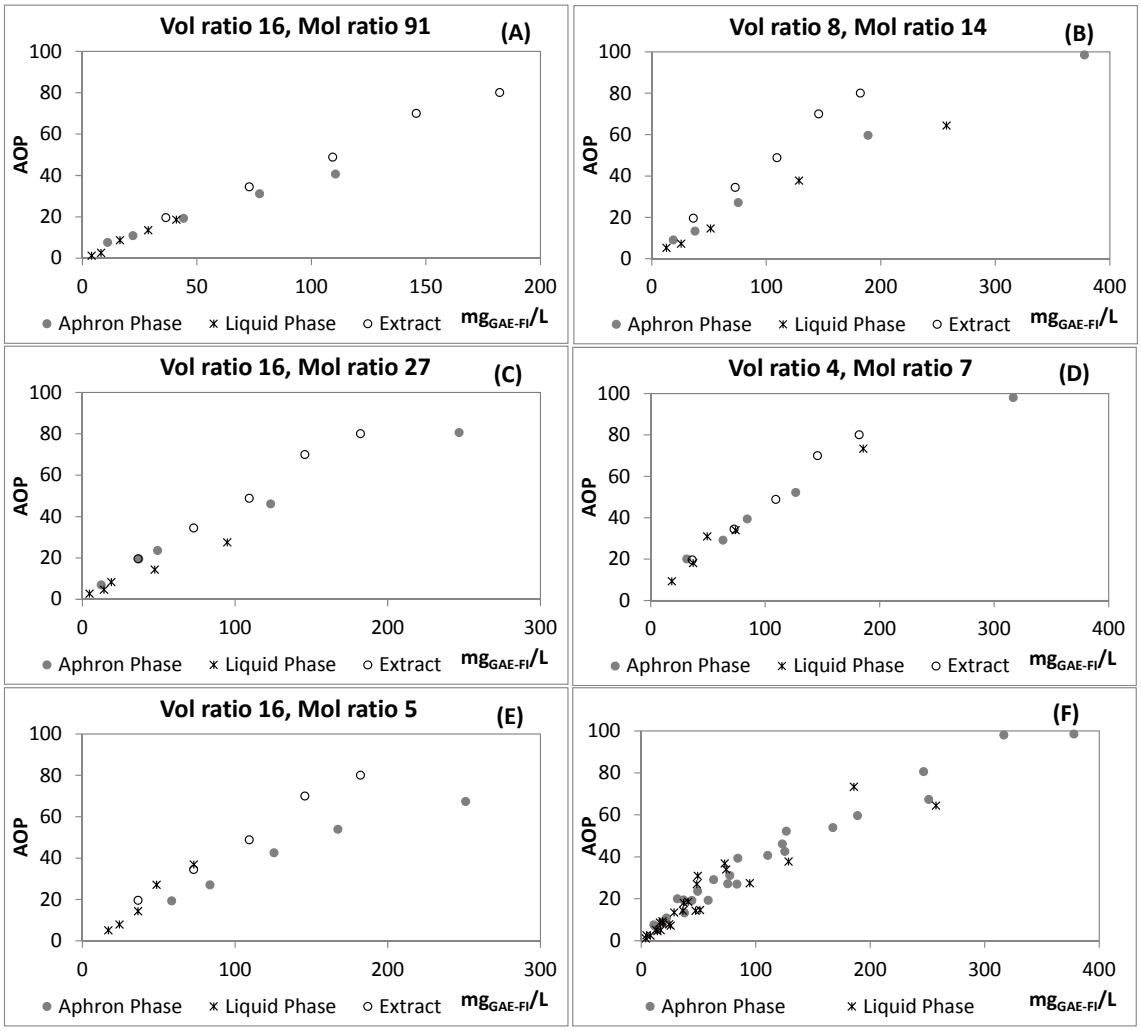


*Figure 3: Influence of separation conditions (in terms of both molar and volumetric ratio) on the specific antioxidant activity (AOP) of the total phenols (expressed as gallic acid equivalents based on Folin index, $GAE_{FI}$). Figure (F) reports all the data of figures (A-E) together.*

*Table 2: Results (in terms of recovery efficiency RE and separation factor SF) for the separation trials carried out at different volumetric ratios ($ml_{CGA}/ml_{extract}$ ) and different molar ratios ($mol_{Tween}/mol_{GAE}$). $V_{AP}$ is the recovered volume of aphron phase; $V_{LP}$ is the recovered volume of liquid phase. GAE: gallic acid equivalents according to total phenol index (TPI) or Folin index (FI). CAE: cinnamic acids equivalents. WAE: wine anthocyans equivalents. Same superscript letters in the same column indicate means not statistically different according to ANOVA and Tukey's post-hoc test.*

| **$mL_{CGA}/mL_{extract}$** | 16 | 8 | 4 | 16 | 8 | 4 |
|---|---|---|---|---|---|---|
| **$Mol_{Tween}/mol_{GAE}$** | 27.2 | 13.6 | 6.8 | 27.2 | 13.6 | 6.8 |
| **$V_{AP}/V_{LP}$** | 1.0 | 0.8 | 0.4 | 1.0 | 0.8 | 0.4 |
| **Measured gas hold-up** | 0.60 | 0.60 | 0.55 | 0.60 | 0.60 | 0.55 |
| | **RE** | **RE** | **RE** | **SF** | **SF** | **SF** |
| **$GAE_{TPI}$** | $75.59^{d}$ | $61.75^{abc}$ | $48.25^{c}$ | $3.00^{c}$ | $1.91^{ab}$ | $2.27^{c}$ |
| **$GAE_{FI}$** | $72.86^{c}$ | $55.40^{a}$ | $41.21^{b}$ | $2.60^{bc}$ | $1.48^{a}$ | $1.71^{b}$ |
| **CAE** | $78.90^{e}$ | $60.66^{ab}$ | $54.56^{d}$ | $3.64^{d}$ | $1.84^{ab}$ | $2.93^{d}$ |
| **WAE** | $69.76^{b}$ | $68.90^{d}$ | $24.56^{a}$ | $2.24^{b}$ | $2.66^{c}$ | $0.79^{a}$ |
| **Glucose** | $73.03^{cd}$ | $66.09^{bcd}$ | $46.99^{bc}$ | $2.62^{bc}$ | $2.32^{bc}$ | $2.18^{bc}$ |
| **Potassium** | $52.38^{a}$ | $66.80^{cd}$ | $43.92^{bc}$ | $1.06^{a}$ | $2.38^{c}$ | $1.91^{bc}$ |

## 4. Conclusions

This study shows the potential of CGA to separate and fractionate phenolic compounds from real plant extracts. Results obtained with the non-ionic food-grade surfactant Tween20, however, revealed a low selectivity under the applied separation conditions and a slighter reduction of the antiradical activity after recovery in the aphron phase. Optimisation of working parameters and trials with different food-grade surfactants could lead to the development of low-cost purification processes for the production of bioactive ingredients with modified solubility properties. Further research is also required to get an insight into the type and stability of the molecular association between the phenolic compounds and the surfactants.

### Acknowledgments

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